Biodiversity and systematics of parasitic Crustacea infesting southern African freshwater fishes

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Abstract

Crustaceans (Arthropoda: Crustacea) are regarded as the most morphologically diverse group in the phylum Arthropoda, and they have the greatest diversity of parasitic forms. Parasitic crustaceans have adapted several morphological and physiological characteristics that enable them to thrive in an array of habitats and hosts. Freshwater parasitic crustaceans are mostly found attached to the external surfaces of their host. These attachment structures are also adapted to the sites of infestation on their hosts. As a result of the pathological impacts of certain parasitic crustaceans on fish, they have been regarded as species of economic and commercial importance.

Individuals from the families Argulidae, Ergasilidae, and Lernaeidae are most frequently reported throughout Africa, including both native and invasive parasite species. In southern Africa, however, these reports proved to be limited compared to other regions, therefore suggesting an underrepresentation of the actual diversity of parasitic crustaceans. In addition to scarce distribution records, only a few integrative studies from southern Africa have been carried out on some of these parasites. It was therefore hypothesised that with broader sampling across several localities and hosts in southern Africa, there will be an increased knowledge of the diversity of parasitic crustacean species present in the different river systems as well as new hosts and locality records, in addition to the possible description of new species. More so, the integration of improved morphological techniques and molecular analyses would provide novel information on the morphological characteristics of these species while adding new DNA sequences to the scanty database.

In collaboration with the Aquatic Research Group of the University of the Free State, Bloemfontein, as well as several ongoing parasitology projects of the Water Research Group of the North-West University, Potchefstroom, this study aimed at linking distribution data of parasitic crustaceans with morphological and molecular data. Native and exotic fishes belonging to six families (Alestidae, Centrarchidae Cichlidae, Clariidae, Cyprinidae, and Hepsetidae) were collected from several localities in southern Africa. With an integration of morphological (light and scanning electron microscopy) and molecular (partial 18S, 28S, and COI genes) techniques, four native species (*Ergasilus mirabilis* Oldewage & van As, 1987; *Lamproglena clariae* Fryer, 1956; *Lamproglena hepseti* van As & van As, 2007; *Lamproglena monodi* Capart, 1944), three known invasive species (*Argulus japonicus* Thiele, 1900; *Lernaea cyprinacea* Linnaeus 1758; *Neoergasilus japonicus* (Harada, 1930)), and one new species *Neoergasilus* sp. nov., were described from the current study.

Previous records of *Ergasilus mirabilis,* a widely distributed species in southern Africa, lacked genetic data. A total of 159 *Clarias gariepinus* (Burchell, 1822) were caught from

various locations in South Africa and Zambia and adult female copepods were collected from two South African localities (Kushokwe Pan and Vaal River) and the Zambezi River in Zambia. Morphological examination confirmed the specimens as *E. mirabilis* with slight variability in the number of spines and setae from the original description. Molecular analyses included partial ribosomal RNA (18S and 28S) and mitochondrial DNA (COI) genes, providing genetic data to support the morphological characteristics of *E. mirabilis*. Additionally, new distribution records *of E. mirabilis* in the Phongolo Floodplain and the Vaal River are reported.

The study further reported on species from the genus *Lamproglena*, which are gill ectoparasites found on African fish hosts in the families Cichlidae, Clariidae, and Hepsetidae. Three *Lamproglena* species (*L. clariae, L. hepseti,* and *L. monodi*) reported from this study are native to southern Africa. *Lamproglena clariae* is reported for the first time from the Phongolo Floodplain and Nyamiti Pan in South Africa, and from West Lunga in Zambezi. This study also provided genetic data using three genetic markers, which is a notable contribution to the limited molecular data available for *Lamproglena* species globally. Additional information on the maxillipeds, antennae, and antennules morphology of *L. hepseti* was also provided.

New distribution records as well as the first molecular characterisation of the invasive fish lice *Argulus japonicus* in Sub-Saharan Africa was also provided from this study. Four fish species: *Clarias gariepinus*; *Cyprinus carpio* Linnaeus, 1758; *Labeo umbratus* (Smith, 1841); *Labeobarbus aeneus* (Burchell, 1822) were caught from seven different sites along the Orange-Vaal River System and found to be hosts for this argulid. With morphological identification and genetic analysis, parasites collected from these sites were positively identified as *A. japonicus.* The presence of this invasive species in the Orange-Vaal River System was confirmed and this study further adds to the available knowledge on the distribution of this species, including the addition of a possible new larval stage, and successfully links genetic data for both larval and adult stages to morphology.

Lernaea cyprinacea, commonly known as an anchor worm, was also reported from this study. This copepod parasite infests various fish species worldwide and has detrimental effects on fish health. The study provided molecular characterisation of *L. cyprinacea* from multiple localities in South Africa. This study provided the first record of *L. cyprinacea* infestation in South Africa on its type host, *Cyprinus carpio*, Linnaeus, 1758 as well as a new host, *Labeobarbus aeneus* (Burchell, 1822), within the Orange-Vaal River System. *Lernaea cyprinacea* was additionally reported for the first time in locations within the Phongolo Floodplain and the Vaal River, and the introduction of this invasive species in the Incomati System was also noted.

Lastly, the study reports on two ergasilid species from the genus *Neoergasilus*: *N. japonicus* and *Neoergasilus* sp. nov. *Neoergasilus japonicus* is an invasive ergasilid with an almost global distribution. In contrast to numerous reports from other continents, the records

from southern Africa seem to be from unpublished abstracts and dissertations. This study provides the first integrative study of *N. japonicus* from Africa and the southern hemisphere and gives the first record of this species from the African tigerfish host, *Hydrocynus vittatus* Castelnau, 1861. Additionally, the novel *Neoergasilus* species not previously known to science is described from the sharptooth catfish, *C. gariepinus*.

The integration of morphological identification with genetic data confirms the validity of the sequences generated and provides valuable insights into all parasite groups presented in the current study. The sequences generated for 18S, 28S, and COI are a significant contribution to future parasitic Crustacea studies in southern Africa and across other regions. This study provides an update on the distribution range of these native and invasive parasitic crustaceans in the various river systems sampled, which would serve as a base for future biodiversity and evolutionary studies. As all river systems and host species in southern Africa were not reported on, more robust sampling of other systems and from other host species is recommended for further integrative studies and evolutionary significance of these parasites.

Keywords: Freshwater fish parasitology, Incomati System, Integrative taxonomy, Mitochondrial DNA gene, Ribosomal RNA genes, Okavango River and Delta, Orange River, Phongolo River, Vaal River, Zambezi River.

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Figure 6.6: Phylogenetic tree of Ergasilidae copepods based on partial 28S rRNA gene alignments. Newly generated *Neoergasilus japonicus* (Harada, 1930) sequences are bold (highlighted in orange), and other published sequences are highlighted in light green (Czech Republic) and blue (South Korea). Nodal supports are placed next to the corresponding branches for Bayesian Inference (>0.7) and Maximum Likelihood (>68%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: BB – Babice, BD – Boskop Dam, CN – China, CZ – Czech Republic, EG – Egypt, HU – Hungary, HV – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, OR – Olifants River, RH – Rohlik, ZA – South Africa.

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Bayesian Inference (>0.7) and Maximum Likelihood (>68%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: AR – Argentina, BD – Boskop Dam, CN – China, KR – South Korea, NI – Nicaragua, OR – Olifants River, US – United States of America, ZA – South Africa.

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– Romania, TW – Takwasa, USA – The United States of America, VR – Vaal River, VRHD – Vaal River Holding Dam, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZR – Zambezi River, ZW – Zimbabwe.

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Chapter 3

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Table 4.3: List of sequences included in the phylogenetic analyses. The taxa in bold are newly generated sequences, all other sequences are from the GenBank databases. *Chonopeltis lisikili* Van As and Van As, 1996 was used as the outgroup for 18S and COI, and *Levisunguis subaequalis* Curran, Overstreet, Collins & Benz, 2014 was used as the outgroup for 28S.

Table 4.4: Measurements for male and female *Argulus japonicus* Thiele, 1900 from selected sites in the current study, from the Orange-Vaal River System in South Africa. Values are given as mean ± standard deviation (with range in parentheses). Abbreviations: %CL of TL – Percentage Carapace Length of Total Length; AbL – Abdomen Length; AbW – Abdomen Width; CL – Carapace Length; CW – Carapace Width; SDi – Sucker Diameter (inner); SDo – Sucker Diameter (outer); SiL – Sinus Length; SthL – Spermathecae Length; SthW – Spermathecae Width; TL – Total Length; TsL – Testes Length; TsW – Testes Width.

Table 4.5: Infestation data of *Argulus japonicus* collected from several hosts and different localities in South Africa. Abbreviations: F – Female; L – Larva; M – Male.

Table 4.6: Comparing the total lengths of all *Argulus japonicus* Thiele, 1900 larval stages from the present study with Tokioka (1936). Abbreviation: L – Larval stage.

Table 4.7: Genetic divergences among aligned 18S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (site localities are included for sequences from the present study). Sequences from the present study in boldface and grey shade. *Chonopeltis lisikili* (MW678625) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; CN – China; F – Female; GD – Gariep Dam; ID – Indonesia; IN – India; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

Table 4.8: Genetic divergences among aligned 28S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (site localities are included for sequences from the present study). Sequences from the present study in boldface and grey shade. *Levisunguis subaequalis* (MN065508) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; CN – China; F – Female; GD – Gariep Dam; ID – Indonesia; IN – India; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

Table 4.9: Genetic divergences among aligned COI sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (site localities are included for

sequences from the present study). Sequences from the present study in boldface and grey shade. *Chonopeltis lisikili* (MW679715) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; E – Egg; F – Female; GD – Gariep Dam; IN – India; JP – Japan; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

Chapter 5

Table 5.1: List of primers used for DNA amplification of South African *Lernaea cyprinacea* Linnaeus, 1758 specimens, with references. Abbreviations: BP – Barberspan; LSR – Lower Sabie River; NP – Nyamiti Pan; TW – Takwasa.

Table 5.2: List of sequences used in the alignment with their host information, GenBank accession number, number of bases pairs (B) and references. Taxa in boldface are newly generated sequences from this study, all other sequences are from GenBank. Outgroup sequences are shaded in grey. Sequences less than 300 bases were not included in the alignment. Abbreviations: BP – Barberspan (Harts River); LSR – Lower Sabie River (Kruger National Park); NP – Nyamiti Pan (Phongolo Floodplain); TW – Takwasa, Venterskroon (Vaal River).

Table 5.3: Infestation data of *Lernaea cyprinacea* Linnaeus, 1758 parasitising three fish species from five localities in South Africa. Abbreviations: MA – Mean Abundance, MI – Mean Intensity, N(E) – number of hosts examined, N(I) – number of hosts infected, P – Prevalence, PC – number of parasites collected, SOI – Site(s) of infection.

Table 5.4: Measurements of body and anchoral processes of *Lernaea cyprinacea* Linnaeus, 1758 (Lcy) specimens collected from this study compared to wild and lab populations of *L. cyprinacea* and the synonymised *L. cruciata* (Lcr) specimens reported by Hua et al. (2019). All measurements for Mean ± SD and range are presented in mm.

Table 5.5: Genetic divergence of aligned 18S sequences used in the *Lernaea cyprinacea* alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study). Sequences from the present study are boldface in a grey shade. *Lamproglena orientalis* Markevich, 1936 was used as the outgroup. Abbreviations: AR – Argentina; AU – Australia; BP – Barberspan (Harts River); CN – China; JP – Japan; LSR – Lower Sabie River (Incomati System, Kruger National Park); NP – Nyamiti Pan (Phongolo Floodplain); TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

Table 5.6: Genetic divergence of aligned 28S sequences used in the *Lernaea cyprinacea* Linnaeus, 1758 alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study). Sequences from the present study are boldface in a grey shade. *Lamproglena orientalis* Markevich, 1936 was used as the outgroup. Abbreviations: AR – Argentina; AU – Australia; BP – Barberspan (Harts River); CN – China; JP – Japan; LSR – Lower Sabie River (Incomati System, Kruger National Park); NP – Nyamiti Pan (Phongolo Floodplain); RO – Romania; TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

Table 5.7: Genetic divergence of aligned COI sequences used in the *Lernaea cyprinacea* Linnaeus, 1758 alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study). Sequences from the present study are boldface in a grey shade. *Sinergasilus polycolpus* (Markevich, 1940) was used as the outgroup. Abbreviations: AU – Australia; BP – Barberspan (Harts River); CN – China; TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

Chapter 6

Table 6.1: An updated global distribution checklist for published records of *Neoergasilus japonicus* (Harada, 1930) with most recent host names and families. Data from the present study presented in bold.

Table 6.2: List of primers used for DNA amplification of South African *Neoergasilus japonicus* (Harada, 1930), with references. Codes: P36R and P46K are specimens from the Olifants River (OR), Kruger National Park; P43R is the specimen from Boskop Dam (BD), Mooi River.

Table 6.3: List of sequences included in the phylogenetic analyses. The taxa in bold are newly generated sequences, all other sequences are from the GenBank and Barcode of Life Database (BOLD) databases. *Lernaea cyprinacea* Linnaeus, 1758 (in grey shade) was used as the outgroup.

Table 6.4: Table of infection data of *Neoergasilus japonicus* (Harada, 1930) sampled from two hosts and three localities in South Africa. Abbreviations: KNP – Kruger National Park, MA – mean abundance, MI – mean intensity, MR – Mooi River, N(E) – number of hosts examined, N(I) – number of hosts Infected, OR – Olifants River, PC – number of parasites collected, P – prevalence, SOI – site of infection, ZA – South Africa.

Table 6.5: Spine-setae formula for swimming legs of *Neoergasilus japonicus* (Harada, 1930) from South Africa. Number of spines in Roman numerals, number of setae in Arabic numerals. Spine with asterisks* represents the generic spatulate spine on the second exopodite of leg 1.

Table 6.6: Genetic divergences among aligned 18S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study and The Czech Republic). Sequences from the present study are presented in bold and grey shade, *Lernaea cyprinacea* (MH982195) was used as the outgroup. Abbreviations: AR – Argentina, BB – Babice, BD – Boskop Dam, CN – China, CZ – Czech Republic, HU – Hungary, HV – Hvezda, JZ – Jezu, KNP – Kruger National Park, KR – South Korea, NI – Nicaragua, OR – Olifants River, RH – Rohlik, VR – Vaal River, ZA – South Africa. *Given as *Ergasilus* sp. on GenBank

Table 6.7: Genetic divergences among aligned 28S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study and The Czech Republic). Sequences from the present study are presented in bold and in grey shade, *Lernaea cyprinacea* (MH982204) was used as the outgroup. Abbreviations: BB – Babice, BD – Boskop Dam, CN – China, CZ – Czech Republic,

HU – Hungary, HV – Hvezda, JZ – Jezu, KNP – Kruger National Park, KR – South Korea, NI – Nicaragua, OR – Olifants River, RH – Rohlik, VR – Vaal River, ZA – South Africa.

Table 6.8: Genetic divergences among aligned COI sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/BOLD/Sequence ID, Taxon and Country (sites are included for sequences from the present study and The Czech Republic). Sequences from the present study are bold in a grey shade, *Lernaea cyprinacea* (MH982220) was used as the outgroup. Abbreviations: AR – Argentina, BD – Boskop Dam, CN – China, KNP – Kruger National Park, KR – South Korea, NI – Nicaragua, OR – Olifants River, US – United States, VR – Vaal River, ZA – South Africa.

Table 6.9: Positions of nucleotide substitution in *Neoergasilus japonicus* (Harada, 1930) from this study used in alignments, for partial ribosomal RNA genes (18S and 28S). Information in bold are positions of variability for newly generated sequences.

Table 6.10: Sites of nucleotide variation in the alignment of partial COI *Neoergasilus* sequences highlighting all silent mutations from newly generated sequences and missense mutations in *Neoergasilus* partial COI alignment from the present study. Invertebrate mitochondrion translation was used, and alignment was without stop codons. Divergent nucleotides in bold, amino acids translations are given. Abbreviations: BD – Boskop Dam (from the Mooi River); KR – South Korea; OR – Olifants River (in the Kruger National Park); US – The United States of America.

Chapter 7

Table 7.1: List of primers with references, annealing temperatures, and the number of cycles used for DNA amplification of South African *Neoergasilus* sp. nov. from the Great Fish River, South Africa. Abbreviations: R indicates specimens extracted using the PCRBio rapid kit extraction method; K indicates specimens extracted using the Machery-Nachel tissue kit extraction method.

Table 7.2: List of sequences included in the phylogenetic analyses. The taxa in bold are newly generated sequences, all other sequences are from the GenBank and Barcode of Life Database (BOLD) databases. *Lernaea cyprinacea* Linnaeus, 1758 (in grey shade) was used as the outgroup.

Table 7.3: Spine-setae formula for swimming legs of *Neoergasilus* sp. nov. from South Africa. Number of spines in Roman numerals, number of setae in Arabic numerals. Spine with asterisks* represents the generic spatulate spine.

Table 7.4: Genetic divergences among aligned 18S sequences expressed as percentage similarity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country. Sequences from the present study are presented in bold and in grey shade, *Lernaea cyprinacea* (MH982204) was used as the outgroup. Abbreviations: AR – Argentina, BB – Babice, CN – China, CZ – Czech Republic, FR – The Great Fish River, HU – Hungary, HZ – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, RH – Rohlik, ZA – South Africa.

Table 7.5: Genetic divergences among aligned 28S sequences expressed as percentage

identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country. Sequences from the present study are presented in bold and in grey shade, *Lernaea cyprinacea* (MH982204) was used as the outgroup. Abbreviations: AR – Argentina, BB – Babice, CN – China, CZ – Czech Republic, EG – Egypt, FR – The Great Fish River, HU – Hungary, HZ – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, RH – Rohlik, ZA – South Africa.

Chapter 8

Table 8.1: List of all parasitic Crustacea sequences generated from this study and selected GenBank and Bold sequences included in the phylogenetic analyses. The taxa in boldface are newly generated sequences. *Levisunguis subaequalis* Curran, Overstreet, Collins & Benz, 2014 was used as the outgroup.

CHAPTER 1

Introduction

1.1. Parasitic Crustacea of freshwater fishes

1.1.1. Background

Crustaceans are a very successful group within the phylum Arthropoda, comprising approximately 60,000 species (Nowak et al., 2020). They are known for their high morphological diversity and include the greatest diversity of parasitic forms (Williams & Bunkley-Williams, 2019). Their success in parasitism may be attributed to a variety of morpho-physiological adaptations, such as reduced setae and body segmentation, loss of appendages that are of little or no use to the parasitic lifestyle, vermiform body development, egg adaptation to harsh conditions, and the ability to survive both on and off a host (Tavares-Dias et al., 2015; Smit et al., 2019). Most parasitic crustaceans of freshwater fishes attach to the external surface of their hosts and can be seen without the use of a microscope. They are characterised by their jointed appendages, two pairs of antennae, three pairs of mouth parts, compound eyes, and a chitinous exoskeleton that is shed through moulting (Smit & Hadfield, 2018). In most groups, taxa differentiation is based on the habitus shape, morphology of the antennae and swimming legs, with differences in the shape, size, segmentation, and ornamentation (with spines and setae) of the latter appendages (Meehean, 1940; Fryer, 1968; Roberts, 1970; Abdelhalim et al., 1993).

1.1.2. Pathology of parasitic crustaceans

The effects of parasitic crustaceans on hosts are characterised by their attachment and feeding activities, which cause various pathological changes to the place of attachment (Fryer, 1968; Tsotetsi et al., 2005; Kilian & Avenant-Oldewage, 2013). Some of them have been implicated as vectors of certain viral infections (Overstreet et al, 2009; Hadfield & Smit, 2019). Freshwater parasitic crustaceans may attach themselves to the body surface, gills, fins, or nostrils of the hosts (Fryer, 1968; Paperna, 1991; Hassanin et al., 2010; Neethling & Avenant-Oldewage, 2016; Shinn et al., 2023). They feed on blood or mucous of the tissue and gill epithelium of the hosts, secreting proteolytic enzymes to aid external digestion (Tam & Avenant-Oldewage, 2009; Walker et al., 2011; AmbuAli et al., 2019). The first pair of legs in some crustaceans is crucial in rasping off skin mucous or gill tissue and moving it toward the parasite's mouth (Roberts, 1970). Alternatively, some have adapted mouth tubes and pre-oral

spines to feed on host epithelial tissue and blood (AmbuAli et al., 2019). Some species attach to hosts' surfaces using modified maxillary suckers or claws (Meehean, 1940; Rushton-Mellor & Boxshall, 1994; van As & van As, 1996; van As et al., 2021), embrace the gill filament with the antennae (Oldewage & van As, 1987) inducing a tourniquet effect, or insert the third segment of the antennae into the host gill filament (Thatcher & Boeger, 1983). A few others burrow into host tissues with their cephalothoraxes completely or partially embedded (Marx & Avenant-Oldewage, 1996; Hossain et al., 2018). The cephalothoracic appendages (such as maxillae, maxillipeds, antennae, and modified cephalic structures) also ensure a firm grip on the hosts. Attachment may result in compression of gill tissue (Oldewage & van As, 1987), host immune responses such as increased production of mucous and rodlet cells (Dezfuli et al., 2003; 2010), and terminal necrosis of the filament making hosts susceptible to secondary infections. The compression of blood vessels may cause hypoxia, with high intensities leading to impaired growth and possibly death (Hoffman, 1977; Dezfuli et al., 2010).

1.1.3. Parasitic crustaceans in Africa

In Africa, most of the parasitic crustaceans that pose a threat to aquaculture and fisheries belong to two subclasses: Branchiura Thorell, 1864 and Copepoda Milne-Edwards, 1840 (Smit & Hadfield, 2018; Hadfield, 2019). Representatives of these parasite groups have been reported to cause severe morbidity and mortality in fish farms, leading to a reduction in production yield, and economic losses (Fryer, 1968; Paperna, 1991; Oldewage & Avenant-Oldewage, 1993; Sahoo et al., 2012; Shinn et al., 2023).

Branchiurans are exclusively parasitic, infecting both marine and freshwater fishes (Smit et al., 2019). Some species can act as vectors or alternative hosts for viruses and fish nematodes (Hadfield & Smit, 2019). There is only one family in this subclass, Argulidae Leach, 1819, which comprises four genera namely: *Argulus* Müller O.F., 1785, *Chonopeltis* Thiele, 1900, *Dipteropeltis* Calman, 1912, and *Dolops* (Audouin, 1837).

The subclass Copepoda is the most diverse crustacean group in terms of morphology, number of species, and host utilisation (Smit et al., 2019), having both free-living, symbiotic, and parasitic forms (Hadfield, 2019). The parasitic forms can be associated with the transmission of infectious hematopoietic necrosis, infectious salmon anaemia, and salmon pancreatic necrosis viruses (Overstreet et al., 2009). In African freshwater systems, the families Ergasilidae and Lernaeidae are the most frequently reported parasitic crustaceans (Fryer, 1960; Fryer, 1968; van As & Basson; 1984; Oldewage & Avenant- Oldewage, 1993; Smit et al., 2017; Shinn et al., 2023).

Argulidae Leach, 1819

The family Argulidae was erected by Leach in 1819 (see Hadfield, 2019). It is the only family in the class Branchiura under the Order Arguloida Yamaguti, 1963. Male and female individuals from the family Argulidae are parasitic on both freshwater and marine host fishes (Neethling & Avenant-Oldewage, 2016). The three widely distributed genera in Africa include *Argulus*, *Dolops*, and *Chonopeltis*, with some species having lower host specificity than others (Avenant-Oldewage & Knight, 2008). According to Fryer (1968), *Argulus japonicus* Thiele, 1900, an invasive species in Africa and elsewhere is frequently parasitic on a wide range of fish species.

Generally, members of the family Argulidae have dorsoventrally flattened bodies partly covered by cephalic shields (Fig. 1.1a–d). The head and thorax are fused into a cephalothorax and there are two pairs of antennae (only one in species of *Chonopeltis* (Fig. 1.1c)) (Neethling & Avenant-Oldewage, 2016). Argulids have compound and nauplius eyes (in all, except *Dolops* (Fig. 1.1b)), a proboscis (in *Argulus* (Fig. 1.1a) and *Dipteropeltis* (Fig. 1.1d)), and the first maxilla is modified to claws (*Dolops*) or suckers (the rest of the genera). Respiratory areas are present on the ventral surface of the carapace lobes (except in *Dolops*), and the thorax has four segments, each with a pair of biramous thoracopods (Neethling & Avenant-Oldewage, 2016).

The life cycle of argulids involves copulation on the host, after which the female argulids detaches to lay eggs on a hard substrate in the water column (Fryer, 1956). Upon hatching, the nauplius larvae must find a host to continue their development. Except for the eggs, all developmental stages of argulids are parasitic, with different species having seven to ten stages (Tokioka, 1936; Fryer, 1965; 1961a; Shimura, 1981; Rushton-Mellor & Boxshall, 1994). More information on the developmental stages of *A. japonicus* will be discussed in Chapter 4.

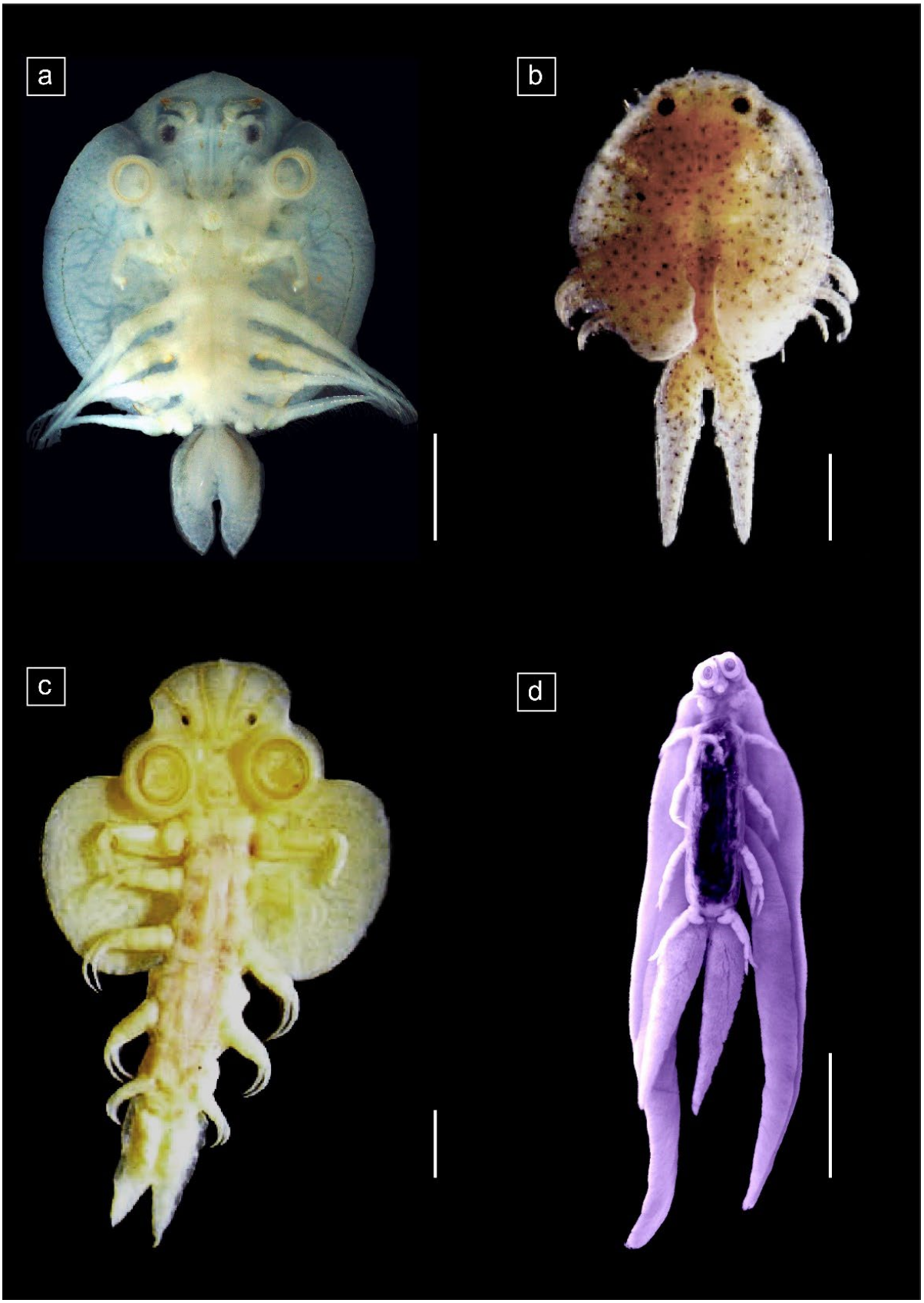


Figure 1.1: Light photomicrographs of representatives of the family Argulidae. (a) *Argulus japonicus* Thiele, 1900; (b) *Dolops geayi* (Bouvier, 1897); (c) *Chonopeltis australis* Boxshall, 1976; (d) *Dipteropeltis hirundo* Calman, 1912. Scale bars: (a, c) 1 mm; (b) 2 mm; (d) 5 mm.

Ergasilidae Burmeister, 1835

The Ergasilidae (Order: Cyclopoida Burmeister, 1835), is one of the largest families and mainly infects bony freshwater fishes, with few species found on brackish and marine hosts (Kabata, 1979). Among the ergasilids, only the adult females are parasitic, while the naupliar and copepodid stages are free-living (Paperna, 1991). This unique periodic parasitic life cycle allows for a broader distribution compared to most other parasitic copepod lineages (Kabata, 1979). According to Walter and Boxshall (2023a) there are currently 30 accepted genera. Thirteen of the 30 genera of Ergasilidae are monotypic. *Ergasilus* is the most speciated among the polytypic genera, with 162 species to date (Walter & Boxshall, 2023b). Other polytypic genera include *Acusicola* Cressey, 1970 (17 species) (Walter & Boxshall, 2023c), *Paraergasilus* Markevich, 1937 (16 species) (Walter & Boxshall, 2023d), *Dermoergasilus* Ho & Do, 1982 (12 species) (Walter & Boxshall, 2023e), *Neoergasilus* Yin, 1956 (nine species) (Walter & Boxshall, 2023f), and *Therodamas* Krøyer, 1863 (eight species) (Walter & Boxshall, 2023g). The remaining genera have between two to six species each.

In Africa, three native ergasilids (*Dermoergasilus*, *Ergasilus*, and *Paraergasilus*) and one non-native Ergasilidae genera (*Neoergasilus*) have been reported (Oldewage & van As, 1988; Shinn et al., 2023; Truter et al., 2023). The morphology of the female (Fig. 1.2a) and male (Fig. 1.2b) ergasilid is characterised by a cyclopiform body with a swelling in the prosome somites of females (Abdelhalim et al., 1993). Females in this family lack maxillipeds but have well-defined antennae, maxillules, mandibles, and maxillae. Ergasilids typically have five pairs of swimming legs, with 3-segmented exopodites and endopodites in legs 1–3, and leg 4 differing in number of segments (see Yin, 1956).

The life cycle of ergasilids typically involves six naupliar stages, five copepodid stages and adults, with mating occurring after the final moult between the fifth copepodid stage and the adult. After copulation, males die, and the females become infective and seek a host; only the mature females are parasitic. Inside the body of the females, the eggs develop and pass onto the membranous egg sacs, extruding from the genital complex. Fertilised eggs are released into the water column where the nauplii hatch and undergo maturation (Wilson 1911; Abdelhalim et al., 1991). Abdelhalim et al. (1991) provided detailed information on the life cycle of *Ergasilus sieboldi* von Nordmann, 1832, including structural development across different developmental stages.

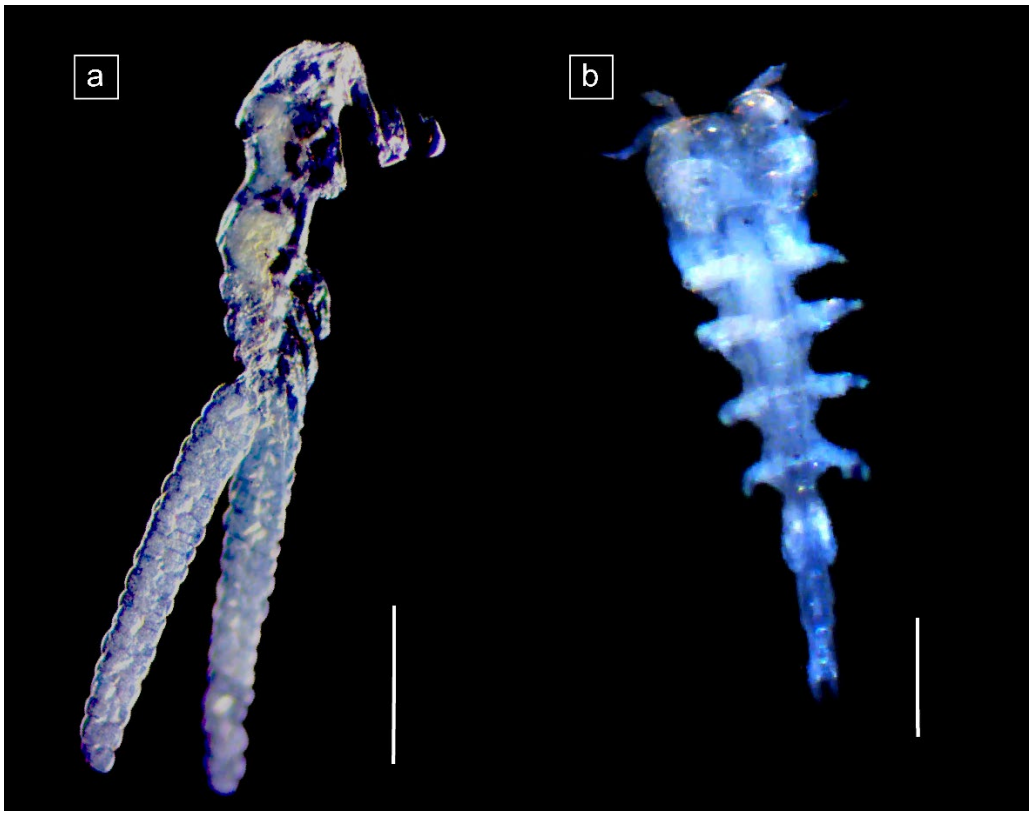


Figure 1.2: Light photomicrographs of female (a) and male (b) representatives of the family Ergasilidae (a) *Ergasilus mirabilis* Oldewage & van As, 1987; (b) *Ergasilus* sp. Scale bars: (a) 0.5 mm; (b) 0.2 mm.

Lernaeidae Cobbold, 1879

The family Lernaeidae Cobbold, 1879 (Cyclopoida) is named after the genus *Lernaea*, which was first described by Linnaeus in 1758, based on specimens collected from the common carp (*Cyprinus carpio*) in Sweden (see Hadfield, 2019). The taxonomy of this family has, over time, been revised and these revisions were based on morphological and molecular data (Fryer, 1956; Robinson & Avenant-Oldewage, 1996; Hua et al., 2019; Zhu et al., 2021). Ho (1998) provided an extensive cladistic analysis of the Lernaeidae, revealing two monophyletic lineages: the subfamilies Lernaeinae and Lamprogleninae, each consisting of seven genera. The biogeography and distribution of the Lernaeidae have also been studied, with Ho (1998) reporting that the descendants of the lernaeid lineage were more widely distributed than those of the lamproglenid lineage, which is confined to Asia and Africa. Currently, there are 19 genera in the family Lernaeidae (Walter & Boxshall, 2023h).

The overall distinguishing morphological features of members of this family include a sharply demarcated thorax from the abdomen, with an abdomen that is narrower than the thorax (Fryer, 1961b; 1968). The egg strings are attached laterally or dorsolateral to the genital double somite (Fryer, 1961b). The antennules (first antennae) are slender and elongate or short and stout, sometimes prehensile, and the antennae (second antennae) are uniramous

and sometimes prehensile (Ibraheem & Izawa, 2000). Some species have adapted protrusions on the cephalothorax to aid in their attachment or grip to host tissues (*Lamproglena* (Fig. 1.3a)) (van As & van As, 2007), while others have modified holdfast structures, such as anchors (*Lernaea* (Fig. 1.3b)) (Robinson & Avenant-Oldewage, 1996).

This parasitic copepod has a direct life cycle consisting of eggs hatching into nauplius larvae and copepodites before maturing into adults (Hossain et al., 2018). There are three naupliar stages that are typically six-legged and non-parasitic (Hossain et al., 2018). In favourable conditions and upon attachment to a host, these nauplii will undergo five moults into different copepodite stages (Hossain et al., 2018). Unlike some other copepod taxa, the copepodites of *L. cyprinacea* are reported as parasitic and are usually found attached to the host gills, but not embedded in the tissue (Hossain et al., 2018). At the fourth copepodite stage, the parasites become sexually mature. The males fertilise the eggs of the females, detach from the host and die. In a single year, female *L. cyprinacea* may have three to four reproductive cycles of about 250 to 340 eggs per cycle (Hossain et al., 2018). Within twenty-four hours after the female *L. cyprinacea* is embedded in the host's tissue, females mature and begin to release eggs from their egg strings.

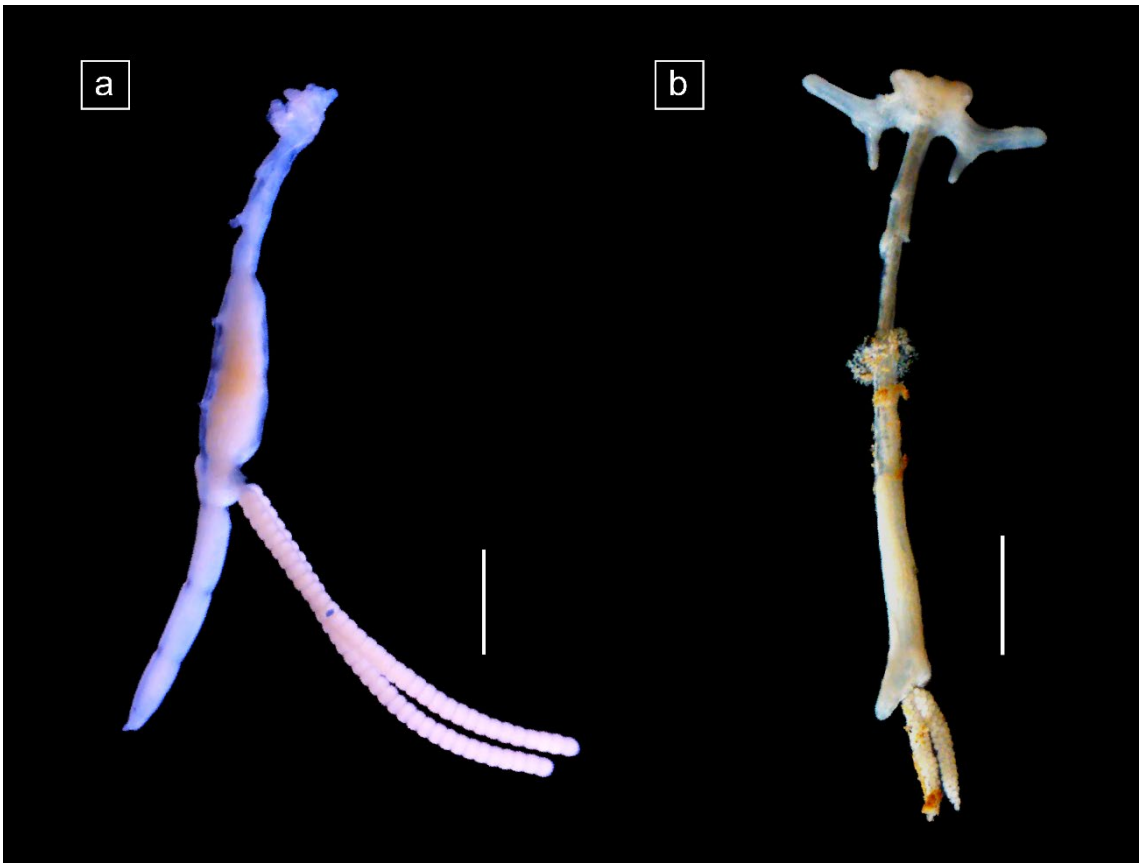


Figure 1.3: Light photomicrographs of representatives of the family Lernaeidae Cobbold, 1879 (a) *Lamproglena clariae* Fryer, 1956; (b) *Lernaea cyprinacea* Linnaeus, 1758. Scale bars: (a) 1 mm; (b) 2 mm.

1.1.4. Aquatic alien and invasive species biodiversity in southern Africa

The introduction of alien fish species for angling and sports fishing, biocontrol, and aquaculture, as well as habitat modification and pollution, have had a significant impact on the biodiversity of freshwater species in southern Africa (Ellender et al., 2014). These activities have led to the co- introduction of parasite fauna, predation on native species, competition for food and space, and colonisation of water bodies by invasive species (Ellender & Weyl, 2014; Ellender et al., 2014; Smit et al., 2017; Weyl et al., 2020; Truter et al., 2023).

Over the years, updated accounts of introduced fishes (and parasite species) in Africa have been given in detail by Bruton and Merron (1985), De Moor and Bruton (1988), Smit et al. (2017), and Weyl et al. (2020). This information included possible mechanisms responsible for the introduction of fishes, current impact levels, as well as distribution records across South Africa. According to Smit et al. (2017) and Weyl et al. (2020), at that time, only two parasitic Crustacea species, *Argulus japonicus* (Argulidae) (introduced from Asia) and *Lernaea cyprinacea* (Lernaeidae) (introduced from Europe), were officially reported as invasive in South Africa.

1.2. Problem statement and hypotheses of the study

Only 14% of the current 100,000–110,000 species of aquatic arthropods are crustaceans (Thorp & Rogers, 2015). These authors stated that there is a significant underrepresentation of their diversity due to limited taxonomical studies focused on these crustaceans, and limited research in several geographic regions, especially the tropics. Furthermore, the decline in taxonomists and scarcity of funds for museum expeditions and biodiversity research has had a significant impact on diversity research (Thorp & Rogers, 2015), with some regions having been more impacted compared to others.

Over the years, there have been several reports on parasitic crustaceans (native and invasive) in Africa (Kruger et al., 1983; Oldewage & van As, 1988; Douëllou & Erlwanger, 1994; Avenant-Oldewage, 2001; Barson et al., 2008; Welicky et al., 2017; Smit et al., 2017; Chakona et al., 2019; Gobbin et al., 2021; Malki et al., 2021; Shinn et al., 2023), with only a few of these reporting genetic data (Welicky et al., 2017; Chakona et al., 2019). The recent reports of infections, from new localities and first-time host mentions across other continents (Batool et al., 2018; Zhu et al., 2021; Alekseev et al., 2021; Amriana et al., 2021; Kvach et al., 2021; Nagasawa, 2021; Waicheim et al., 2021), may raise concerns for biodiversity and conservation researchers about the adequacy of sampling and reporting in Africa, particularly southern Africa. Recent reports within southern Africa suggest that the distribution range of native and invasive parasite species is continuously increasing (see Truter et al., 2023), so continued sampling and reporting of these fish parasites are necessary for proper biodiversity

assessments to be achieved.

Parasitic crustaceans are often neglected since trained and experienced taxonomists are typically needed for their identification, as larval forms are often morphologically different to adults, and they have a high level of morphological and ecological diversity (Radulovici et al., 2009; Marco-Herrero et al., 2021). The use of DNA barcoding can, however, aid in identification when these problems arise. Furthermore, while some parasite taxa may be morphologically distinct, molecular techniques have been found to provide valuable information on parasite identification, origin, and evolution. The incorporation of molecular techniques in parasitological studies in Africa (especially southern Africa) has received more attention in the past decade, albeit slowly compared to the rest of the world (possibly due to insufficient funds, technical knowledge, or a lack of interest). This scarcity of molecular data may also be due to difficulties in isolating DNA from the parasites, which also could be linked to the type of preservatives used for specimens and how long specimens have been held in collections. There is generally scarce genetic data (for ribosomal RNA genes and mitochondrial DNA genes) within parasitic Crustacea, much less so from southern Africa. Some of the parasitic Crustacea genera do not have any genetic information for the mitochondrial gene region (COI) globally, and often available genetic data are not associated with peer reviewed publications.

It was therefore hypothesised that with more sampling effort and spread, the knowledge of invasive parasitic Crustacea species in southern Africa will increase and there would be new host and locality records, with the possibility of new species discoveries. Furthermore, with the use of integrated taxonomy (using improved morphological techniques such as differential interference contrast (DIC) microscopy and scanning electron microscopy (SEM), as well as molecular techniques), novel information on known parasites would be assembled.

1.3. General aims and objectives

In collaboration with the Aquatic Research Group of the University of the Free State, Bloemfontein, and several ongoing parasitology projects of the Water Research Group of the North-West University, Potchefstroom, parasites belonging to three families (Argulidae, Ergasilidae, and Lernaeidae) were collected. The overarching aim of this study was to increase the knowledge of the biodiversity of native and invasive parasitic Crustacea in southern Africa, with the application of an integrative approach to parasite characterisation. The specific aims were as follows:

Aim 1: To provide an integrative approach to characterisation of *Ergasilus mirabilis* Oldewage & van As, 1987, from southern Africa (almost four decades after its description)

Aim 2: To carry out an integrative approach for characterisation of three African *Lamproglena* von Nordmann, 1832 species, providing novel sequences for accurately identified specimens and further highlighting distinct differences among these three *Lamproglena* species.

Aim 3: To report new locality records for *Argulus japonicus* Thiele 1900; providing the first molecular data for this species from sub-Saharan Africa, as well as to propose an additional *A. japonicus* larval stage (along with morphological descriptive notes of the other developmental stages) as a contribution to the already existing and reliable database.

Aim 4: To molecularly characterise *Lernaea cyprinacea* Linnaeus 1758 specimens collected from several regions in South Africa, document the variability in the lengths and shapes of the anchor processes; and report new distributions and host records in South Africa.

Aim 5: To provide the first integrative study of *Neoergasilus japonicus* (Harada, 1930) from Africa and the southern hemisphere, giving the first record of *N. japonicus* from an alestid host, the tigerfish, *Hydrocynus vittatus* Castelnau, 1861 as well as new locality records from southern Africa.

Aim 6: To characterise a species morphologically and molecularly from the genus *Neoergasilus* previously not known to science.

The following objectives were set out in order to achieve the above six aims of this study:

- To collect, classify, and compare recovered parasitic crustaceans.
- To revise the known species of parasitic Crustacea collected.
- To describe any new species encountered.
- To provide detailed information on the hosts and distribution.
- To use differential interference contrast (DIC) microscopy and scanning electron microscopy (SEM) for morphological characterisation.
- To molecularly characterise each species with partial 18S, 28S, and COI genes as the molecular markers.
- To complete phylogenetic analyses on each of the different parasitic genera collected.

1.4. Thesis layout

This study focused on five genera of parasitic Crustacea: *Argulus* Müller, 1785 (Branchiura: Argulidae); *Ergasilus* von Nordmann, 1832 and *Neoergasilus* Yin, 1956 (Copepoda: Ergasilidae); *Lamproglena* von Nordmann, 1832; and *Lernaea* Linnaeus, 1758 (Copepoda: Lernaeidae).

Following the current introductory chapter (1), the thesis presents six result chapters largely grouped as native and invasive parasite genera, followed by the description of a new

species, and concludes with a general discussion, conclusion, and recommendations chapter. All references used in this thesis are listed in the respective chapters. A breakdown of the chapters is given below.

Parasites collected in this study that are native to southern Africa will be discussed in Chapter 2 (*Ergasilus mirabilis*) and Chapter 3 (three *Lamproglena* species). The next three chapters will deal with invasive parasite species namely: *Argulus japonicus* (Chapter 4), *Lernaea cyprinacea* (Chapter 5), and *Neoergasilus japonicus* (Chapter 6). The final results chapter is on the description of a new species *Neoergasilus* sp. nov. (Chapter 7). This thesis ends with a general discussion, conclusion and recommendations in Chapter 8, along with all appendices for the respective chapters.

1.5. Project outputs

1.5.1. Publications

***Fikiye, P.P.,** Smit, N. J., Van As, L. L., Truter, M., & Hadfield, K. A. (2023). Integrative Morphological and genetic characterisation of the fish parasitic copepod *Ergasilus mirabilis* Oldewage & van As, 1987: Insights into host specificity and distribution in southern Africa. *Diversity*, 15(9): 965. https://doi.org/10.3390/d15090965

*Fikiye P. P. is the same person as Ayawei P. P.

1.5.2. Conference contributions

Some of the research compiled throughout the duration of this project has been presented at a number of national and international conferences. These conference contributions included both oral and poster presentations and details for each are listed below. Underlined bold text denotes that the author of this thesis presented the work at these conferences.

Fikiye*, P. P., Smit, N. J., Van As, L. L., Truter, M., & Hadfield, K. A. First integrated taxonomy study for characterisation of gill copepod *Ergasilus mirabilis* Oldewage & van As, 1987 (Ergasilidae: Cyclopoida). *51st Annual Conference of the Parasitological Society of Southern Africa (PARSA),* 26 Degrees South, Muldersdrift, South Africa. (September 2023) (Poster presentation).

Ayawei, P.P., Smit, N. J., van As, L.L. & Hadfield, K. A. (2022). An integrated study on parasitic Crustacea infesting selected freshwater fishes from southern Africa. *4th International Congress on Parasites of Wildlife and 50th Annual Conference of the Parasitological Society of Southern Africa (PARSA)*, *Kruger National Park, South Africa*. (September 2022) (Oral presentation).

Ayawei, P.P., Smit, N. J., van As, L.L. & Hadfield, K. A. (2022). Molecular and morphological characterisation of three species of *Lamproglena* Von Nordmann, 1832 from southern Africa. *4th International Congress on Parasites of Wildlife and 50th Annual Conference of the Parasitological Society of Southern Africa (PARSA)*, *Kruger National Park, South Africa*. (September 2022) (Poster presentation).

Ayawei, P.P., Smit, N. J., van As, L.L. & Hadfield, K. A. (2022). Biodiversity, and integrative systematics of parasitic Crustacea among selected commercial fish species in southern Africa. *59th Southern African Society of Aquatic Scientists (SASAqS) Conference*. (June 2022) (Oral presentation).

Ayawei, P.P., Smit, N. J., van As, L.L. & Hadfield, K. A. (2021). First molecular characterisation of *Lamproglena* von Nordmann, 1832 in Africa. *Southern African Society of Aquatic Scientists (SASAqS) Congress 2021*. (November 2021) (Poster - online).

Ayawei, P.P., Smit, N. J., van As, L.L. & Hadfield, K. A. (2021). Morphological and molecular characterization of *Argulus japonicus* from the Orange-Vaal River System. *Virtual 49th Annual Parasitological Society of southern Africa (PARSA) Conference*. (September 2021) (Oral - online).

*Fikiye P. P. is the same person as Ayawei P. P.

1.5.3. Annual institutional progress symposia

Ayawei, P.P., Smit, N. J. & Hadfield, K. A. (2019). Biodiversity of parasitic Crustacea infesting *Clarias gariepinus* and their role as potential bioindicators of aquatic health. *2nd Unit for Environmental Sciences and Management (UESM) Prestigious PhD Conference, North-West University, Potchefstroom, South Africa*. (August 2019) (Poster presentation).

1.5.4. Awards

a. Awardee of the best senior category poster presentation at the 51st Annual Conference of the Parasitological Society of Southern Africa (PARSA), (September 2023) entitled “First integrated taxonomy study for characterisation of gill copepod *Ergasilus mirabilis* Oldewage & van As, 1987 (Ergasilidae: Cyclopoida)”.

b. Awardee of the top-rated poster presentation at the Southern African Society of Aquatic Scientists (SASAqS) Congress 2021 (November 2021) (Virtual Conference) entitled “First molecular characterisation of *Lamproglena* von Nordmann, 1832 in Africa”.

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CHAPTER 2

First integrated characterisation of the gill copepod *Ergasilus mirabilis* Oldewage & van As, 1987 (Ergasilidae: Cyclopoida), using morphological and molecular techniques

2.1. Abstract

Ergasilids are external parasites typically found on the gills and fins of their fish hosts. They represent an economically important parasitic Crustacea group and some representatives of the genus *Ergasilus* have been reported as detrimental to fish populations. In Africa, there are 19 reported *Ergasilus* species. Of those, *Ergasilus mirabilis* Oldewage & van As, 1987 is one of the least host-specific species with a wide distribution range in southern Africa. Following its description in 1987, the most recent record of this parasite was in 1994 by Douëllou and Erlwanger, from Lake Kariba, Zimbabwe. As with most other species of *Ergasilus*, there are no genetic data available to support the morphology of this species. Forming part of an ongoing project, 157 *Clarias gariepinus* (Burchell, 1822) were caught in several localities around South Africa and Zambia. Adult female copepods were collected from two localities in South Africa (the Kushokwe Pan in the Phongolo Floodplain and the Vaal River) and from the Zambezi River, in Zambia. Fish were dissected and gills screened following standard techniques. Parasites were morphologically examined using light and scanning electron microscopy and identified as *E. mirabilis*. Molecular analyses were carried out using partial ribosomal RNAs genes (18S and 28S) and partial mitochondrial DNA (COI) gene. This study is, therefore, the first integrative study of this African species, *E. mirabilis,* and provides supporting genetic data for partial 18S, 28S, and COI gene regions. Additionally, new distribution records of *E. mirabilis* in the Phongolo Floodplain and the Vaal River (South Africa) and the upper Zambezi River System (Zambia) are given, and the species from Africa are also revised (including hosts, distribution, and genetic information).

2.2. Introduction

Species from the genus *Ergasilus* are parasitic copepods in the family Ergasilidae (Cyclopoida: Copepoda) and are a widely distributed group of parasites infesting African freshwater fishes (Fryer, 1968; Oldewage & van As, 1988a, b; Avenant-Oldewage & Oldewage, 1993; Oldewage & Avenant-Oldewage, 1993). They are typically found attached to a host's gills, fins, and occasionally in the urinary bladder (Fryer, 1968; Rosim

et al., 2013). The family comprises 30 accepted genera and one genus inquirendum, *Macrobrachinus* Hesse, 1871 (Walter & Boxshall, 2023a). Due to their economic importance in the global aquaculture and fisheries industry, and their significance in biodiversity studies, there have been numerous publications regarding the taxonomy, feeding, pathology, and lifecycle of ergasilids over the years (Einszporn, 1965; Abdelhalim et al., 1991, 1993; Kim, 2004; Piasecki et al., 2004; Kilian & Avenant-Oldewage, 2013; Boxshall, 2016; Jiménez-Garciá & Suárez-Morales, 2017; Varella et al., 2019; Waicheim et al., 2021).

Members of the Ergasilidae are characterised by the loss of the maxilliped in females, and well-defined antennae, mandibles, maxillules, and maxillae (Kabata, 1979). The general body morphology of a typical ergasilid, whether male or female, is cyclopiform with a swelling in the prosome somites of females (Kabata, 1979; Abdelhalim et al., 1993). These parasites possess five pairs of swimming legs comprising segmented exopods and endopods in legs 1–4.

The first genus to be described from this family was *Ergasilus* von Nordmann, 1832 with the description of two species: *Ergasilus gibbus* von Nordmann, 1832 and *Ergasilus sieboldi* von Nordmann, 1832. Of the 30 accepted genera in this family, four are known from Africa, *viz. Dermoergasilus* Ho & Do, 1982 (brackish and marine); *Ergasilus* (freshwater, brackish and marine); *Neoergasilus* Yin, 1956 (freshwater) and *Paraergasilus* Markewitsch, 1937 (freshwater) (Fryer, 1968; Oldewage & van As, 1988 a, b; Oldewage & Avenant-Oldewage,1993; Berrouk et al., 2018, 2020). The genus *Ergasilus* is the most speciose in Africa. There are currently 162 accepted *Ergasilus* species (Walter & Boxshall, 2023b), 16 of which have been recorded in Africa by 2022, namely: *Ergasilus brevimanus* (Sars , 1909); *Ergasilus cunningtoni* Capart, 1944; *Ergasilus egyptiacus* Abdel-Hady, Bayoumy & Osman, 2008; *Ergasilus flaccidus* Fryer, 1965; *Ergasilus ilani* Oldewage & van As, 1988; *Ergasilus inflatipes* Cressey & Collette, 1970; *Ergasilus kandti* van Douwe, 1912; *Ergasilus lamellifer* Fryer, 1965; *Ergasilus latus* Fryer, 1961; *Ergasilus lizae* Krøyer, 1863; *Ergasilus macrodactylogyrus* (Sars, 1909); *Ergasilus megacheir* (Sars, 1909); *Ergasilus mirabilis* Oldewage & van As, 1987; *Ergasilus nodosus* Wilson, 1928; *Ergasilus sarsi* Capart, 1944; and *Ergasilus sieboldi* Krøyer, 1863. Most recently, three new species have been described from Lake Tanganyika, Burundi, namely: *Ergasilus carparti* Míč, Řehulková & Seifertová, 2023; *Ergasilus parasarsi* Míč, Řehulková & Seifertová, 2023; and *Ergasilus parvus* Míč, Řehulková & Seifertová, 2023 (see Míč et al., 2023), bringing the total to 19 *Eragasilus* species in Africa. Updated information on the hosts, distribution, and available molecular data for all of these African *Ergasilus* species is summarised in Table 2.1. One freshwater species, *E. mirabilis*, is reported to parasitise a wide range of hosts and is widely distributed in southern Africa. Following its description in 1987, there had been only four subsequent reports of this parasite from southern Africa, the latest being in 1994 by Douëllou and Erlwanger (1994).

As with several other *Ergasilus* species, there is no genetic information available for *E. mirabilis*. Globally, only 17 of the 162 species of *Ergasilus* have genetic data available on GenBank. From the 19 species found in Africa, there are five submissions for 18S and five for 28S (from five host species) from sub-Saharan Africa (Burundi) (Míč et al., 2023), and a single submission for partial 28S from northern Africa (Egypt) (Abdel-Radi et al., 2022). Two other submissions of *E. sieboldi* are available for partial 18S and 28S genes from the Czech Republic (Kvach et al., 2021), and an unpublished submission of *E. lizae* from Canada in the Barcode of Life Database (BOLD, 2023).

Almost four decades after its discovery, the current study thus aims to provide an integrative characterisation of *E. mirabilis,* from southern Africa, using morphological and molecular techniques, with partial 18S, 28S, and COI genes as the molecular markers.

2.3. Materials and methods

2.3.1. Sampling

As part of a larger parasitology project, a total of 157 *Clarias gariepinus* specimens were caught from several localities in southern Africa (Fig. 2.1) between 2018 and 2020, using various sampling methods: rod and reel, baited longlines, gill nets, and fyke nets (see Truter et al., 2023). This study received necessary ethical clearance (Ethics No. NWU-00159-18-A5) and permits: Ezemvelo KZN Wildlife (KwaZulu-Natal, permit Nos. OP 1075/2017, OP 1582/2018), Department of Rural, Environmental and Agricultural Development (North West, permit no. HO 20/02/18-057 NW), the Department of Economic, Small Business Development, Tourism and Environmental Affairs (DESTEA, Free State, permit no. JM 4066/2018), the Department of Economic Development, Environmental Affairs and Tourism (Eastern Cape, permit no. CRO 20/18CR, CRO 22/18CR) and CapeNature (Western Cape, permit no. CN44-31-6790), as well as permission for joint research in the Upper Zambezi Basin, Zambia. Host nomenclature is from FishBase (Froese & Pauly, 2023) (www.fishbase.org), and host and parasite authorities (except for the synonymy) are not included in the references.

Table 2.1: Updated information for all 19 African *Ergasilus* species with information on host species, host families, distribution, and available GenBank data. Information from the present study is represented in bold. Abbreviations: TH – Type Host, TLOC – Type Locality.

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
Ergasilus brevimanus (Sars, 1909)	**TH:** Unknown	**TLOC:** Mbete, south shore of Lake Tanganyika		Freshwater		Sars (1909)
		Lake Malawi		Freshwater		Sars (1909)
Ergasilus caparti Míč, Řehulková & Seifertová, 2023	**TH:** *Neolamprologus brichardi* (Poll, 1974)	**TLOC:** Magara, Lake Tanganyika, Burundi	Cichlidae	Freshwater		Míč et al. (2023)
	Eretmodus marksmithi Burgess, 2012; *Lamprologus callipterus* Boulenger, 1906; *Neolamprologus mondabu* (Boulenger, 1906); *Perissodus microlepis* Boulenger, 1898; *Spathodus erythrodon* Boulenger, 1900	Burundi: Mukuruka, Mvugo, Nyaruhongoka (Lake Tanganyika)	Cichlidae	Freshwater	OQ407469 (18S); OQ407474 (28S)	Míč et al. (2023)
Ergasilus cunningtoni Capart, 1944	**TH:** *Campylomormyrus elephas* (Boulenger, 1898)	**TLOC:** Lake Tumba, Ubangi River, Democratic Republic of the Congo	Mormyridae	Freshwater		Capart (1944)
	Cyphomyrus psittacus (Boulenger, 1897); Distichodus atroventralis Boulenger, 1898; *Marcusenius greshoffii* (Schilthuis, 1891); *Marcusenius moorii* (Günther, 1867); *Mormyrops nigricans* Boulenger, 1899; *Petrocephalus grandoculis* Boulenger, 1916; *Pollimyrus isidori* (Valenciennes, 1847); *Pterochromis congicus* (Boulenger, 1897), *Schilbe laticeps* (Boulenger, 1899); *Schilbe tumbanus* (Pellegrin, 1926), *Synodontis nigriventris* David, 1936; *Tylochromis microdon* Regan, 1920	Democratic Republic of the Congo: Lake Tumba, Ubangi River, Ikela, Tshuapa River & Mokombe River	Cichlidae; Distichodontidae; Mochokidae; Mormyridae; Schilbeidae	Freshwater		Fryer (1964, 1967)
	Brycinus leuciscus (Günther, 1867); *Brycinus nurse* (Rüppell, 1832); *Distichodus rostratus* Günther, 1864; *Pellonula leonensis* Boulenger, 1916	Ghana: Lake Volta	Alestidae; Distichodontidae; Dorosomatidae	Freshwater		Paperna (1969)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	Brycinus nurse (Rüppell, 1832); *Enteromius macrops* (Boulenger, 1911); *Hydrocynus vittatus* Castelnau, 1861; *Mormyrops anguilloides* (Linnaeus, 1758); *Mormyrus macrophthalmus* Günther, 1866; *Raiamas senegalensis* (Steindachner, 1870)	Nigeria: Galma River, Zaria	Alestidae; Cyprinidae; Mormyridae	Freshwater		Shotter (1977)
	Chrysichthys auratus (Geoffroy Saint-Hilaire, 1809)	Nigeria: Tiga Lake, Kano	Claroteidae	Freshwater		Ndifon & Jimeta (1990)
Ergasilus egyptiacus Abdel-Hady, Bayoumy & Osman, 2008	**TH:** *Coptodon zillii* (Gervais, 1848)	**TLOC:** Lake Temsah	Cichlidae	Freshwater		Abdel-Hady et al. (2008)
Ergasilus flaccidus Fryer, 1965	**TH:** *Oreochromis tanganicae* (Günther, 1894)	**TLOC:** Lake Tanganyika	Cichlidae	Freshwater		Fryer (1965)
Ergasilus ilani Oldewage & van As, 1988	**TH:** *Mugil cephalus* Linnaeus, 1758	**TLOC:** Mgobezeleni Estuary, Sodwana Bay, South Africa	Mugilidae	Brackish; Freshwater		Oldewage & van As (1988a)
	Mugil cephalus Linnaeus, 1758	South Africa: Kowie River Estuary, Eastern Cape	Mugilidae	Brackish; Freshwater		Oldewage & van As (1988b)
	Chelon richardsonii (Smith, 1846)	South Africa: Berg River and Verlorevlei River, Western Cape	Mugilidae	Freashwater		Oldewage & van As (1988b)
Ergasilus inflatipes Cressey in Cressey & Collette, 1970	**TH:** *Strongylura senegalensis* (Valenciennes, 1864)	**TLOC:** Volta River, Ghana	Belonidae	Freshwater		Cressey & Collette (1970)
	Strongylura senegalensis (Valenciennes, 1864)	Ivory Coast: Ébrié Lagoon	Belonidae	Brackish; Marine		Cressey & Collette (1970)
Ergasilus kandti van Douwe, 1912	**TH:** Unknown	**TLOC:** Lake Albert		Freshwater		van Douwe (1912)
	Pseudosimochromis curvifrons (Poll, 1942)	Lake Tanganyika	Cichlidae	Freshwater		Capart (1944)
	Lates niloticus (Linnaeus, 1758)	Mali: Niger River	Latidae			Capart (1956)
	Pterochromis congicus (Boulenger, 1897)	Democratic Republic of the Congo: Lake Tumba, Ubangi River	Cichlidae	Freshwater		Fryer (1964)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	Limnotilapia dardennii (Boulenger, 1899); *Lamprologus lemairii* Boulenger, 1899; *Lates niloticus* (Linnaeus, 1758) *Plecodus paradoxus* Boulenger, 1898; *Oreochromis tanganicae* (Günther, 1894)	Lake Albert & Lake Tanganyika	Cichlidae; Latidae	Freshwater		Fryer (1965)
	Tylochromis bangwelensis Regan, 1920; *Tylochromis mylodon* Regan, 1920;	Democratic Republic of the Congo: Lake Mweru and Luapula River	Cichlidae	Freshwater		Fryer (1967)
	Tylochromis polylepis (Boulenger, 1900)	Tanzania: Malagarasi Delta	Cichlidae	Freshwater		Fryer (1967)
	Citharinus citharus (Geoffroy St. Hilaire, 1809); *Synodontis membranaceus* (Geoffroy Saint-Hilaire, 1809); *Lates niloticus* (Linnaeus, 1758); *Schilbe intermedius* Rüppell, 1832	Ghana: Lake Volta	Citharinidae; Mochokidae; Latidae; Schilbeidae	Freshwater		Paperna (1969)
	Bagrus bajad (Forsskål, 1775); *Lates niloticus* (Linnaeus, 1758)	Lake Albert	Bagridae	Freshwater		Thurston (1970)
	Lates niloticus (Linnaeus, 1758)	Egypt: Lake Nasser	Latidae	Freshwater		Hamouda et al. (2018)
Ergasilus lamellifer Fryer, 1961	**TH:** Various *Haplochromis* species	**TLOC:** Lake Victoria and the Victoria Nile	Cichlidae	Freshwater		Fryer (1961)
	Parailia pellucida (Boulenger, 1901)	Ghana: Lake Volta	Schilbeidae	Freshwater		Paperna (1969)
	Astatoreochromis alluaudi Pellegrin, 1904; *Haplochromis guiarti* (Pellegrin, 1904); *Haplochromis longirostris* (Hilgendorf, 1888); *Haplochromis nuchisquamulatus* (Hilgendorf, 1888); *Haplochromis obesus* (Boulenger, 1906); *Haplochromis obliquidens* (Hilgendorf, 1888); *Haplochromis retrodens* (Hilgendorf, 1888); *Haplochromis bicolor* Boulenger, 1906; *Haplochromis degeni* (Boulenger, 1906)	Lake Victoria and the Victoria Nile	Cichlidae	Freshwater		Thurston (1970)
	Haplochromis spp.; *Haplochromis heusinkveldi* Witte & Witte-Maas, 1987;	Lake Victoria	Cichlidae	Freshwater		Witte & van Oijen (1990)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	Haplochromis hiatus Hoogerhoud & Witte, 1981; *Haplochromis iris* Hoogerhoud & Witte, 1981; *Haplochromis macrognathus* Regan, 1922; *Haplochromis ptistes* Greenwood & Barel, 1978; *Haplochromis pyrrhocephalus* Witte & Witte-Maas, 1987; *Haplochromis teegelaari* Greenwood & Barel, 1978					
	Haplochromis nyererei Witte-Maas & Witte, 1985	Tanzania: Makobe Island in the western Speke Gulf, Lake Victoria	Cichlidae	Freshwater		Maan et al. (2006)
	Haplochromis nyererei Witte-Maas & Witte, 1985; *Haplochromis pundamilia* (Seehausen & Bouton, 1998)	Makobe Island, south-eastern Lake Victoria	Cichlidae	Freshwater		Maan et al. (2008)
	Haplochromis chilotes (Boulenger, 1911); *Haplochromis mbipi* (Lippitsch & Bouton, 1998); *Haplochromis nyererei* Witte-Maas & Witte, 1985; *Haplochromis omnicaeruleus* (Seehausen & Bouton, 1998); *Haplochromis pundamilia* (Seehausen & Bouton, 1998); *Haplochromis rufocaudalis* (Seehausen & Bouton, 1998); *Haplochromis sauvagei* (Pfeffer, 1896); *Neochromis* sp.; *Pundamilia* sp.	Tanzania: Lake Victoria	Cichlidae	Freshwater		Karvonen et al. (2018); Gobbin et al. (2021)
	Clarias gariepinus (Burchell, 1822); *Haplochromis* spp.; *Oreochromis esculentus* (Graham, 1928); *Protopterus aethiopicus* Heckel, 1851	Kenya: Lake Kanyaboli	Cichlidae; Clariidae; Protopteridae	Freshwater		Mwamburi et al. (2018)
	Oreochromis niloticus (Linnaeus, 1758)	Kenya: Lake Victoria	Cichlidae	Freshwater		Mwainge et al. (2021); Outa et al. (2021)
Ergasilus latus Fryer, 1960	**TH:** *Oreochromis niloticus* (Linnaeus, 1758); *Sarotherodon galilaeus* (Linnaeus, 1758)	**TLOC:** Lake Turkana, Kenya	Cichlidae	Freshwater		Fryer (1960)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	Sarotherodon nigripinnis (Guichenot, 1861); *Pelmatolapia cabrae* (Boulenger, 1899)	Kitona, Moanda and Bulambemba, near the Congo River mouth; Nile River	Cichlidae	Brackish; Freshwater		Fryer (1963, 1967)
	Coptodon guineensis (Günther, 1862); *Coptodon zillii* (Gervais, 1848); *Oreochromis niloticus* (Linnaeus, 1758); *Sarotherodon melanotheron* Rüppell, 1852	Ghana: Volta Basin and Peshi Lagoon	Cichlidae	Brackish; Freshwater		Paperna (1969)
	Auchenoglanis occidentalis (Valenciennes, 1840); *Oreochromis niloticus* (Linnaeus, 1758); *Sarotherodon galilaeus* (Linnaeus, 1758); *Schilbe mystus* (Linnaeus, 1758); *Coptodon zillii* (Gervais, 1848)	Nigeria: Galma River	Claroteidae; Cichlidae; Schilbeidae	Freshwater		Shotter (1977)
	Chrysichthys nigrodigitatus (Lacepède, 1803)	Nigeria: Cross River estuary	Claroteidae	Brackish		Obiekezie et al. (1988)
	Mugil cephalus Linnaeus, 1758; *Neochelon falcipinnis* (Valenciennes, 1 836)	Republic of Benin: Ganvie, Djdje and Zogbo, Lake Nokoue Lagoon	Mugilidae	Brackish		Aladetohun et al. (2013a)
	Mugil cephalus Linnaeus, 1758; *Neochelon falcipinnis* (Valenciennes, 1 836)	Nigeria: Makoko, Mcquin and University of Lagos lagoon	Mugilidae	Brackish		Aladetohun et al. (2013b)
	Sarotherodon melanotheron Rüppell, 1 852	Ghana: Oyibi, Fosu, Apabaka, Kpeshie, Sakumo, and Keta Lagoons	Cichlidae	Brackish		Rokicki et al. (2016)
	Lates niloticus (Linnaeus, 1758)	Egypt: Lake Nasser	Latidae	Freshwater		Hamouda et al. (2018)
	Sarotherodon melanotheron Rüppell, 1 852	Côte d'Ivoire: Ebrie Lagoon	Cichlidae	Brackish		Adou et al. (2021)
Ergasilus lizae Krøyer, 1863 **Syn:** *Ergasilus nanus* Beneden, 1870	**TH:** *Mugil liza* Valenciennes, 1836	**TLOC:** New Orleans, USA	Mugilidae	Marine		Krøyer (1863)
	Alosa fallax (Lacepéde, 1803); *Barbus barbus* (Linnaeus, 1758); *Chelon ramada* (Risso, 1827); *Chelon saliens* (Risso, 1810); *Mugil cephalus* Linnaeus, 1758; *Solea solea* (Linnaeus, 1758)	Tunisia: Gulf of Gabès & Lake Ichkeul	Alosidae; Cyprinidae; Mugilidae; Soleidae	Brackish; Marine		Raïbaut et al. (1971)
	Mugil cephalus Linnaeus, 1759	Algeria: Gulf of Annaba, East coast	Mugilidae	Marine		Boualleg et al. (2011)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	Mugil cephalus Linnaeus, 1758; *Neochelon falcipinnis* (Valenciennes, 1836)	Republic of Benin: Ganvie, Djdje and Zogbo, Lake Nokoue Lagoon	Mugilidae	Brackish		Aladetohun et al. (2013a)
	Mugil cephalus Linnaeus, 1758; *Neochelon falcipinnis* (Valenciennes, 1836)	Nigeria: Makoko, Mcquin and University of Lagos lagoon	Mugilidae	Brackish		Aladetohun et al. (2013b)
	Synodontis schall (Bloch & Schneider, 1801)	Nigeria: Nsidung beach, Cross River Estuary	Mochokidae	Brackish		Eyo & Effanga (2018)
	Clarias gariepinus (Burchell, 1822)	Nigeria: Lake Gerio, Yola, Adamawa	Clariidae	Freshwater		Amos et al. (2018)
	Coptodon zillii (Gervais, 1848)	Egypt: Lake Maruit	Cichlidae	Freshwater		Mitwally et al. (2023)
Ergasilus macrodactylogyrus (Sars, 1909)	**TH:** Unknown	**TLOC:** Sumbu, south-western shore of Lake Tanganyika		Freshwater		Sars (1909)
	Brycinus imberi (Peters, 1852); *Haplochromis* spp.; *Lethrinops* spp.; *Tilapia* spp.	Lake Malawi	Alestidae; Cichlidae	Freshwater		Fryer (1956)
	Eretmodus marksmithi Burgess, 2012; *Gnathochromis permaxillaris* (David, 1936); *Lamprologus callipterus* Boulenger, 1906; *Perissodus microlepis* Boulenger, 1898; *Tanganicodus irsacae* Poll, 1950	Burundi: Magara, Mvugo, Nyaruhongoka (Lake Tanganyika)	Cichlidae	Freshwater	OQ407465 (18S) OQ407470 (28S)	Míč et al. (2023)
Ergasilus megacheir (Sars, 1909)	**TH:** Unknown	**TLOC:** Sumbu, south-western shore of Lake Tanganyika		Freshwater		Sars (1909)
	Pseudosimochromis curvifrons (Poll, 1942)	Lake Tanganyika	Cichlidae	Freshwater		Capart (1944)
	Pterochromis congicus (Boulenger, 1877)	Democratic Republic of the Congo: Lake Tumba	Cichlidae	Freshwater		Fryer (1964)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	Bathybates fasciatus Boulenger, 1901; *Bathybates minor* Boulenger, 1906; *Cyphotilapia frontosa* (Boulenger, 1906); *Haplotaxodon microlepis* Boulenger, 1906; *Limnotilapia dardennii* (Boulenger, 1899); *Plecodus paradoxus* Boulenger 1898; *Synodontis granulosus* Boulenger, 1900; *Synodontis multipunctatus* Boulenger, 1898	Lake Tanganyika	Cichlidae; Mochokidae	Freshwater		Fryer (1965)
	Shuja horei (Günther, 1894); *Simochromis diagramma* (Günther, 1894)	Burundi: Magara, Nyaruhongoka (Lake Tanganyika)	Cichlidae	Freshwater	OQ407466 (18S) OQ407471 (28S)	Míč et al. (2023)
Ergasilus mirabilis Oldewage & van As, 1987	**TH:** *Synodontis leopardinus* Pellegrin, 1914	**TLOC:** Pongola flood plains on the Makatini Flats, South Africa	Mochokidae	Freshwater		Oldewage & van As (1987a)
	Brycinus imberi (Peters, 1852); *Clarias gariepinus* (Burchell, 1822); *Clarias ngamensis* Castelnau, 1861; *Enteromius afrohamiltoni* (Crass, 1960); *Glossogobius giuris* (Hamilton, 1822); *Hydrocynus vittatus* Castelnau, 1861; *Labeo rosae* Steindachner, 1894; *Schilbe intermedius* Rüppell, 1832; *Synodontis zambezensis* Peters, 1852	South Africa: Limpopo River & Pongola River System	Alestidae; Clariidae; Cyprinidae; Gobiidae; Schilbeidae	Freshwater		Oldewage & van As (1988b)
	Clarias gariepinus (Burchell, 1822); *Clarias ngamensis* Castelnau, 1861; *Hemichromis elongatus* (Guichenot, 1861); *Hepsetus odoe* (Bloch, 1794); *Marcusenius macrolepidotus* (Peters, 1852); *Schilbe intermedius* Rüppell, 1832; *Schilbe mystus* (Linnaeus, 1758); *Synodontis leopardinus* Pellegrin, 1914; *Synodontis macrostigma* Boulenger, 1911; *Synodontis nigromaculatus* Boulenger, 1905	Namibia: Zambezi River, Caprivi	Cichlidae; Clariidae; Hepsetidae; Mochokidae; Mormyridae; Schilbeidae	Freshwater		Oldewage & van As (1988b)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	Synodontis zambezensis Peters, 1852	Lake Malawi	Mochokidae	Freshwater		Oldewage & van As (1988b)
	Cyphomyrus discorhynchus (Peters, 1852)	Zimbabwe: Lake Kariba	Mormyridae	Freshwater		Oldewage & van As (1988b)
	Clarias gariepinus (Burchell, 1822); *Marcusenius macrolepidotus* (Peters, 1852); *Petrocephalus catostoma* (Günther, 1866); *Synodontis nigromaculatus* Boulenger, 1905	Namibia: Kwando River, Caprivi	Clariidae; Mochokidae ; Mormyridae	Freshwater		Avenant-Oldewage & Oldewage (1993)
	Cyphomyrus discorhynchus (Peters, 1852)	Zimbabwe: Lake Kariba	Mormyridae	Freshwater		Douëllou & Erlwanger (1994)
	***Clarias gariepinus* (Burchell, 1822)**	**South Africa: Kushokwe Pan**	**Clariidae**	**Freshwater**		**Present study**
	***Clarias gariepinus* (Burchell, 1822)**	**South Africa: Vaal River**	**Clariidae**	**Freshwater**	**P38K_VR (18S); P38Ka_VR (28S); P38kb_VR (COI)**	**Present study**
	***Clarias gariepinus* (Burchell, 1822)**	**Zambia: Zambezi River**	**Clariidae**	**Freshwater**	**P49K_72ZR (18S); P49Ka_72ZR (28S); P49Kb_72ZR (COI)**	**Present study**
Ergasilus nodosus Wilson, 1924	**TH:** *Bagrus bajad* (Forsskål, 1775)	**TLOC:** White Nile, Omdurman, Sudan	Bagridae	Freshwater		Wilson (1924)
	Bagrus sp.	Ghana: Sielo Tuni Stream	Bagridae	Freshwater		Fryer (1964)
Ergasilus parasarsi Míč, Řehulková & Seifertová, 2023	**TH:** *Simochromis diagramma* (Günther, 1894)	**TLOC:** Magara, Lake Tanganyika, Burundi	Cichlidae	Freshwater		Míč et al. (2023)
	Eretmodus marksmithi Burgess, 2012; *Gnathochromis permaxillaris* (David, 1936); *Lamprologus callipterus* Boulenger, 1906; *Ophthalmotilapia nasuta* (Poll & Matthes, 1962); *Perissodus microlepis* Boulenger, 1898; *Tanganicodus irsacae* Poll, 1950	Burundi: Mukuruka, Nyaruhongoka (Lake Tanganyika)	Cichlidae	Freshwater	OQ407467 (18S) OQ407473 (28S)	Míč et al. (2023)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	TH: *Spathodus erythrodon* Boulenger, 1900	**TLOC:** Magara, Lake Tanganyika, Burundi	Cichlidae	Freshwater		Míč et al. (2023)
Ergasilus parvus Míč, Řehulková & Seifertová, 2023	*Bathybates ferox* Boulenger, 1898; *Eretmodus marksmithi* Burgess, 2012; *Lamprologus callipterus Boulenger, 1906; Neolamprologus brichardi* (Poll, 1974); *Neolamprologus mondabu* (Boulenger, 1906)	Burundi: Bujumbura fish market, Nyaruhongoka (Lake Tanganyika)	Cichlidae	Freshwater	OQ407468 (18S) OQ407472 (28S)	Míč et al. (2023)
	TH: *Tylochromis mylodon* Regan, 1920	**TLOC:** Katanga, Democratic Republic of the Congo	Cichlidae	Freshwater		Capart (1944)
	Marcusenius macrolepidotus (Peters, 1852); *Synodontis nigromaculatus* Boulenger, 1905; *Clarias ngamensis* Castelnau, 1861	Lake Bangwelu	Clariidae; Mochokidae; Mormyridae	Freshwater		Fryer (1959)
	Thoracochromis moeruensis (Boulenger, 1899); Tylochromis bangwelensis Regan, 1920; Tylochromis mylodon Regan, 1920	Democratic Republic of the Congo: Lake Mweru and Luapula River	Cichlidae	Freshwater		Fryer (1967)
	Clarias gariepinus (Burchell, 1822)	Ghana: Mawli River	Clariidae	Freshwater		Paperna (1969)
Ergasilus sarsi Capart, 1944	*Clarias anguillaris* (Linnaeus, 1758); *Heterobranchus bidorsalis* Geoffroy Saint-Hilaire, 1809	Nigeria: River Galma, small lakes around Zaria	Clariidae	Freshwater		Shotter (1977)
	Clarias gariepinus (Burchell, 1822)	Nigeria: Bagauda fish farm, Kano	Clariidae	Freshwater		Bichi & Yelwa (2010)
	Lamprichthys tanganicanus (Boulenger, 1898)	Democratic Republic Congo: Lake Tanganyika	Procatopodidae	Freshwater		Kilian & Avenant-Oldewage (2013)
	Oreochromis niloticus (Linnaeus, 1758)	Egypt: Mariotteya Stream	Cichlidae	Freshwater		Mahmoud et al. (2014)
	Oreochromis niloticus (Linnaeus, 1758)	Egypt: River Nile Branch (Bahr Nashart), Drainage canal (Damroo Drainage canal) and Fish farm	Cichlidae	Freshwater		El-Seify et al. (2018)
Ergasilus sieboldi von Nordmann,	**TH:** pike, bream and carp	**TLOC:** Europe	Cyprinidae; Percidae	Marine		von Nordmann (1832)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
1832 **Syn:** *Ergasilus baicalensis* Messjatzeff, 1928 **Syn:** *Ergasilus depressus* Sars, 1863 **Syn:** *Ergasilus esocis* Sumpf, 1871 **Syn:** *Ergasilus hoferi* Borodin, 1915 **Syn:** *Ergasilus surbecki* Baumann, 1913 **Syn:** *Ergasilus trisetaceus* von Nordmann, 1832	*Cyprinus carpio* (Linnaeus, 1758)	Algeria: Foum El Khanga reservoir, Souk Ahras	Cyprinidae	Freshwater		Boucenna et al. (2015)
	Luciobarbus callensis (Valenciennes, 1 842)	Algeria: Beni-Haroun Dam, Mila city	Cyprinidae	Freshwater		Boucenna et al. (2018)
	Bagrus bajad (Fabricius, 1775)	Egypt: Lake Nasser	Bagridae	Freshwater		Hamouda (2018)
	Sparus aurata Linnaeus, 1758	Egypt: Semi-intensive marine fish farms	Sparidae	Marine	OM812074 (28S)	Abdel-Radi et al. (2022)
	Carassius carassius (Linnaeus, 1758)	Algeria: Beni-Haroun Dam, Mila city	Cyprinidae	Freshwater		Berrouk et al. (2018, 2022)

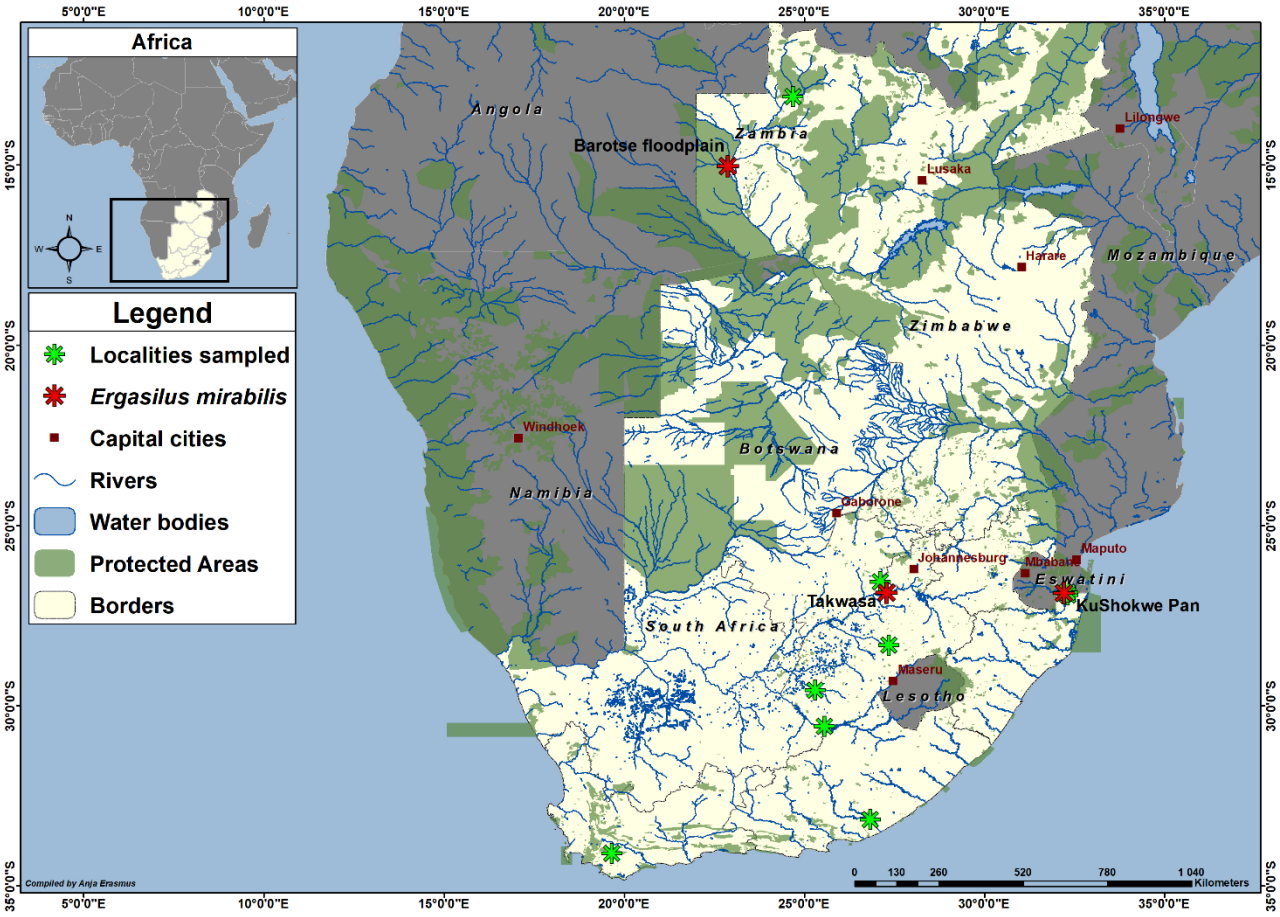


Figure 2.1: Map of all sampling localities from this study, with red star icons representing sites where adult female ergasilids were collected.

2.3.2. Morphological analysis

Fish gills were removed and screened for parasites with the aid of a Zeiss Stemi 305 compact stereomicroscope (Zeiss, Oberkochen, Germany). Copepods were collected using tweezers and fine brushes and preserved in 70% ethanol for morphological and molecular analysis.

Macromorphology of specimens was studied with the aid of dissection microscopes (Zeiss Stemi 305 compact and Zeiss Stemi 508 stereomicroscopes (Zeiss, Oberkochen, Germany)). Photomicrographs were taken using a ZEISS Axiocam ERc 55 camera attached to the Zeiss Stemi 508 stereomicroscope. Specimens were measured in millimetres using Labscope Mat Ver. 2.8.4 microscope software and expressed as mean ± standard deviation (with range in parenthesis). Measurements of total length were from the anterior end of the cephalosome to the posterior margin of the caudal rami.

Preserved specimens were cleared in lactic acid for 60 minutes, stained with lignin pink for 40–60 minutes, and dissected. Specimens were temporarily mounted with glycerine and studied using bright field and phase-contrast microscopy functions of Nikon Eclipse *Ni* microscope (Nikon Instruments, Tokyo, Japan), further applying the z-dimensional stacking (Z-stack) function for differential interference contrast (DIC) micrographs of different

structures. Pencil drawings of specimens and dissected appendages were made with the aid of a drawing tube attached to the Nikon Eclipse *Ni* microscope. Electronic inking was completed in Adobe Illustrator (Creative Cloud, 2022) using a Wacom Intuos 4 pro graphic tablet (PTK-640). Terminologies for the description of body somites and cephalic appendages in this manuscript follow Boxshall (2016).

For scanning electron microscopy (SEM), 13 adult females were studied. Specimens were dehydrated through a graded ethanol series for 10 minutes per concentration of ethanol (70%, 80%, 90%, 96%, 100%, 100%). Ethanol was removed through a series of graded HMDS (1,1,1,3,3,3-Hexamethyldisilazane) (Merck, Germany) at five minutes per 30%, 50%, 70%, 100%, 100% concentration, and allowed to dry. Dried specimens were mounted on aluminium stubs using carbon tape, sputter coated in gold/palladium (SPI-module, SPI Supplies, Pennsylvania, USA or Emscope SC500, Quorum Technologies, Lewes, UK), and observed using a JEOL Winsem JSM IT 200 InTouchScope™. Photomicrographs of selected features were taken at 5Kva.

2.3.3. Infestation rates

Infestation levels were expressed as prevalence (P), mean abundance (MA) and mean intensity (MI), following definitions from Bush et al. (1997); calculations for each are provided in parentheses.

2.3.4. Molecular analysis

Non-ovigerous females (Zambezi River) and egg strings (Vaal River) were used for genomic DNA extraction. The Macherey-Nagel tissue kit (GmbH & Co. KG, Sandton, South Africa) manufacturer's protocol was followed, with a pre-lysis period of 3–4 hours. For DNA amplifications, two ribosomal RNA gene regions (18S and 28S) and one mitochondrial DNA gene region (Cytochrome *c* Oxidase I) (COI) were targeted for partial gene amplification. Primers prepared by Song et al. (2008) were used for 18S (18SF and 18SR) and 28S (28SF and 28SR) Polymerase Chain Reactions (PCR), and the universal mitochondrial primers LCO1490 and HCO2198 (Folmer et al., 1994) were used for COI reactions (see Table 2.2). Each amplification reaction was carried out in 25 µl volumes using: 12.5 µl of DreamTaq PCR Master Mix (2X) (ThermoFischer Scientific, Waltham, Massachusetts, USA), 1.25 µl of 10 µM of each primer, 3 µl of DNA product and 7 µl of double distilled water for 18S, 28S, and COI reactions. Thermocycling conditions followed Song et al. (2008) for 18S and 28S, and Hayes et al. (2021) for COI. Positive PCR products were verified by 1% agarose gel electrophoresis and sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa) for purification and sequencing in both directions.

Table 2.2: List of primers used for DNA amplification of *Ergasilus mirabilis* Oldewage & van As, 1987 with sequences and references used in the amplification of partial 18S, 28S, and COI genes in this study.

Gene regions	Primers	Sequences	References
18S	18SF	5′-AAG GTG TGM CCT ATC AAC T-3′	Song et al. (2008)
	18SR	5′-TTA CTT CCT CTA AAC GCT C-3′	
28S	28SF	5′-ACA ACT GTG ATG CCC TTA G-3′	
	28SR	5′-TGG TCC GTG TTT CAA GAC G-3′	
COI	LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer et al. (1994)
	HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	

Newly generated sequences were compared with closely related sequences from the family Ergasilidae, available on GenBank using the nucleotide Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov/). Only GenBank sequences associated with peer-reviewed publications were used in the alignment with newly generated sequences. *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup for all three gene regions (Table 2.3). For the COI gene, however, unpublished submissions for *Ergasilus* species occurring in Africa were selected from the Barcode of Life Database (BOLD, 2023) and included in the COI alignments with the GenBank and newly generated sequences (see Table 2.3). Using Geneious Prime v. 2022.2.2 (Biomatters, Auckland, New Zealand), newly generated forward and reverse sequences were assembled, aligned, edited, and trimmed. Resultant sequences (contigs) will be submitted to GenBank.

Following the implemented default parameters of MAFFT v7.490 (Katoh et al., 2002; Katoh & Standley, 2013), alignments for novel sequences and selected GenBank and BOLD sequences (for COI) were generated and trimmed. Genetic divergences among aligned specimens were calculated in Geneious Prime v. 2022.2.2 and expressed as percentage similarities and differences in the number of base pairs. An estimation of the best nucleotide substitution model for each dataset was determined using the Akaike Information Criterion (AIC) implemented in the jModelTest 2.1.4 (Posada, 2008; Darriba et al., 2012). The suggested model for all datasets (18S, 28S, COI) was the general time-reversible model incorporating invariant sites and gamma distributed among-site rate variations (GTR+I+G).

Table 2.3: List of GenBank and Barcode of Life Database (BOLD) Ergasilidae sequences included in the phylogenetic analyses. The sequences from taxa in boldface were generated from the present study, all other sequences are GenBank sequences, with the exception of the sequence from the taxon with an asterisk* that was retrieved from BOLD (2023). *Lernaea cyprinacea* Linneaus, 1758 (in grey shade) was used as the outgroup.

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Acusicola margulisae	*Amphilophus citrinellus*, *Parachromis managuensis*, *Oreochromis* sp., *Poecilia exicana*	Nicaragua	MN852694	MN852851	MN854870	Santacruz et al. (2020)
Ergasilus anchoratus	*Pseudobagrus fulvidraco*	China	DQ107564	DQ107528		Song et al. (2008)
Ergasilus briani	*Misgurnus anguillicaudatus*	China	DQ107572	DQ107532		Song et al. (2008)
Ergasilus caparti	*Neolamprologus brichardi*	Burundi	OQ407469	OQ407474		Míč et al. (2023)
Ergasilus hypomesi	*Acanthogobius hasta*	China	DQ107573	DQ107539		Song et al. (2008)
**Ergasius lizae*	*Fundulus diaphanus*	Canada			ECTCR024-14	BOLD (2023)
Ergasilus macrodactylus	*Gnathochromis permaxillaris*	Burundi	OQ407465	OQ407470		Míč et al. (2023)
Ergasilus megacheir	*Simochromis diagramma*	Burundi	OQ407466	OQ407471		Míč et al. (2023)
Ergasilus mirabilis	***Clarias gariepinus***	**Vaal River, South Africa**	**P38K_38VR**	**P38Ka_38VR**	**P38Kb_38VR**	**Present study**
Ergasilus mirabilis	***Clarias gariepinus***	**Zambezi River, Zambia**	**P49K_72ZR**	**P49Ka_72ZR**	**P49Kb_72ZR**	**Present study**
Ergasilus parasarsi	*Simochromis diagramma*	Burundi	OQ407467	OQ407473		Míč et al. (2023)
Ergasilus parvus	*Spathodus erythrodon*	Burundi	OQ407468	OQ407472		Míč et al. (2023)
***Ergasilus parasiluri*	*Tachysurus fulvidraco*	China	DQ107567	DQ107536		Song et al. (2008)
Ergasilus peregrinus	*Siniperca chuatsi*	China	DQ107577	DQ107531		Song et al. (2008)

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Ergasilus scalaris	*Tachysurus dumerili*	China	DQ107565	DQ107538		Song et al. (2008)
Ergasilus sieboldi	*Perca fluviatilis*	Czech Republic	MW810238	MW810242		Kvach et al. (2021)
Ergasilus sieboldi	*Sparus aurata*	Egypt		OM812074		Abdel-Radi et al. (2022)
Ergasilus sp.	Free-living	South Korea			KR049035	Baek et al. (2016)
Ergasilus sp.	*Mugil liza*	Argentina			KU557411	Castro-Romero et al. (2016)
Ergasilus tumidus	*Acanthorhodeus taenianalis*	China	DQ107569	DQ107535		Song et al. (2008)
Ergasilus wilsoni	Free-living	South Korea			KR049036	Baek et al. (2016)
Ergasilus yaluzangbus	*Gymnocypris stewartii*	China	DQ107578	DQ107540		Song et al. (2008)
***Ergasilus yandemontei*	*Odontesthes hatcheri*	Argentina	MT969345			Waicheim et al. (2021)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MH167969	MH167967		Ondračková et al. (2019)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MH167970	MH167968		Ondračková et al. (2019)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MW810236	MW810240		Kvach et al. (2021)
Neoergasilus japonicus	*Lepomis gibbosus, Scardinius erythrophthalmus*	Czech Republic	MW810237	MW810241		Kvach et al. (2021)
****Neoergasilus japonicus*	Collected by plankton net	USA			MZ964935	Vasquez et al. (2021)
Neoergasilus japonicus	Free-living	South Korea			KR049037	Baek et al. (2016)
Paraergasilus brevidigitus	*Cyprinus carpio*	China	DQ107576	DQ107530		Song et al. (2008)
Paraergasilus longidigitus	*Abramis brama, Perca fluviatilis, Scardinius erythrophthalmu*	Czech Republic	MW810239	MW810243		Kvach et al. (2021)
Paraergasilus medius	*Ctenopharyngodon idellus*	China	DQ107574	DQ107529		Song et al. (2008)

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Sinergasilus major	*Ctenopharyngodon idella*	China	DQ107560	DQ107524		Song et al. (2008)
Sinergasilus major	*Silurus glanis*	Hungary	MZ047814	MZ047815		Dos Santos et al. (2021)
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	China	DQ107563	DQ107525		Song et al. (2008)
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	China			KR263117	Feng et al. (2016)
Sinergasilus undulatus	*Cyprinus carpio*	China	DQ107561	DQ107526		Song et al. (2008)
Sinergasilus undulatus	*Cyprinus carpio*	China			MW080644	Hua et al. (2021)
Lernaea cyprinacea	*Carassius auratus, Cyprinus carpio, Chanodichthys ilishaeformis*	China	MH982195	MH982204	MH982220	Hua et al. (2019)

* Taxon from the Barcode of Life Database (BOLD);
** *Ergasilus yandemontei* (Published on GenBank as *Ergasilus* sp.); *Ergasilus parasiluri* (published on GenBank as its synonym *Pseudergasilus parasiluri*);
*** Only able to access abstract and sequences that were published as supplementary data.

For phylogenetic analyses, Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were run using this model of nucleotide evolution. Bayesian Inference analyses were executed on computational resource CIPRES Science Gateway v 3.3 (Miller et al., 2010) (https://www.phylo.org/), adapting MrBayes v. 3.2.7a. set parameters (Ronquist et al., 2012), running two independent Markov Chain Monte Carlo (MCMC) runs of four chains for 10 million generations and sampling tree topologies every 1000 generations. Burn-in parameters were set to the first 25,000 generations. Maximum Likelihood analyses were run using PhyML v. 3.0 (Guindon et al., 2010), on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) with estimated model parameters and bootstrap values of 1000 repetitions. Nodal support for ML analyses was estimated at 100 bootstrap repetitions. Phylogenetic trees for BI and ML outputs were visualised in FigTree v 1.4.4 software (Rambaut, 2012).

2.4. Results

2.4.1. Taxonomy

Order Cyclopoida Burmeister, 1834

Family Ergasilidae von Nordmann, 1832

Genus *Ergasilus* von Nordmann, 1832

Restricted synonymy.

Ergasilus von Nordmann, 1832: 160–168.—Wilson, 1911: 326–328.—Sars, 1918: 198–199.—Yamaguti, 1963: 28.—Roberts, 1970: 134–161.—Kabata, 1979: 87–89.—Oldewage & van As, 1988c: 42–46.—Abdelhalim, Lewis, & Boxshall, 1993: 45–52.—Amado & Rocha, 2001: 149–157.

Ergasiloides Sars, 1909: 63–64

Limnoncaea Kokubo, 1914: 537–538.—Ohtsuka, Ho & Nagasawa, 2004a: 472–473.—Ohtsuka, Ho, Nagasawa, Morozinska-Gogol, & Piasecki, 2004b: 35–44

Markewitschia Yamaguti, 1963: 36

Megabrachinus Hesse, 1871: 38–40

Pseudergasilus Yamaguti, 1936: 3

Type species. *Ergasilus gibbus* von Nordmann, 1832 and *Ergasilus sieboldi* von Nordmann, 1832.

Generic remarks.

Individuals from the genus *Ergasilus* are characterised by an elongate cyclopoid body form. Antennules are usually six-segmented and ornamented with setae, although a few species have five-segmented antennules i.e., *E. flaccidus*, *E. ilani*, *E. inflatipes*, *E. nodosus* from Africa; *E. pitalicus* Thatcher, 1984 from Brazil and *E. wilsoni* Markewitsch, 1933 from the Black Sea. The antennae of *Ergasilus* species are typically devoid of any cuticular covering and its terminal segment is sclerotised and with a single point. The fourth swimming legs usually have only two segmented exopodites.

The other ergasilid genera reported from Africa are *Dermoergasilus, Neoergasilus* and *Paraergasilus* (Fryer, 1968; Oldewage & van As, 1988b; Oldewage & Avenant-Oldewage, 1993). In addition to the features listed above, individuals of the genus *Ergasilus* are further differentiated from these other genera by the following characteristics: *Dermoergasilus* has a characteristic cuticular membranous covering on the antennae, which is absent in *Ergasilus.* The antennae of *Neoergasilus* are short and strongly curved as opposed to the usual long slender antennae of *Ergasilus.* Additionally, the first legs of *Neoergasilus* have a triangular protrusion at the posterior margin of the basiopodite (in between the exopod and the endopod), and the second segment of the exopod is characterised by a spatulate spine extending parallel

to the length of the third exopod segment. All these features of leg 1 are absent from the genus *Ergasilus.* Lastly, the antennae of the genus *Ergasilus* is characterised by a single claw, compared to *Paraergasilus* which has three prongs for its terminal antennal segment.

***Ergasilus mirabilis* Oldewage & van As, 1987**

Figs. 2.2–2.7

Ergasilus mirabilis Oldewage & van As, 1987a: 62–65.—Oldewage & van As, 1987b: 313–317.—Oldewage & van As, 1988b: 177–187.—Oldewage & van As, 1988c: 42–46.—Douëllou & Erlwanger, 1994: 233–242.

Type host. Synodontis leopardinus Pellegrin, 1914

Other hosts. Brycinus imberi (Peters, 1852); *Clarias gariepinus* (Burchell, 1822); *Clarias ngamensis* Castelnau, 1861; *Cyphomyrus discorhynchus* (Peters, 1852); *Enteromius afrohamiltoni* Crass, 1960; *Glossogobius giuris* (Hamilton, 1822); *Hemichromis elongatus* (Guichenot, 1861); *Hepsetus cuvieri* (Castelnau, 1861); *Hippopotamyrus discorhynchus* (Peters, 1852); *Hydrocynus vittatus* Castelnau, 1861; *Labeo rosae* Steindachner, 1894; *Marcusenius macrolepidotus* (Peters, 1852); *Petrocephalus catostoma* (Günther, 1866); *Schilbe intermedius* Rüppell, 1832; *Schilbe mystus* (Linnaeus, 1758); *Synodontis macrostigma* Boulenger, 1911; *Synodontis nigromaculatus* Boulenger, 1905; *Synodontis zambezensis* Peters, 1851.

Type locality. Phongolo River, northern Natal, South Africa.

Other localities. Mozambique—Lake Malawi; South Africa—**Kushokwe Pan (present study)**, Limpopo River; **Vaal River (present study)**; Namibia—the Zambezi region (previously known as Caprivi strip): Chobe River, Kwando River, Lake Liambezi, Lake Lisikili, Zambezi River; Zambia—**Barotse Floodplain (present study)**; Zimbabwe—Lake Kariba (Oldewage & van As, 1988b; Oldewage & Avenant-Oldewage,1993; Avenant-Oldewage & Oldewage, 1993).

Material examined.

A total of 184 ergasilids (151 adult females and 33 copepodites/males) were examined; 13 used for SEM; eight for dissection; eight adult females and five egg strings used for DNA extraction; 10 specimens will be deposited in the collections of the National Museum, Bloemfontein, South Africa; the rest remain in the possession of the Water Research Group, NWU, Potchefstroom, South Africa.

Zambia. 164 copepods were collected from the Barotseland Floodplain, Zambezi River, North-western Province, Zambia (15°12′01.59″S 22°58′09.27″E), from four *Clarias gariepinus*, collected by M. Truter (2019).

South Africa. 17 copepods were collected from the Vaal River (Takwasa Youth Camp), Venterskroon, North West Province, South Africa (26°52'02.7"S 27°17'36.0"E) from nine *Clarias gariepinus*, collected by M. Truter (2019). Another three copepods were collected from the KuShokwe Pan, Phongolo Floodplain in Ndumo Game Reserve, KwaZulu-Natal Province, South Africa (26°52'19.5"S 32°12'53.1"E) from three *Clarias gariepinus*, collected by M. Truter (2018).

Representative DNA sequences. Only extractions from the Vaal River, South Africa, and the Zambezi River, Zambia were successfully amplified. Sequences, with the numbers of base pairs (bp), will be submitted to GenBank as follows: (18S) – 1,367 & 1,373 bp long sequences of two specimens; (28S) – 668 & 694 bp long sequences of two specimens; (COI) – 692 & 693 bp long sequences of two specimens.

Infestation rates. From all the localities sampled, *E. mirabilis* was only collected from three sites and the infestation rates (of copepodites and adults) are given as follows:

South Africa. Kushokwe Pan – prevalence 20% (3/15), mean intensity 1 (3/3), mean abundance 0.2 (3/15); Vaal River – prevalence 50% (9/18), mean intensity 1.8 (17/9), mean abundance 0.9 (17/18).

Zambia. Barotse Floodplain – prevalence 23.5% (4/17), mean intensity 41 (164/4), mean abundance 9.6 (164/17).

Description of adult female (Figs. 2.2–2.7).

Measurements (n=20) are given as: total length (anterior margin of prosome to posterior margin of caudal rami, excluding caudal rami seta) 1.35 ± 0.14 (1.05–1.58) mm, cephalosome length 0.51 ± 0.07 (0.36–0.63) mm, cephalosome width 0.42 ± 0.04 (0.34–0.50) mm.

Body cyclopiform (Figs. 2.2a, 2.4a, 2.5a, 2.7a). Prosome comprising cephalosome, thorax with four pedigerous somites, urosome comprising reduced fifth pedigerous somite, non-pedigerous genital double-somite, and three free abdominal somites. Cephalosome (Figs. 2.2a, 2.4a, 2.4b) quadrangular in shape, almost as broad as long. Dorsolateral depression between cephalosome and first thoracic segment present; first thoracic segment and cephalosome not fused. Ornamentation present on dorsal side of cephalosome (Figs. 2.2a, 2.4b, 2.7b), comprises an inverted T-structure situated post-medially, between two oval sculptures situated anteriorly and posteriorly on cephalosome; paired eyespots and depression of antennae attachment visible above anterior oval ornamentation; paired sensory pores and papillae observed between inverted T and posterior oval sculpture with numerous sensory papillae and pores scattered over dorsal surface of cephalosome. Thorax five-

segmented (Figs. 2.2a, 2.4a, 2.4c). Segments one to four wider than long, progressively smaller, fifth segment reduced. Paired sensory papillae observed mid-dorsally on segments two to four (Figs. 2.4c, d), 2–4 sensory papillae on dorsolateral margins of segments two to four (Figs. 2.4c, e). Genital double-somite (Figs. 2.3a, 2.7c) 1.50 times as wide as long, five times as long as first abdominal somite, bearing a pair of multiseriate egg sacs dorsally (Figs. 2.2a, 2.4a, 2.5a). Two robust spines situated dorsolaterally, close to egg sac attachment pore (Fig. 2.6d). Abdomen (Figs. 2.3a, 2.7c) three-segmented, first abdominal somite widest, second somite shortest, third somite incised dorsoventrally forming attachment for caudal rami. All abdominal somites with a posterior row of ventral spinules. Caudal rami elongated, approximately twice as long as wide with four setae: one long median seta with an array of spines (Figs. 2.6e, f), a single shorter dorsolateral seta, 0.2 times as long as the median seta, and two even shorter ventrolateral setae, 0.1 times as long as the median seta. Two sensory pores, and spinules on the posterior-ventral margins on each ramus.

Antennule (Figs. 2.2c, 2.6a) six-segmented, armed with long and short setae, bearing a ring of spines on dorsal surface of first antennular segment (Fig. 2.6b). Sensory pores at proximal and distal dorsolateral margin of second antennular segment, setal formula from proximal to distal segments given as: 2–11–3–3–2–6. Antenna (Fig. 2.2b) four-segmented, slender, smooth, and unarmed; second segment longest; third segment sickle-shaped; fourth segment greatly reduced; terminal claw curved and sharply pointed.

Mouth tube positioned ventrally on cephalosome with row of spines on lateral side (Fig. 2.5b); labrum with studs towards posterior margin (Fig. 2.5c). Mandible (Figs. 2.2d, 2.5e) comprises two stout segments with three blades; endopod splits into a shorter anteriorly toothed blade and longer medial blade ornamented with teeth along anterior and posterior margin; distal blade (exopod) ornamented with teeth on posterior margin. Maxillule (Figs. 2.2f, 2.5d) ornamented with spines on dorsal surface, reduced to two-segmented lobe having two simple setae on distal margin of exopod and a single simple seta on distal margin of endopod. Maxilla (Figs. 2.2e, 2.5c) three-segmented with terminal process of numerous teeth on convex margin of distal segment, single seta on medial segment, proximal segment ornamented with large pore.

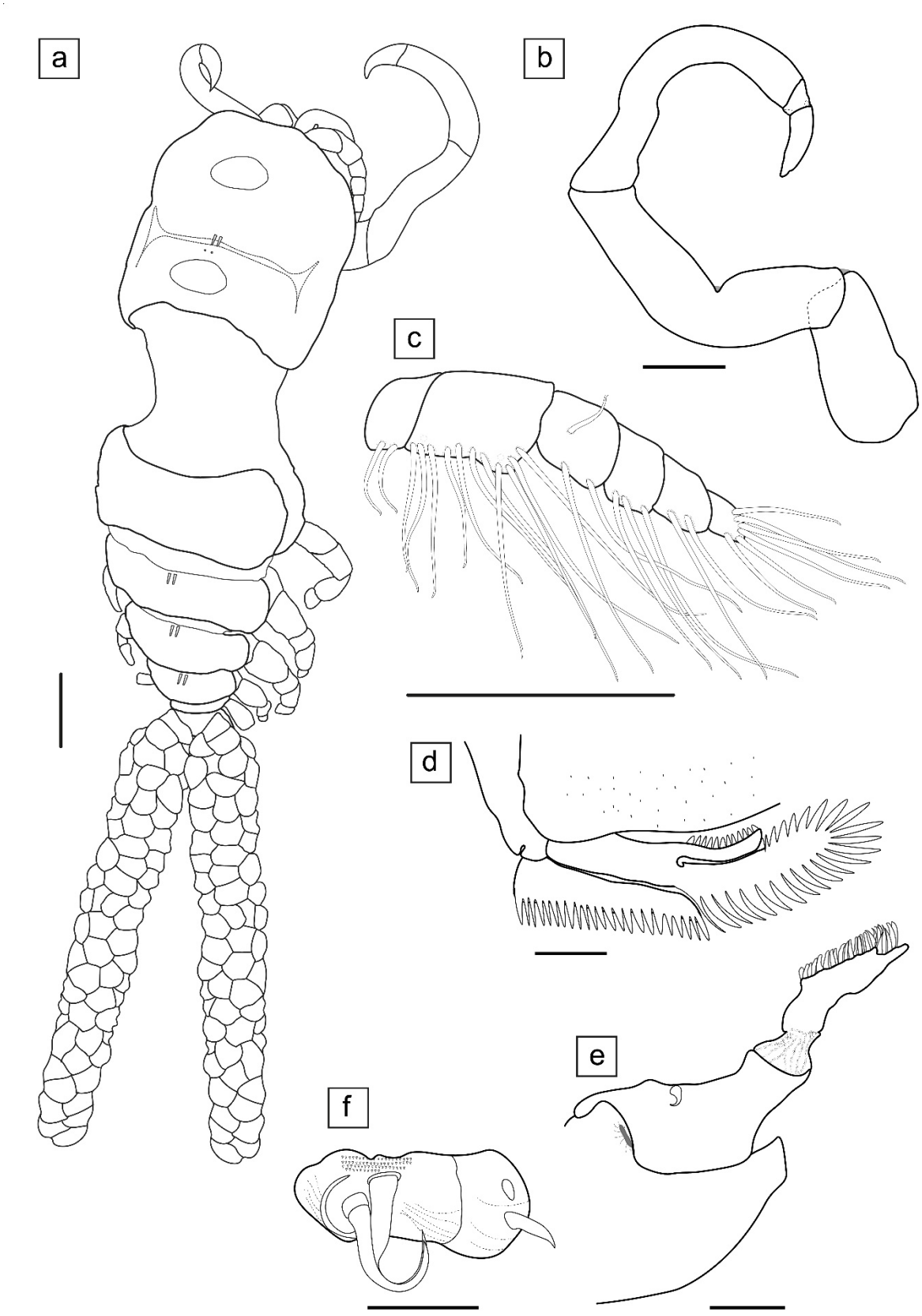


Figure 2.2: Illustrations of adult female *Ergasilus mirabilis* Oldewage & van As, 1987. (a) full image, dorsal view; (b) antenna; c) antennule; d) mandible; e) maxilla; f) maxillule. Scale bars: (a–c) 100 µm; (d–f) 10 µm.

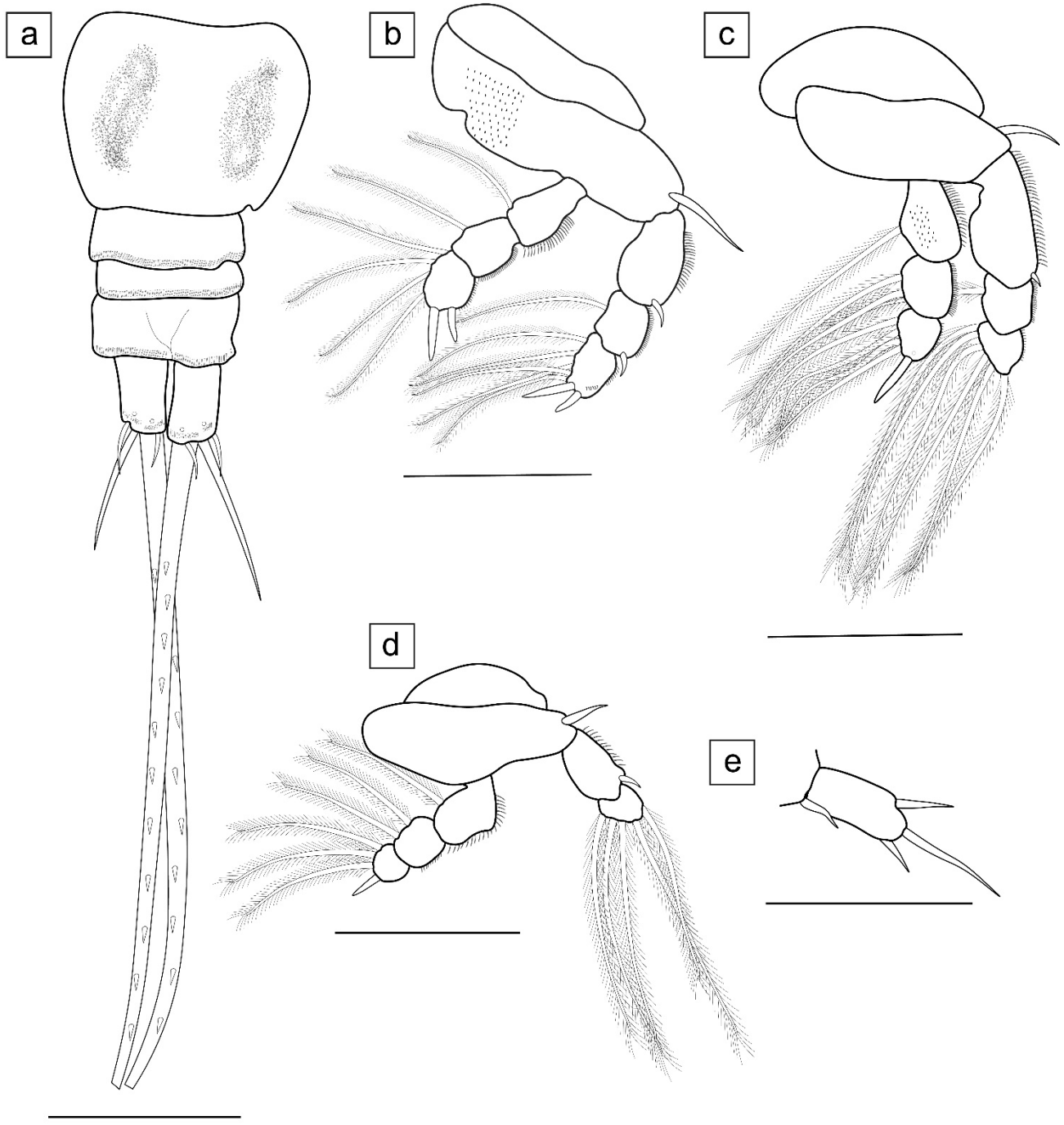


Figure 2.3: Illustrations of adult female *Ergasilus mirabilis* Oldewage & van As, 1987. (a) genital double somite, three abdominal somites and caudal rami; (b) leg 1; (c) leg 2; (d) leg 4; (e) leg 5. Scale bars: (a–e) 100 µm.

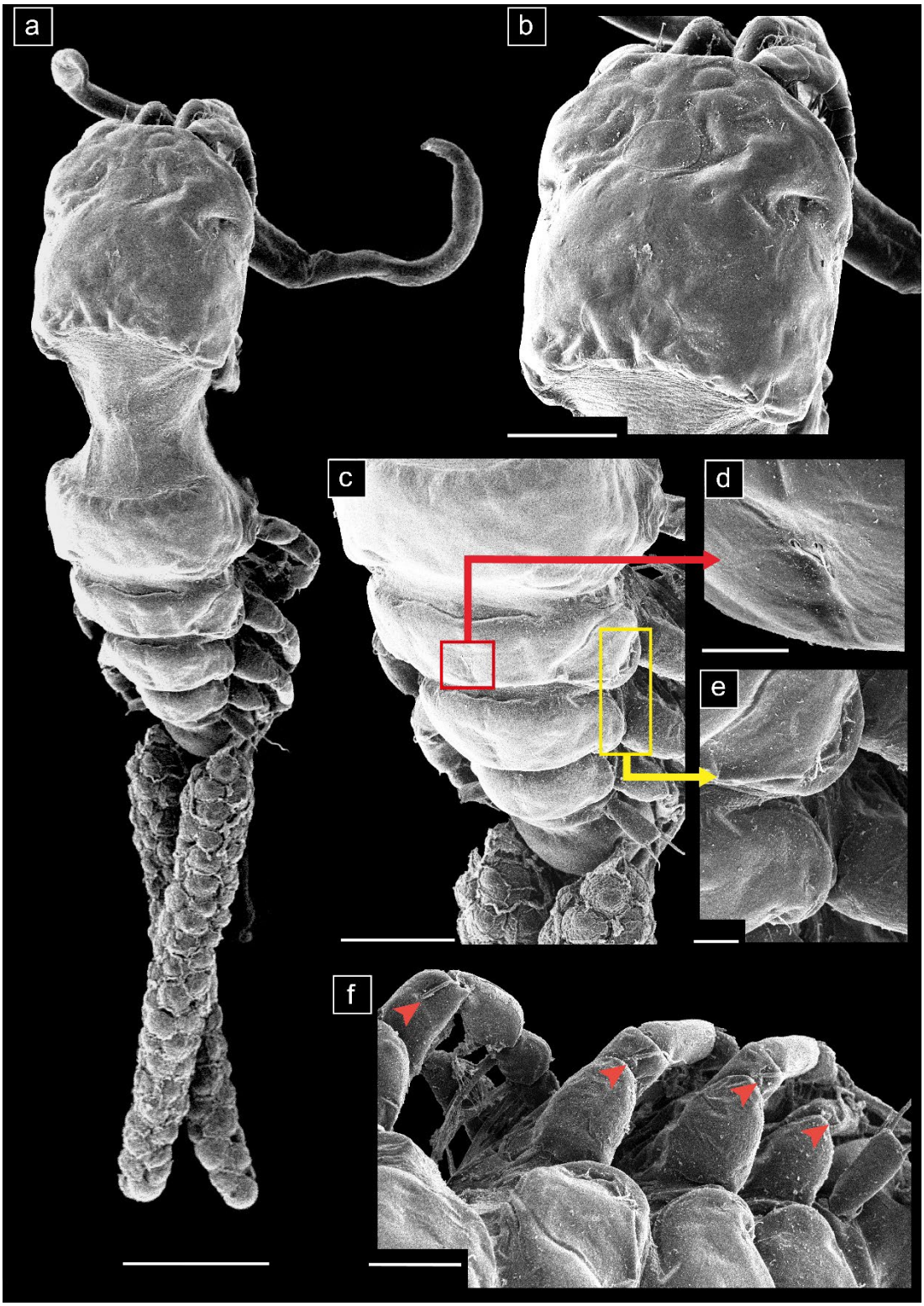


Figure 2.4: Scanning electron microscope photomicrographs of adult females *Ergasilus mirabilis* Oldewage & van As, 1987 (Zambezi River representatives) showing features from the dorsal view (a) full image, dorsal view; (b) cephalosome showing ornamentation, sensory pores and sensory papillae; (c) thoracic segments highlighting paired mid-dorsal sensory papillae on segments 2–4 (red circle) and dorsolateral sensory papillae (yellow circle); (d) zoomed in paired mid-dorsal sensory papillae; (e) zoomed in dorsolateral sensory papillae; (f) simple setae on basiopodite of legs 1–4 highlighted with red arrowheads. Scale bars: (a–c, f) 100 µm; (d–e) 50 µm.

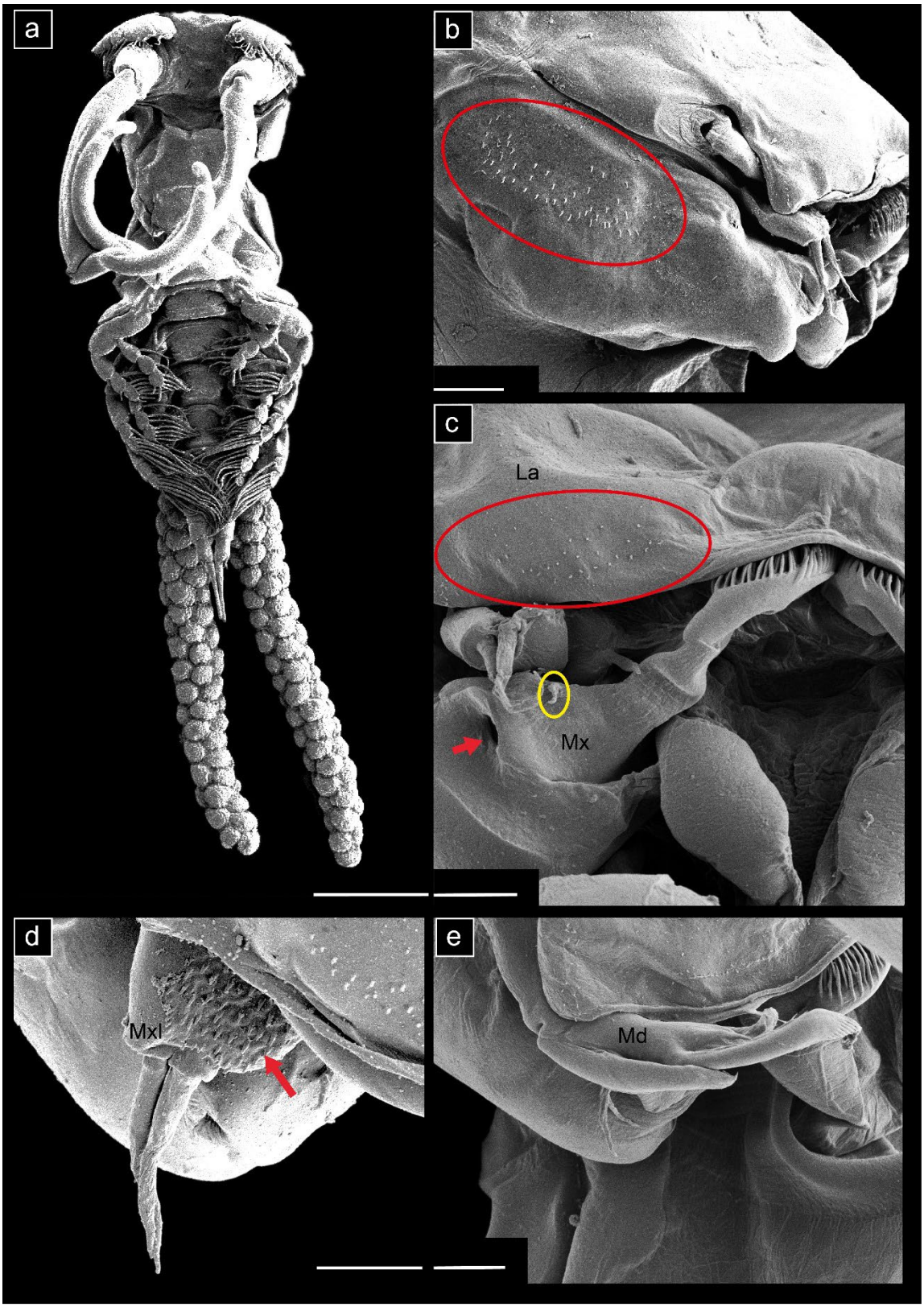


Figure 2.5: Scanning electron microscope photomicrographs of the full ventral image (a) and mouth parts (b–e) of *Ergasilus mirabilis* Oldewage & van As, 1987. (a) Full ventral image; (b) Mouth tube with lateral spines (red circle); (c) Studded labrum (red circle), maxilla with maxillary pore (red arrow) and single maxillary seta (yellow circle); (d) maxillule with rows of spines (red arrow); (e) mandible. Scale bars: (a) 200 µm; (b) 20 µm; (c–e) 10 µm. Abbreviations: La – labrum; Md – mandible; Mx – maxilla; Mxl – maxillule.

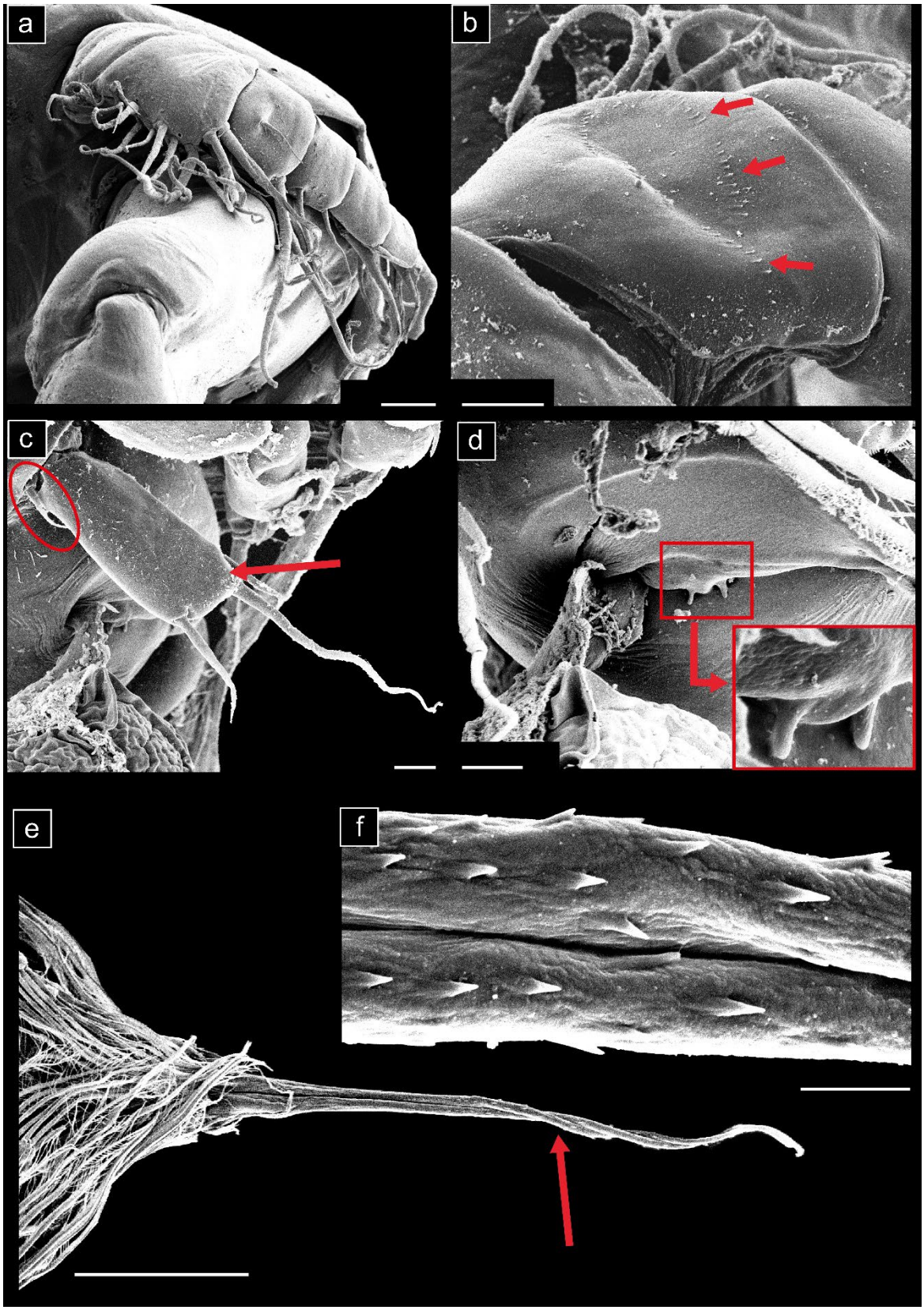


Figure 2.6: Scanning electron microscope photomicrographs of *Ergasilus mirabilis* Oldewage & van As, 1987. (a) antennule; (b) first antennular segment with ring of spines (red arrows); (c) leg 5 (red arrow) with basal seta (red circle); (d) Two robust spines situated dorsolaterally on genital somite; (e) Elongated median setae (red arrow) of caudal rami; (f) Zoomed in median setae showing array of spines. Scale bars: (a) 20 µm; (b–d) 10 µm; (e) 100 µm; (f) 5 µm.

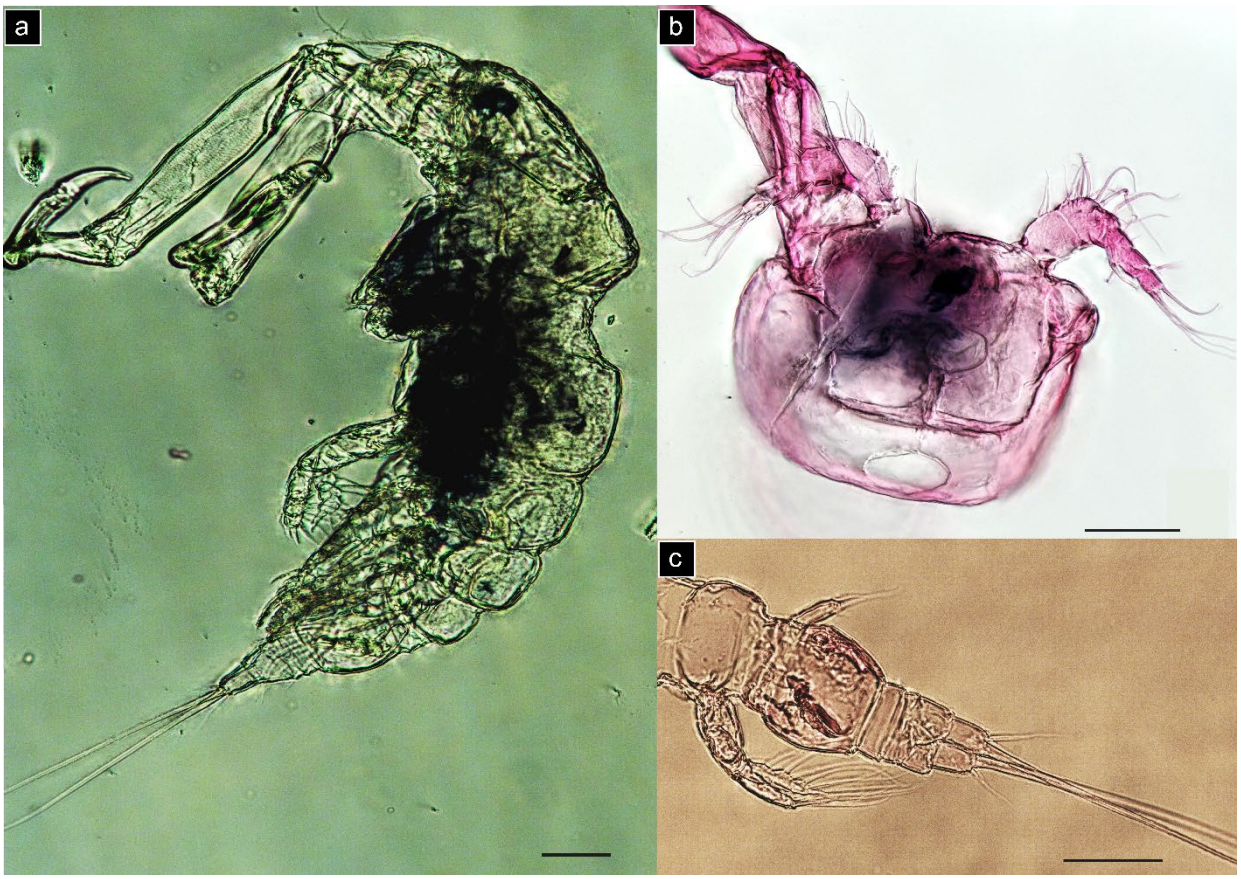


Figure 2.7: Bright-field (a, c) and phase-contrast (b) photomicrographs of adult female *Ergasilus mirabilis* Oldewage & van As, 1987 applying the z-dimensional stacking function. (a) full image, lateral view of cleared *E. mirabilis* showing eye spot and gut pigmentation; (b) dissected off cephalosome, cleared and stained in lignin pink, showing antenna, antennules, eye spot and cephalic ornamentation (two oval structures and an inverted T structure); (c) urosome with fourth and fifth thoracic segments; fourth and fifth legs; and caudal rami, emphasising the length of the median caudal seta. Scale bars: (a–c) 100 µm.

Legs 1–4 (Figs. 2.3b–d, 2.4f) consistent for the genus *Ergasilus*. Setae for legs 1–4 plumose except basiopodites ornamented with short simple setae; legs 2 and 3 with similar armature formula. Spinules present on lateral margins of exo- and endopodites of legs 1–4. Armature of legs 1–4 given in Table 2.4. Leg 5 with 4 setae; one short seta at the base of the segment, three terminal setae of unequal length on free segment, median seta the longest with an array of spines (Figs. 2.3e, 2.6c).

Table 2.4: Spine-setae formula on swimming legs of *Ergasilus mirabilis* Oldewage & van As, 1987. Number of spines in Roman numerals, number of setae in Arabic numerals.

	Coxa	Basis	Exopod	Endopod
Leg 1	0-0	I-0	I-0; I-1; II-5	0-1; 0-1; II-4
Leg 2	0-0	I-0	I-0; 0-1; 0-6	0-1; 0-2; I-4
Leg 3	0-0	I-0	I-0; 0-1; 0-6	0-1; 0-2; I-4
Leg 4	0-0	I-0	I-0; 0-5	0-1; 0-2; I-3

Male. Not described.

Variability.

Compared with the original description by Oldewage and van As (1987a), specimens from this study showed some variability in the number of antennular setation, mandible dentation, spines on the mouth tube and maxillules, as well as number of spines and setae on legs 1–5 and the genital somite (see Remarks for details).

Remarks.

The specimens from this study were identified as *Ergasilus mirabilis* based on a combination of specific morphological characteristics. Representative specimens from South Africa (Kushokwe Pan in the Pongola Floodplain, and the Vaal River) and Zambia (Zambezi River) were morphologically similar when comparing data from SEM and light microscopy. They were characterised by a quadrangular-shaped cephalosome with two oval structures, positioned anteriorly and posteriorly, respectively, to an inverted T structure; paired sensory papillae on the cephalosome; as well as the six-segmented antennules armed with setae, four-segmented smooth antennae, and paired sensory papillae observed dorsomedially on the thoracic somites 2–4.

On the cephalosome, numerous sensory pores and papillae were observed on specimens from this study (Fig. 2.4b). Compared with the original description, the first antennular segment was reported without any ornamentation and the number of setae found on the segments of the antennule were 19 in total (0-7-4-2-2-4). From the current study, however, two short simple setae and a ring of spines were observed (Figs. 2.2c, 2.6b) and an average number of 27 setae (2-11-3-3-2-6) was reported for all antennular segments (Fig. 2.2c). Drawings of the mandible from Oldewage and van As (1987a) showed denticulation on

the medial blade across all margins (anterior and posterior), however, this was not observed on all margins in the current specimens (Figs. 2.2d). Additionally, several rows of spines were observed on the lateral and dorsal axis of the mouth tube and maxillules, respectively. Five thoracic segments are reported from this study (Figs. 2.2a, 2.4c, 2.7a), whereas six segments were described in Oldewage and van As (1987a), which included the genital double-somite. In the present study, the genital double-somite is separated from the thoracic segments following nomenclature by Boxshall (2016). Two robust spines, not previously reported, were also observed on the genital somite, situated close to the egg string attachment pore (Fig. 2.6d). Comparing leg armature, the basiopodite of legs 1–4 observed from the present study possessed a single simple seta each (Fig. 2.4f); however, the presence of setae on the basiopodite of the legs were not mentioned in the original description. The third exopodite of Leg 1 from the current study had two spines and five plumose setae (Fig. 2.3b); whereas the original description reported six plumose setae and no spines. Furthermore, legs 2 and 3 of the specimens described in this study have similar spine-setae formulas, which is not the case with *E. mirabilis* from the original description. From the current study, Leg 4 has one, two, and three setae on the first, second, and third endopodal segments, respectively, with a spine on the third endopoal segment (Fig. 2.3d). The original description, however, gives no setae on the first and second endopodites, with six setae and no spines on the third endopodite. Leg 5 in the present study has four setae (Fig. 2.3e, 2.6c) and the original description gives only two setae for leg 5.

Compared to all other species from Africa, *E. mirabilis* is most similar to *E. cunningtoni* (description of *E. cunningtoni* is given by Capart, 1944). The cephalosome of *E. cunningtoni* is shorter than the sum of its thoracic segments and it possesses similar cephalothoracic ornamentation as *E. mirabilis.* However, and in accordance with the original description of *E. mirabilis*, the species described in this study also differs from *E. cunningtoni* in having a more quadrangular cephalosome than the triangular shape seen with *E. cunningtoni*. The digitiform process observed on the antennae of *E. cunningtoni* is absent in the species described in this study. Additionally, the second proximal segment of the antennae of *E. cunningtoni* have a definite notch which is absent in *E. mirabilis.*

With reference to the clariid host, *E. sarsi* is the only African species that has been reported from *C. gariepinus* apart from *E. mirabilis* (description of *E. sarsi* is given Capart, 1944). The smooth antennae and ornamentation on the cephalosome are similar to *E. mirabilis,* however, the triangular shaped cephalosome and possession of only two abdominal segments differentiates it from *E. mirabilis.*

2.4.2. Molecular analysis

A total of six sequences were generated from this study, two each for partial 18S, 28S, and COI gene regions, with representatives from the Vaal and Zambezi rivers, respectively. The BI tree topology showed the strongest evolutionary relationship as it had stronger support for its branch lengths.

For the 18S phylogenetic analyses, alignments of GenBank and novel sequences gave a length of 1,398 base pairs. Newly generated partial 18S sequences from the Vaal River (South Africa) and Zambezi River (Zambia) were 100% identical and most similar to the African sequences from Lake Tanganyika, with percentage similarity ranging from 99.60–99.70% (3–4 bp) (see Table 2.5). Bayesian Inference and Maximum Likelihood analyses yielded similar phylogenetic tree topologies. Newly generated sequences clustered as sister taxa with all Tanganyikan sequences: *E. caparti, E. macrodactylus, E. megacheir, E. parasarsi,* and *E. parvus* (Fig. 2.8), further confirming its identity as a different species within the genus *Ergasilus*, but also showing an evolutionary relationship with the African species compared to the other species.

Nucleotide alignments for the partial 28S gene region resulted in a length of 752 base pairs. Similar to the evolutionary relationship of the 18S gene, sequences from the Vaal and Zambezi rivers were 100% identical, and most similar to the African sequences from Lake Tanganyika with a percentage similarity range of 93.11–95.10% (32–45 bp) (see Table 2.6). Tree topologies for BI and ML analyses were similar. All newly generated sequences clustered as sister taxa with all Tanganyikan sequences separate from the *E. sieboldi* sequence from Egypt, which claded with the other available *E. sieboldi* sequence from the Czech Republic (Fig. 2.9). This phylogenetic relationship confirms the identity of the newly generated sequences as a different species from its congeners in the genus *Ergasilus.*

With the COI analyses, a total of 12 sequences were aligned with an invertebrate mitochondrial translation for the COI gene region, resulting in an alignment length of 692 base pairs. The sequences used included selected GenBank sequences and a BOLD sequence (*E. lizae,* an ergasilid also found in Africa) submitted from Canada. Newly generated partial COI sequences showed a 98.55% similarity (10 bp). From the translations, the codons having these 10 nucleotide differences all translated to the same amino acids (silent mutations) (see Appendix Table A1). Newly generated sequences differed by over 100 bases from all other COI Ergasilidae sequences in the alignment (see Table 2.7). Some of these nucleotide differences were silent mutations and others were missense mutations. Phylogenetic tree topologies for BI and ML analyses were similar; novel sequences of *E. mirabilis* clustered in a clade with *E. lizae* (Fig. 2.10).

Table 2.5: Genetic divergences among aligned 18S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country. Sequences from the present study in boldface and grey shade. *Lernaea cyprinacea* (MH982195) was used as the outgroup. Abbreviations: AR – Argentina, BI – Burundi; CN – China, CZ – Czech Republic, HU – Hungary, KR – South Korea, NI – Nicaragua, ZA – South Africa (Vaal River), ZM – Zambia (Zambezi River).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	**P38K_38VR *Ergasilus mirabilis* ZA**		**0**	**33**	**25**	**24**	**3**	**22**	**3**	**4**	**4**	**31**	**3**	**39**	**32**	**16**	**24**	**26**	**26**	**25**	**25**	**27**	**23**	**25**	**25**	**28**	**30**	**33**	**31**	**30**	**136**
2	**P49K_72ZR *Ergasilus mirabilis* ZM**	**100**		**37**	**29**	**28**	**3**	**26**	**3**	**4**	**4**	**35**	**3**	**43**	**36**	**19**	**28**	**29**	**27**	**25**	**27**	**27**	**25**	**30**	**26**	**33**	**35**	**37**	**35**	**34**	**139**
3	MN852694 *Acusicola margulisae* NI	**97.59**	**97.31**		39	43	23	36	23	22	22	47	23	45	50	36	43	48	33	37	34	36	35	38	38	38	38	48	45	44	143
4	DQ107564 *Ergasilus anchoratus* CN	**98.17**	**97.89**	97.16		32	13	25	13	14	14	35	13	39	29	27	32	41	34	30	31	32	29	11	11	14	15	35	33	34	139
5	DQ107572 *Ergasilus briani* CN	**98.24**	**97.96**	96.87	97.67		20	14	20	21	21	20	20	38	41	23	4	39	38	12	13	14	11	34	34	35	37	39	37	36	143
6	OQ407469 *Ergasilus caparti* BI	**99.7**	**99.7**	97.69	98.69	97.99		13	0	1	1	25	0	28	18	15	20	16	16	18	20	20	18	12	12	14	15	15	17	16	98
7	DQ107573 *Ergasilus hypomesi* CN	**98.39**	**98.11**	97.38	98.18	98.98	98.69		13	14	14	20	13	32	35	21	14	37	32	12	13	14	11	26	26	27	29	37	35	34	141
8	OQ407465 *Ergasilus macrodactylus* BI	**99.7**	**99.7**	97.69	98.69	97.99	100	98.69		1	1	25	0	28	18	15	20	16	16	18	20	20	18	12	12	14	15	15	17	16	98
9	OQ407466 *Ergasilus megacheir* BI	**99.6**	**99.6**	97.79	98.59	97.89	99.9	98.59	99.9		0	26	1	29	19	14	21	15	17	19	21	21	19	13	13	15	16	16	18	17	97
10	OQ407467 *Ergasilus parasarsi* BI	**99.6**	**99.6**	97.79	98.59	97.89	99.9	98.59	99.9	100		26	1	29	19	14	21	15	17	19	21	21	19	13	13	15	16	16	18	17	97
11	DQ107567 *Ergasilus parasiluri* CN	**97.73**	**97.45**	96.58	97.45	98.54	97.49	98.54	97.49	97.39	97.39		25	43	44	30	20	42	38	22	22	22	22	37	37	38	40	44	42	41	150
12	OQ407468 *Ergasilus parvus* BI	**99.7**	**99.7**	97.69	98.69	97.99	100	98.69	100	99.9	99.9	97.49		28	18	15	20	16	16	18	20	20	18	12	12	14	15	15	17	16	98
13	DQ107577 *Ergasilus peregrinus* CN	**97.15**	**96.87**	96.72	97.16	97.23	97.19	97.67	97.19	97.09	97.09	96.87	97.19		51	36	37	43	44	33	34	35	32	41	41	40	40	40	38	37	148
14	DQ107565 *Ergasilus scalaris* CN	**97.66**	**97.38**	96.36	97.89	97.01	98.19	97.45	98.19	98.09	98.09	96.8	98.19	96.28		34	41	48	43	39	41	41	39	30	30	31	32	45	43	42	145
15	MW810238 *Ergasilus sieboldi* CZ	**98.83**	**98.62**	97.38	98.03	98.32	98.49	98.47	98.49	98.59	98.59	97.82	98.49	97.38	97.52		23	24	30	21	22	23	20	26	25	27	30	35	31	30	136
16	DQ107569 *Ergasilus tumidus* CN	**98.24**	**97.96**	96.87	97.67	99.71	97.99	98.98	97.99	97.89	97.89	98.54	97.99	97.3	97.01	98.32		39	38	12	13	14	11	34	34	35	37	39	37	36	144
17	DQ107578 *Ergasilus yaluzangbus* CN	**98.1**	**97.89**	96.5	97.01	97.16	98.39	97.31	98.39	98.49	98.49	96.94	98.39	96.87	96.5	98.25	97.16		38	33	33	35	31	37	36	39	42	43	39	39	140
18	*MT969345 *Ergasilus yandemontei* AR	**98.11**	**98.04**	97.59	97.52	97.22	98.42	97.67	98.42	98.32	98.32	97.22	98.42	96.77	96.84	97.82	97.22	97.22		31	31	31	31	33	33	36	39	39	36	35	141
19	MW810236 *Neoergasilus japonicus* CZ	**98.16**	**98.16**	97.28	97.79	99.12	98.19	99.12	98.19	98.09	98.09	98.38	98.19	97.57	97.13	98.45	99.12	97.57	97.72		2	2	0	28	28	29	29	41	37	36	139
20	MH167969 *Neoergasilus japonicus* CZ	**98.15**	**98.01**	97.49	97.71	99.04	97.99	99.04	97.99	97.89	97.89	98.38	97.99	97.49	96.97	98.38	99.04	97.57	97.71	99.85		0	2	27	27	28	28	40	35	35	136
21	MW810237 *Neoergasilus japonicus* CZ	**98.01**	**98.01**	97.35	97.64	98.97	97.99	98.97	97.99	97.89	97.89	98.38	97.99	97.42	96.98	98.31	98.97	97.42	97.72	99.85	100		2	28	28	29	29	41	37	36	139
22	MH167970 *Neoergasilus japonicus* CZ	**98.3**	**98.16**	97.42	97.86	99.19	98.19	99.19	98.19	98.09	98.09	98.38	98.19	97.64	97.12	98.52	99.19	97.71	97.71	100	99.85	99.85		27	27	28	28	40	35	35	136
23	DQ107560 *Sinergasilus major* CN	**98.17**	**97.82**	97.23	99.2	97.52	98.8	98.11	98.8	98.69	98.69	97.31	98.8	97.01	97.82	98.11	97.52	97.31	97.59	97.94	98.01	97.94	98.01		0	3	7	38	37	36	138
24	MZ047814 *Sinergasilus major* HU	**98.17**	**98.1**	97.22	99.2	97.51	98.8	98.1	98.8	98.69	98.69	97.3	98.8	97	97.81	98.17	97.51	97.37	97.59	97.94	98.01	97.94	98.01	100		3	7	37	36	35	138
25	DQ107563 *Sinergasilus polycolpus* CN	**97.95**	**97.6**	97.23	98.98	97.45	98.59	98.03	98.59	98.49	98.49	97.23	98.59	97.09	97.74	98.03	97.45	97.16	97.37	97.87	97.94	97.87	97.94	99.78	99.78		6	40	39	38	137
26	DQ107561 *Sinergasilus undulatus* CN	**97.81**	**97.45**	97.23	98.91	97.31	98.49	97.89	98.49	98.39	98.39	97.09	98.49	97.09	97.67	97.82	97.31	96.94	97.14	97.87	97.94	97.87	97.94	99.49	99.49	99.56		43	42	41	141
27	DQ107576 *Paraergasilus brevidigitus* CN	**97.59**	**97.31**	96.5	97.45	97.16	98.49	97.3	98.49	98.39	98.39	96.8	98.49	97.08	96.72	97.45	97.16	96.87	97.14	96.98	97.05	96.98	97.05	97.23	97.3	97.09	96.87		8	7	145
28	MW810239 *Paraergasilus longidigitus* CZ	**97.73**	**97.45**	96.72	97.59	97.3	98.29	97.45	98.29	98.19	98.19	96.94	98.29	97.23	96.87	97.74	97.3	97.16	97.37	97.28	97.42	97.28	97.42	97.31	97.37	97.16	96.94	99.42		3	142
29	DQ107574 *Paraergasilus medius* CN	**97.81**	**97.52**	96.79	97.52	97.38	98.39	97.52	98.39	98.29	98.29	97.01	98.39	97.3	96.94	97.81	97.38	97.16	97.44	97.35	97.42	97.35	97.42	97.38	97.44	97.23	97.01	99.49	99.78		142
30	MH982195 *Lernaea cyprinacea* CN	**90.17**	**89.98**	89.69	89.98	89.69	90.4	89.83	90.4	90.5	90.5	89.19	90.4	89.33	89.55	90.19	89.62	89.91	89.63	89.95	90.15	89.95	90.15	90.05	90.03	90.12	89.83	89.55	89.76	89.76	

Table 2.6: Genetic divergences among aligned 28S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country. Sequences from the present study in boldface and grey shade. *Lernaea cyprinacea* (MH982204) was used as the outgroup. Abbreviations: CN – China, CZ – Czech Republic, EG – Egypt, HU – Hungary, KR – South Korea, NI – Nicaragua, ZA – South Africa (Vaal River), ZM – Zambia (Zambezi River).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	**P38Ka_38VR *Ergasilus mirabilis* ZA**		**0**	**71**	**85**	**66**	**32**	**65**	**43**	**41**	**44**	**72**	**45**	**78**	**62**	**57**	**67**	**61**	**81**	**76**	**73**	**74**	**74**	**68**	**70**	**69**	**97**	**79**	**84**	**84**	**218**
2	**P49Ka_72ZR *Ergasilus mirabilis* ZM**	**100**		**71**	**85**	**67**	**32**	**66**	**43**	**41**	**44**	**73**	**45**	**78**	**62**	**57**	**68**	**61**	**82**	**76**	**73**	**74**	**74**	**69**	**70**	**70**	**98**	**79**	**85**	**85**	**218**
3	MN852851 *Acusicola margulisae* NI	**88.87**	**88.91**		71	50	66	43	70	70	73	49	72	51	54	50	53	49	79	64	62	62	63	45	49	49	82	67	71	71	202
4	DQ107528 *Ergasilus anchoratus* CN	**87.29**	**87.72**	88.89		74	77	63	80	80	85	78	84	76	73	69	78	64	100	81	80	80	81	72	72	71	25	9	12	11	228
5	DQ107532 *Ergasilus briani* CN	**90.15**	**90.33**	92.18	89.31		67	32	66	69	71	18	70	23	43	37	19	43	77	46	45	45	46	47	51	55	87	71	74	75	213
6	OQ407474 *Ergasilus caparti* BI	**95.1**	**95.1**	89.56	88.23	89.77		59	13	14	18	72	17	73	65	59	69	57	73	72	70	70	71	66	66	69	91	73	78	76	210
7	DQ107539 *Ergasilus hypomesi* CN	**90.3**	**90.48**	93.27	90.9	95.38	90.99		63	66	64	32	67	35	40	35	35	41	68	43	41	41	42	47	45	49	75	55	62	62	215
8	OQ407470 *Ergasilus macrodactylus* BI	**93.42**	**93.42**	88.92	87.77	89.92	98.01	90.38		9	15	72	6	73	70	62	68	57	71	74	73	73	74	67	68	72	90	76	79	79	205
9	OQ407471 *Ergasilus megacheir* BI	**93.72**	**93.72**	88.92	87.77	89.47	97.86	89.92	98.62		19	75	13	76	72	64	72	60	73	77	76	76	77	68	69	72	90	76	79	78	206
10	OQ407473 *Ergasilus parasarsi* BI	**93.26**	**93.26**	88.45	87	89.16	97.24	90.23	97.7	97.09		71	15	74	72	67	74	65	70	70	69	69	70	74	76	77	95	81	84	82	206
11	DQ107536 *Ergasilus parasiluri* CN	**89.24**	**89.45**	92.32	88.71	97.4	88.99	95.37	88.99	88.53	89.14		72	9	72	40	25	45	74	40	38	38	39	56	58	60	91	75	78	79	206
12	OQ407472 *Ergasilus parvus* BI	**93.11**	**93.11**	88.61	87.16	89.31	97.4	89.77	99.08	98.01	97.7	88.99		75	72	66	72	60	74	76	75	75	76	72	73	77	94	80	83	83	204
13	DQ107538 *Ergasilus scalaris* CN	**88.34**	**88.73**	92.01	89	96.67	88.84	94.93	88.84	88.38	88.69	98.7	88.53		72	40	30	46	73	40	36	38	37	55	56	59	91	74	78	79	205
14	MW810242 *Ergasilus sieboldi* CZ	**90.37**	**90.37**	91.25	88.68	93.33	89.83	93.8	89.05	88.73	88.26	92.86	88.42	92.86		10	44	46	66	53	51	51	52	49	49	50	90	71	76	79	215
15	OM812074 *Ergasilus sieboldi* EG	**91.01**	**91.01**	91.76	89.13	94.17	90.62	94.49	90.14	89.83	89.35	93.69	89.51	93.69	98.42		36	44	62	46	44	44	45	44	44	48	83	66	69	71	210
16	DQ107535 *Ergasilus tumidus* CN	**90**	**90.19**	91.71	88.73	97.25	89.47	94.94	89.62	89.01	88.7	96.38	89.01	95.66	93.18	94.33		46	74	43	41	41	42	52	52	58	91	75	78	77	213
17	DQ107531 *Ergasilus peregrinus* CN	**90.88**	**91.18**	92.32	90.74	93.78	91.28	94.07	91.28	90.83	90.06	93.48	90.83	93.33	92.86	93.06	93.34		66	59	58	58	59	43	46	42	80	59	66	69	207
18	DQ107540 *Ergasilus yaluzangbus* CN	**87.96**	**88.22**	87.73	85.65	88.95	88.91	90.24	89.21	88.91	89.36	89.37	88.75	89.51	89.81	90.28	89.38	90.52		78	75	77	76	71	74	73	111	94	98	97	212
19	MW810240 *Neoergasilus japonicus* CZ	**88.6**	**88.6**	90	87.75	93.04	89.07	93.57	88.76	88.3	89.37	94.02	88.46	94.02	91.82	92.8	93.57	91.14	88.29		2	2	1	55	60	55	92	80	81	79	216
20	MH167967 *Neoergasilus japonicus* CZ	**88.32**	**88.32**	89.92	87.18	92.79	88.8	93.43	88.32	87.84	88.96	93.9	88	94.22	91.84	92.85	93.43	90.69	88.08	99.76		0	1	54	58	54	91	79	80	78	212
21	MW810241 *Neoergasilus japonicus* CZ	**88.82**	**88.82**	90.24	87.9	93.19	89.3	93.8	88.84	88.38	89.45	94.24	88.53	94.24	92.06	93.04	93.8	91.21	88.44	99.77	100		1	54	58	54	91	79	80	78	215
22	MH167968 *Neoergasilus japonicus* CZ	**88.16**	**88.16**	89.76	87.02	92.63	88.64	93.27	88.16	87.68	88.8	93.74	87.84	94.06	91.68	92.68	93.27	90.53	87.92	99.92	99.84	99.84		55	59	55	92	80	81	79	213
23	DQ107530 *Paraergasilus brevidigitus* CN	**89.84**	**90.03**	92.95	89.58	93.2	89.91	93.2	89.76	89.6	88.69	91.88	88.99	92.03	92.39	93.06	92.47	93.77	89.8	91.67	91.33	91.82	91.17		9	8	84	67	71	71	205
24	MW810243 *Paraergasilus longidigitus* CZ	**89.36**	**89.36**	92.27	89.04	92.24	89.91	93.15	89.6	89.45	88.38	91.16	88.84	91.46	92.33	93	92.09	92.99	88.82	90.93	90.69	91.16	90.53	98.62		15	83	66	70	70	205
25	DQ107529 *Paraergasilus medius* CN	**89.69**	**89.88**	92.32	89.73	92.04	89.45	92.91	88.99	88.99	88.23	91.3	88.23	91.45	92.24	92.43	91.61	93.91	89.51	91.67	91.33	91.82	91.17	98.83	97.7		83	64	70	72	207
26	DQ107524 *Sinergasilus major* CN	**85.5**	**85.84**	87.17	96.37	87.43	86.09	89.16	86.24	86.24	85.47	86.83	85.63	86.83	86.05	86.93	86.85	88.42	84.07	86.08	85.42	86.23	85.26	87.84	87.37	87.99		21	19	22	239
27	MZ047815 *Sinergasilus major* HU	**87.92**	**87.92**	89.38	98.62	89.14	88.84	91.59	88.38	88.38	87.61	88.51	87.77	88.67	88.91	89.52	88.53	90.96	85.74	87.75	87.34	87.9	87.18	89.74	89.89	90.2	96.77		8	11	228
28	DQ107525 *Sinergasilus polycolpus* CN	**87.44**	**87.72**	88.89	98.26	89.31	88.07	91.04	87.92	87.92	87.16	88.71	87.31	88.71	88.22	89.13	88.73	90.45	85.94	87.75	87.18	87.9	87.02	89.73	89.35	89.87	97.24	98.77		9	227
29	DQ107526 *Sinergasilus undulatus* CN	**87.44**	**87.72**	88.89	98.4	89.16	88.38	91.04	87.92	88.07	87.46	88.57	87.31	88.57	87.75	88.82	88.87	90.01	86.08	88.05	87.5	88.2	87.34	89.73	89.35	89.58	96.8	98.31	98.69		229
30	MH982204 *Lernaea cyprinacea* CN	**69.12**	**69.12**	70.47	67.71	69.83	70.25	69.59	70.96	70.82	70.82	70.82	71.1	70.96	68.89	69.16	69.83	70.68	70.1	69.48	68.69	69.55	68.54	70.96	70.96	70.68	66.15	67.71	67.85	67.56	

Table 2.7: Genetic divergences among aligned COI sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/BOLD/Sequence ID, Taxon and Country. Sequences from the present study in boldface and grey shade. *Lernaea cyprinacea* (MH982220) was used as the outgroup. The taxon with an asterisk* was retrieved from the Barcode of Life Database (BOLD). Abbreviations: AR – Argentina, CA – Canada, CN – China, KR – South Korea, NI – Nicaragua, US – United States of America, ZA – South Africa (Vaal River), ZM – Zambia (Zambezi River).

		1	2	3	4	5	6	7	8	9	10	11	12
1	**P38Kb_38VR *Ergasilus mirabilis* ZA**		**10**	**132**	**139**	**122**	**122**	**139**	**114**	**133**	**134**	**149**	**150**
2	**P49Kb_72ZR Ergasilus mirabilis ZM**	**98.55**		**130**	**139**	**123**	**122**	**138**	**117**	**134**	**135**	**146**	**153**
3	ECTCR024-14 *Ergasilus lizae* CA*	**79.94**	**80.24**		134	118	114	137	109	125	133	126	144
4	KU557411_*Ergasilus* sp. AR	**79.32**	**79.32**	79.54		128	124	154	112	126	133	137	142
5	KR049035 *Ergasilus* sp. KR	**79.04**	**78.87**	79.73	78.01		14	115	108	107	102	117	138
6	KR049036 *Ergasilus wilsoni* KR	**79.04**	**79.04**	80.41	78.69	97.59		114	107	105	99	118	138
7	MN854870 *Acusicola margulisae* NI	**79.88**	**80.03**	79.18	77.08	80.24	80.41		103	118	133	138	149
8	KR049037 *Neoergasilus japonicus* KR	**80.41**	**79.90**	81.27	80.76	81.44	81.62	82.30		40	112	115	131
9	MZ964935 *Neoergasilus japonicus* US	**79.79**	**79.64**	81.00	80.76	81.62	81.96	82.07	93.13		124	130	136
10	KR263117 *Sinergasilus polycolpus* CN	**80.64**	**80.52**	79.79	80.21	82.47	82.99	80.75	80.76	81.16		107	139
11	MW080644 *Sinergasilus undulatus* CN	**78.47**	**78.93**	80.85	79.61	79.90	79.73	80.03	80.24	80.24	84.6		144
12	MH982220 *Lernaea cyprinacea* CN	**73.12**	**72.63**	73.38	73.61	72.51	72.51	73.25	73.90	74.86	75.1	74.2	

* Taxon from the Barcode of Life Database (BOLD)

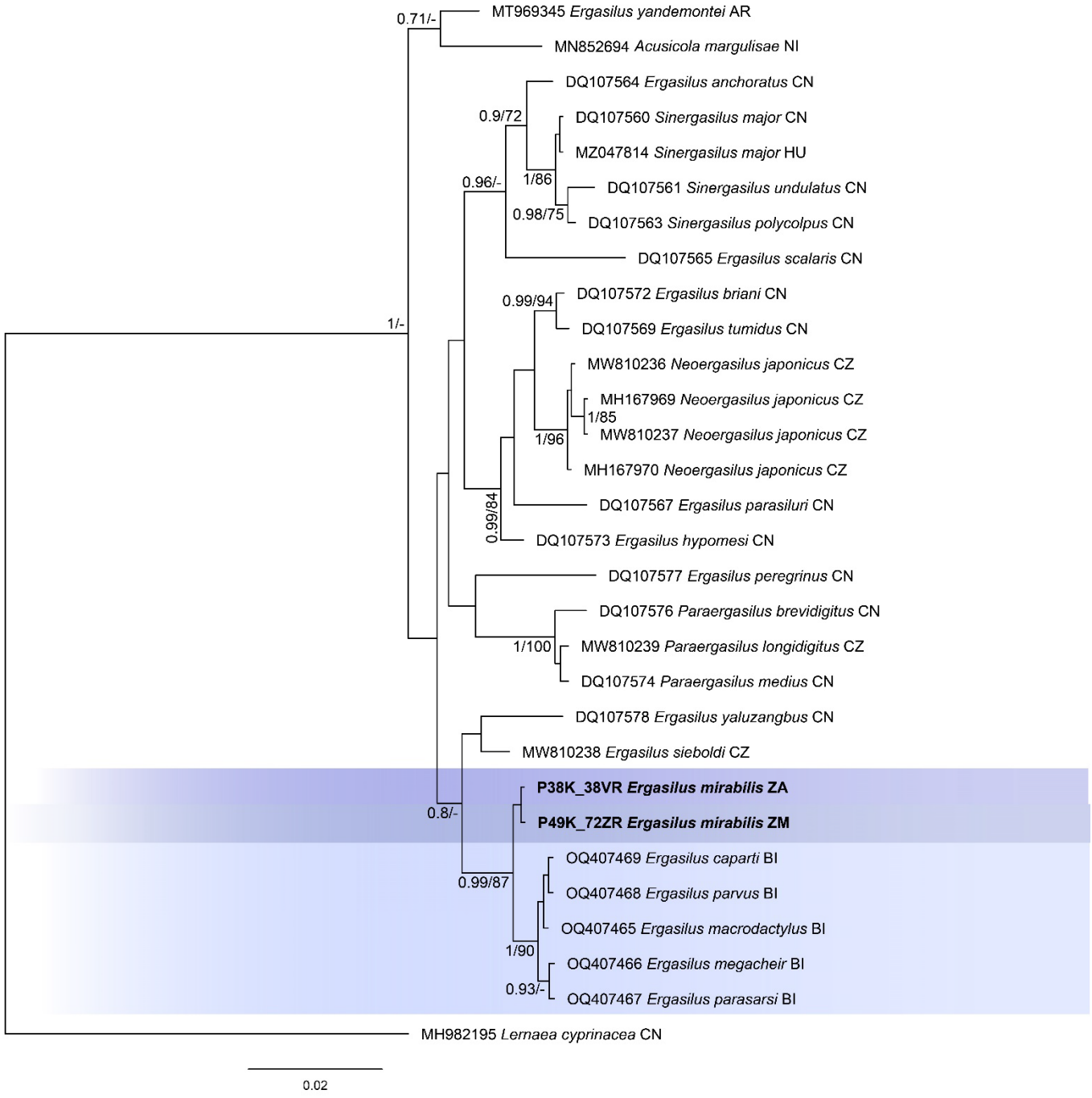


Figure 2.8: Phylogenetic tree of the Ergasilidae based on partial 18S rRNA gene alignments. Newly generated sequences appear in boldface in a gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: AR – Argentina, BI – Burundi, CN – China, CZ – Czech Republic, HU – Hungary, NI – Nicaragua, ZA – South Africa (Vaal River), ZM – Zambia (Zambezi River).

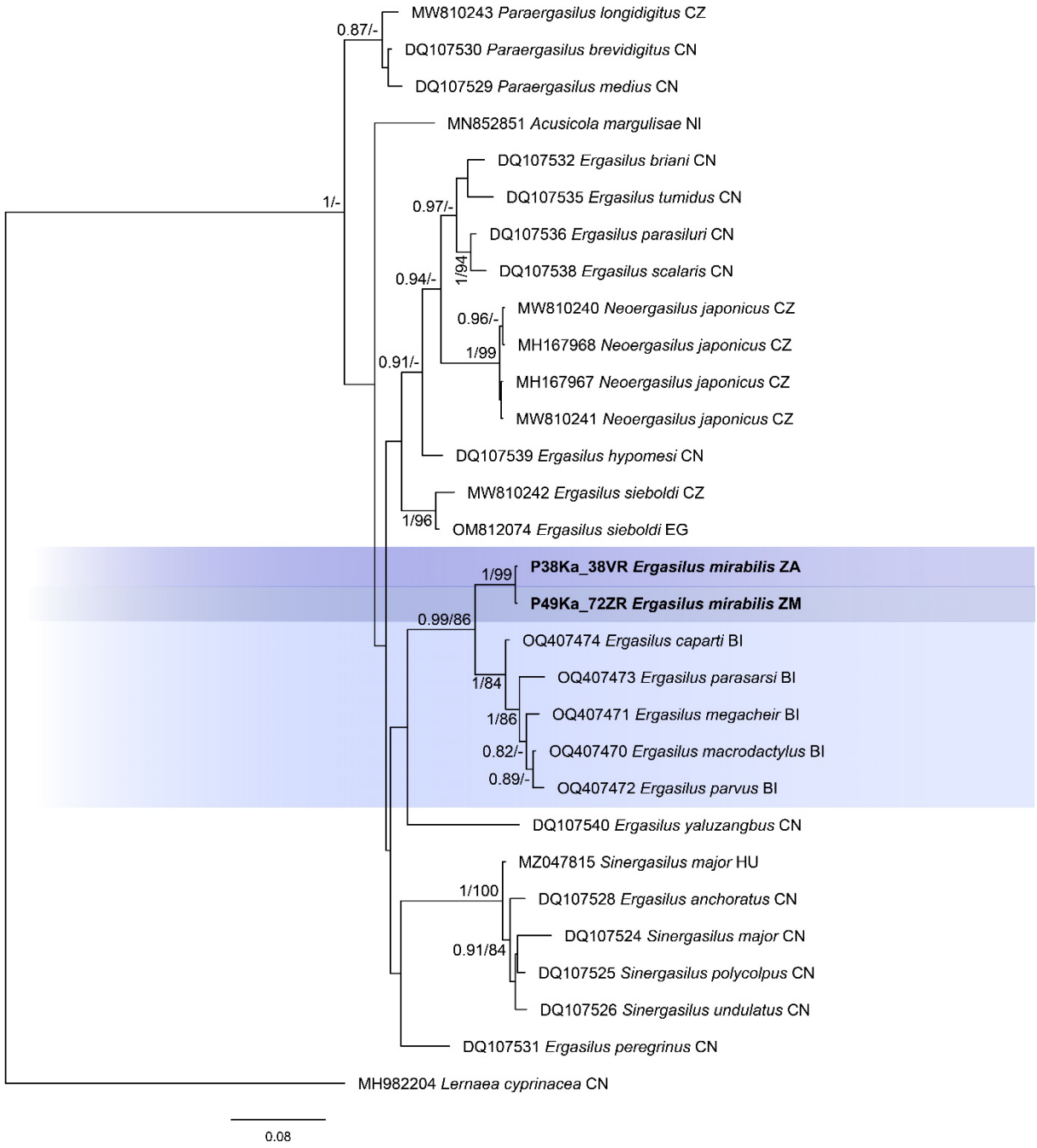


Figure 2.9: Phylogenetic tree of the Ergasilidae based on partial 28S rRNA gene alignments. Newly generated sequences appear in boldface in a gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: BI – Burundi, CN – China, CZ – Czech Republic, EG – Egypt, HU – Hungary, NI – Nicaragua, ZA – South Africa (Vaal River), ZM – Zambia – (Zambezi River).

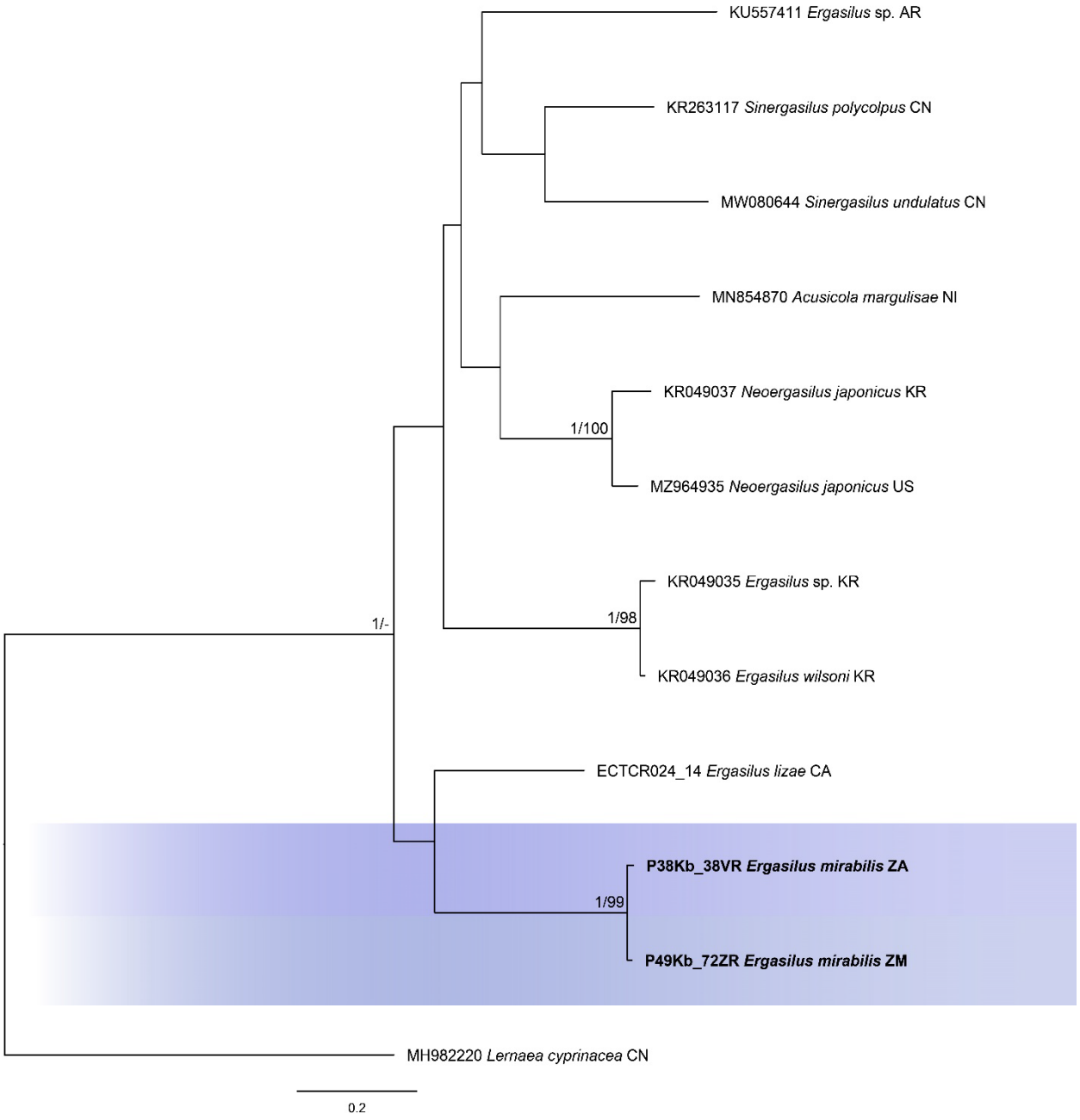


Figure 2.10: Phylogenetic tree of Ergasilidae based on partial COI mitochondrial gene alignments. Newly generated sequences are bold in a gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: AR – Argentina, CA – Canada, CN – China, KR – South Korea, NI – Nicaragua, US – United States of America, ZA – South Africa (Vaal River), ZM – Zambia (Zambezi River).

2.5. Discussion

Ergasilus mirabilis was originally described from the leopard squeaker *Synodontis leopardinus* in the Phongolo River, northern Natal, South Africa (Oldewage & van As, 1987a), with a report on some of its sensory structures and attachment to its host (Oldewage & van As, 1987b). According to FishBase (Froese & Pauly, 2023), however, the distribution of the leopard squeaker is in the Kunene, Okavango, and (upper) Zambezi rivers; it has not been reported in the Phongolo River. There is, therefore, the possibility of type host misidentification as the only other *Synodontis* species found in the Phongolo System is the plain squeaker, *Synodontis zambezensis* Peters, 1852 (see Skelton, 2001; Acosta et al., 2020). Furthermore, in almost a decade of fish sampling by the Water Research Group of the North-West University (Acosta et al., 2020), there has been no occurrence of the leopard squeaker in the Phongolo System. That notwithstanding, a year after its description, *E. mirabilis* was reported on 16 fish species across several regions in southern Africa, including the leopard squeaker from the Phongolo System as well as from the Zambezi River (Oldewage & van As, 1988b) (see Table 2.1). It is thus difficult to conclude if this report in 1988 was also a case of misidentification because the authors also reported *E. mirabilis* from other *Synodontis* species in the Zambezi River System (Oldewage & van As, 1988b) or if there is a variation of the plain squeaker from the Phongolo that may look similar to the leopard squeaker.

Among the 16 hosts reported in 1988 was the sharptooth catfish, *C. gariepinus* from the Zambezi River System (Oldewage & van As, 1988b). Avenant-Oldewage and Oldewage (1993) also reported *E. mirabilis* from *Marcusenius macrolepidotus*, *Synodontis nigromaculatus*, and *C. gariepinus* in the Kwando River, Caprivi, Namibia, which is a tributary of the Zambezi River. The present study confirms *C. gariepinus* as a host for *E. mirabilis* and supports the distribution record from the Zambezi River System. To the best of our knowledge, the current study adds the Kushokwe Pan as a new site in the Phongolo System. Additionally, Oldewage and van As (1988b) reported that there were no confirmed records of *E. mirabilis* in the Orange-Vaal River System at the time. The current study, therefore, gives the first record of this ergasilid species in the Vaal River in South Africa.

Ergasilus mirabilis is able to parasitise bottom feeders, pelagic species, predators, and scavengers because of its firm attachment to the host's gill filaments (Oldewage & van As, 1988b). Parasite intensity may vary depending on factors such as host preference and availability, seasonal variation, and environmental conditions (Avenant-Oldewage & Oldewage, 1993; Aladetohun et al., 2013a; Arif & Sheriff, 2022). As with other ergasilids, the attachment and feeding activities of these parasites can impact host tissue, interfere with respiration, cause irritations, and make fish susceptible to secondary infections (Fryer, 1968; Paperna & Zwerner, 1976a; Dezfuli et al., 2010; Kilian & Avenant-Oldewage, 2013). Avenant-Oldewage and Oldewage (1993) reported that *H. cuvieri* and *S. zambeziensis*, which were

previously reported as hosts for *E. mirabilis*, were present in the Kwando River System, Namibia, yet no parasite infestation by *E. mirabilis* was recorded. They thus suggested that host preference in *Ergasilus* might be multifactorial and not solely dependent on the availability of host species in a river system (Avenant-Oldewage & Oldewage, 1993). As the present study reports parasitisation for only the catfish, it is impossible to affirm this possibility of host preference by *E. mirabilis.* Following the published host range, it seems *E. mirabilis* is a generalist parasite, but more studies on several host species across several regions are recommended.

Heavy infestation of fishes has previously been reported for some *Ergasilus* species. Paperna and Zwerner (1976a, b) for instance, reported infestations of up to 2,757 *Ergasilus labracis* Krøyer, 1863 individuals on a single host, as well as several developmental stages of *E. labracis* on striped bass*, Morone saxatilis* (Walbaum, 1792), respectively. Abdel-Radi et al. (2022) also reported severe parasitisation of, and mortality in, cultured sea bream by *E. sieboldi*, which is a challenge in aquaculture. Although the prevalence of infestation from this study was low (10.06%), the mean intensity of parasitisation was 11.5 with up to 160 ergasilids collected (the highest record for *E. mirabilis*) from a single catfish host. Oldewage and van As (1987b) recorded infestation of approximately seven parasites per host in what they considered a “non-hyperparasitisation” period. From their study, they reported proliferation of gill tissues and distortion in gill structure, which they attributed to the parasite's feeding and attachment, respectively. In the Kwando River System, Namibia, an infestation prevalence of 81% by *E. mirabilis* was recorded from *C. gariepinus* (Avenant-Oldewage & Oldewage, 1993). The authors suggested that *C. gariepinus* might have been the preferred host of the four infected and 21 examined fishes in the system. Additionally, a total of 106 *E. mirabilis* was also reported from a specimen of *Cyphomyrus discorhynchus* (Peters, 1852) (syn. *Hippopotamyrus discorhynchus* (Peters, 1852)) by Douëllou and Erlwanger (1994) in Lake Kariba, Zimbabwe. Even though higher infestation levels have been reported for other *Ergasilus* species compared to *E. mirabilis*, future studies are recommended to investigate the potential for high infectivity by *E. mirabilis,* especially if fishes would be reared in capture environments, since all the currently available records of parasitisation by *E. mirabilis* are from natural or wild caught populations (see Paperna, 1975; Abdel-Radi et al., 2022; Shinn et al., 2023).

Morphological characters among specimens from the Vaal River, Kushokwe Pan, and the Zambezi River in this study, showed very little variation and were identified as *E. mirabilis*. When compared with the original description, certain subtle variations were observed which may be due to a number of limitations: slight mutation over time and across regions; subspecies variation (Mugridge et al., 1982); and observational errors (Hayden & Rogers, 1998), as seen with other ergasilid genera. Boxshall (2016) highlighted inconsistencies

as he compared swimming legs in original descriptions of *Ergasilus xenomelanirisi* Carvalho, 1955 and *Ergasilus jiangxiensis* Liu, 1998 with known Ergasilidae characteristics. He also explained that details such as antennular setation may differ from older descriptions because setae could have broken off or been overlooked, and the aesthetasc setae are difficult to observe. We therefore suggest that variations such as the number of antennular setae, as well as the presence or absence of sensory papillae and pores may be overlooked when confirming a species' identity. To ascertain if these disparities observed are inter- or intraspecific differences, a holistic view of all characters, comparing specimens to original descriptions and type specimens, if available, and noting the variations, is necessary.

Phylogenetic analyses corroborate the identity of this species belonging to the family Ergasilidae. The separate clades formed by newly generated sequences for all datasets (18S, 28S, and COI partial gene regions) further confirm its identity as an *Ergasilus* species distinct from its congeners used in the alignments. As previously reported, less divergence was recorded for the ribosomal RNA genes than for the faster evolving mitochondrial DNA gene, COI (Cristescu & Hebert, 2002). In the ribosomal RNA phylogenetic analyses, the Tanganyika sequences were, however, the closest evolutionarily to specimens from this study, forming a sub-Saharan evolutionary clade. *Ergasilus lizae*, although submitted from Canada, formed a sister clade with newly generated COI sequences. The study shows the possible evolutionary relationship between species ancestry and geographical distribution but with the limited amount of genetic data available, this concept cannot be further explored. That notwithstanding, since the specimens from the Vaal and Zambezi rivers, which are two completely different river systems in southern Africa, were similar molecularly (100% identical with the ribosomal genes), the molecular analysis from this study supports the distribution reports and affirms the status of *E. mirabilis* as a pan-southern African species.

From this study, the evolutionary positions of certain genera in the family Ergasilidae are consistent with Song et al. (2008): monophyly for both *Sinergasilus* Yin, 1949 and *Paraergasilus*, and polyphyly for *Ergasilus.* There is, however, a need for more genetic and morphological studies for species belonging to the genus *Ergasilus* and ultimately, the family Ergasilidae to enable further evolutionary studies within this family.

2.6. Conclusion

With a combination of taxonomic, morphological, and molecular techniques, the identity of the species from this study is confirmed as *Ergasilus mirabilis.* Phylogenetic analyses of all datasets showed that the newly generated sequences belonged to the family Ergasilidae, but clustered separately in clades with sequences of other *Ergasilus* species. An evolutionary relationship between species ancestry and parasite distribution is suggested among *Ergasilus*

species, as seen with the sub-Saharan species, but more genetic data is needed to further understand this relationship. This study serves as the first integrative study of *E. mirabilis*, using morphological and molecular techniques, with partial 18S, 28S, and COI genes as the molecular markers; moreover, adding six new sequences for an African ergasilid to the very scanty genetic database available in the family Ergasilidae. These novel sequences are the first available sequences for *E. mirabilis,* and the first sequences from the genus *Ergasilus* from southern Africa. The study also adds valuable information on the distribution of this ergasilid, providing a new locality record from the Vaal River System and the Kushokwe Pan in the lower Phongolo River Floodplain, South Africa, and the Barotse Floodplain as a new locality in the upper Zambezi River System, Zambia. These new locality reports suggest that this parasite is most probably present in other southern African rivers systems as well.

2.7. References

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CHAPTER 3

Molecular characterisation of three species of *Lamproglena* von Nordmann, 1832 from southern Africa, with descriptive notes

3.1. Abstract

Members of the genus *Lamproglena* von Nordmann, 1832 are gill ectoparasites of teleost fishes occurring mostly in Africa and Asia, with approximately 13 confirmed species in Africa and seven from southern Africa. The most frequently encountered species in southern Africa are *L. clariae* Fryer, 1956, and *L. monodi* Capart, 1944. In 2007, a new species, *Lamproglena hepseti* van As & van As, 2007 was described from the Okavango Delta, Botswana. The individual *Lamproglena* species are appreciably distinct morphologically but supporting molecular data would aid in taxa comparison as well as phylogenetic and evolutionary studies. Compared to other copepods, molecular studies on *Lamproglena* have received almost no attention. There are currently only three reports on the molecular characterisation of species from this genus, for two Asian species, *Lamproglena chinensis* and *L. orientalis*, and two African species, *L. monodi* and *L. hemprichii*. From 2016 to 2020, fishes from the families Cichlidae, Clariidae, and Hepsetidae were collected from selected rivers in southern Africa. Parasites were removed and morphologically identified using light and scanning electron microscopy. Three species, *L. clariae, L. hepseti,* and *L. monodi* were identified and subsequently sequenced using three genetic markers (partial 18S, 28S, and COI). The integration of morphological identification with genetics verifies the validity of the sequences. This study thus provides the first molecular characterisation of *L. clariae* and *L. hepseti* with descriptive notes, using three different genes (partial 18S, 28S, and COI), and the first partial COI sequence for *L. monodi*. New localities are reported for *L. clariae* in the Phongolo River and Nyamiti Pan (South Africa) and from the West Lunga (Zambia). This study also provides additional information on the maxilliped, antennae, and antennules of *L. hepseti.*

3.2. Introduction

The genus *Lamproglena* von Nordmann, 1832 (Crustacea: Copepoda) is the second largest in the family Lernaeidae Cobbold, 1879 (Cyclopoida), parasitising mostly freshwater fishes, except for two records: *Lamproglena lichiae* von Nordmann, 1832 which was reported on *Scomberoides lysan* (Forsskål, 1775) from the Red Sea (see Piasecki, 1993) and *Lamproglena chinensis* Yü, 1937, reported on *Pampus argenteus* (Euphrasen, 1788) from the

coastal waters of Pakistan (Batool et al., 2018). They are gill ectoparasites of teleost fishes occurring mostly in Africa (Okaeme et al., 1988; Marx & Avenant-Oldewage, 1996; Ibraheem & Izawa, 2000; van As & van As, 2007; Ibraheem et al., 2017; Kunutu et al., 2018; Mabika et al., 2022; Rindoria et al., 2022), Asia (Kuang & Qian, 1985; Kumari et al., 1989; Yambot & Lopez, 1997; Song et al., 2008; Batool et al., 2018), and have also been reported in Europe (Cakic et al., 1998; Galli et al., 2001).

Currently, there are 42 accepted species and one sub-species of *Lamproglena* (Walter & Boxshall, 2023), 38 of which are nominal species according to Kunutu et al. (2018). Two of the four species not included by Kunutu et al. (2018) have been synonymised with species within the same genus: *Lamproglena aubentoni* Dollfus, 1960 and *Lamproglena nyasae* Fryer, 1956 have been synonymised with *Lamproglena hemprichii* von Nordmann, 1832 (see Fryer, 1964) and *Lamproglena monodi* Capart, 1944 (see Fryer, 1959), respectively. Another species, *Lamproglena intercedens* Fryer, 1964, has been transferred to a different genus *Catlaphilla* Tripathi, 1960 (see Ho, 1998), within the family Lernaeidae; and *Lamproglena notopterai* Jafri & Mahar, 2006 should be placed into *species inquirendum* until its identity can be validated by collection and redescription of another specimen. The description for the latter species is considered inadequate, with illustrations lacking details of the taxonomic features (including cephalic appendages, legs, and abdominal structures) as given in other *Lamproglena* species descriptions (see Jafri & Mahar, 2006).

In Africa, 13 species have been reported from Botswana, Egypt, Nigeria, South Africa, Tanzania, Zambia, and Zimbabwe (see checklist in Table 3.1). Seven of these African species have been reported from southern Africa, namely: *Lamproglena barbicola* Fryer, 1961; *Lamproglena clariae* Fryer, 1956; *Lamproglena cornuta* Fryer, 1965; *Lamproglena hemprichii*; *Lamproglena hepseti* van As & van As, 2007; *Lamproglena hoi* Dippenaar, Luus-Powell & Roux, 2001; and *Lamproglena monodi* Capart, 1944 (see Table 3.1 for distribution records). Some species are more host specific, for instance, *Lamproglena hepseti*, parasitising a single host species *Hepsetus cuvieri* (Castelnau, 1861) the southern African pike; while others tend to be less host specific, such as *L. monodi*, parasitising a range of fish species in the family Cichlidae (Fryer, 1968).

The morphology of individual *Lamproglena* species is appreciably distinctive; however, the addition of molecular data can support morphology and distribution records that aid in taxa comparison and in studying the evolutionary relationships of the species. Compared to other parasitic copepods, molecular studies on *Lamproglena* have received very little attention. At the time of writing, there are only three reports on the molecular characterisation of this genus from Asia (Song et al., 2008), Egypt and Kenya (Rindoria et al., 2022), and Zimbabwe (Mabika et al., 2022). The report by Song et al. (2008) provided 12 sequences for two Asian species, *L. chinensis* and *L. orientalis* Markevich, 1936 for 18S and 28S ribosomal RNA genes.

Rindoria et al. (2022) provided ribosomal RNA data (18S and 28S) for *L. monodi* species from El-mina and Sharqia, Egypt, and the Kibos fish farm in Kenya. Mabika et al. (2022) reported on *L. hemprichii* from Lake Kariba in Zimbabwe, giving data for 18S and 28S gene regions. There is currently no genetic data available for the mitochondrial DNA gene region, COI for *Lamproglena* species. The aim of the present study was to use an integrative approach for characterisation of three African *Lamproglena* species using morphological and molecular techniques (partial 18S, 28S, and COI gene regions), to provide novel sequences for accurately identified specimens and further highlight distinct differences among these three *Lamproglena* species.

3.3. Materials and methods

3.3.1. Sampling

This study forms part of a larger parasitology project from the North-West University, Potchefstroom (Ethics numbers: NWU-00159-18-A5, NWU-00600-19-A5), and a collaborative study with the University of the Free State, Bloemfontein (Ethics Number: UFS-AED2016/0127), South Africa. A total of 206 fishes, comprising eight species, from the families Cichlidae, Clariidae, and Hepsetidae were caught using rod and reel, hook and line, nets, and electrofishing. From 2016–2020, sampling was carried out across selected sites in the Phongolo Floodplain (Nyamiti Pan, Phongolo and Usuthu rivers) and the Orange-Vaal River System (Boskop Dam, Mooi River and Vaal River), South Africa; the Kabompo River, Zambezi River, Zambia; and the Okavango River and Delta, Botswana (Fig. 3.1). Using standard techniques, fish were euthanised and gills dissected. The gills were screened for the presence of parasitic copepods with the aid of a Zeiss Stemi 305, and 508, and a Wild Heerbrugg M5A stereo microscope. Copepods were removed from hosts' gills using fine needles and tweezers and preserved in 70% and 96% ethanol for morphological and molecular analyses.

Table 3.1: Global distribution records of the 13 African *Lamproglena* von Nordmann, 1832 species. Data from the present study presented in boldface. Abbreviations: TH – Type Host, TLOC – Type Locality.

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
Lamproglena angusta Wilson, 1924	**TH:** *Malapterurus electricus* (Gmelin, 1789)	**TLOC:** Nile River	Malapteruridae	Freshwater		Wilson (1924); Fryer (1968)
Lamproglena barbicola Fryer, 1961	**TH:** *Labeobarbus altianalis* (Boulenger, 1900)	**TLOC:** Lake Victoria	Cyprinidae	Freshwater		Fryer (1961); Fryer (1968)
		Nile River		Freshwater		Fryer (1968)
Lamproglena clariae Fryer, 1956	**TH:** *Clariidae* spp.; *Clarias gariepinus* (Burchell, 1822)	**TLOC:** Banga River (part of Luweya River), Lake Malawi System (Malawi)	Clariidae	Freshwater		Fryer (1956)
	Clarias gariepinus (Burchell, 1822); Clariidae sp.; *Heterobranchus longifilis* Valenciennes, 1840	Tanzania: Lake Victoria; Malagarasi swamps	Clariidae	Freshwater		Fryer (1961)
	Clarias gariepinus (Burchell, 1822)	Sudan: White Nile, near Khartoum	Clariidae	Freshwater		Fryer (1965a)
		DRC: Congo River; Malawi: Lake Malawi; Lake Victoria; Nile River; Zambezi River	Clariidae	Freshwater		Fryer (1968)
	Clarias gariepinus (Burchell, 1822)	Uganda: Edward-George Lake System, Kazinga Channel	Clariidae	Freshwater		Thurston (1970)
	Clarias anguillaris (Linnaeus, 1758)	Nigeria: Galma River, lakes around Zaria	Clariidae	Freshwater		Shotter (1977)
	Clarias gariepinus (Burchell, 1822)	South Africa: Olifants River, Kruger National Park	Clariidae	Freshwater		Marx & Avenant-Oldewage (1996)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
Lamproglena clariae Fryer, 1956	*Clarias gariepinus* (Burchell, 1822)	Namibia: Cuando River, Caprivi	Clariidae	Freshwater		Marx & Avenant-Oldewage (1996)
	Clarias gariepinus (Burchell, 1822)	South Africa: Vaal River System (Bloemhof Dam, Deneysville, Douglas Wier, Grootdraai Dam, Vaal Dam, Vaal-Harts Wier, Yellowfish Paradise)	Clariidae	Freshwater		Tsotetsi et al. (2004, 2005); Madanire-Moyo & Avenant-Oldewage (2013); Moll & Avenant-Oldewage (2017); Gilbert et al. (2020); Pretorius & Avenant-Oldewage (2022, 2023); Ndaba et al. (2022); Latief et al. (2023)
	Clarias gariepinus (Burchell, 1822); *Clarias ngamensis* Castelnau, 1861	Botswana: Okavango River and Delta	Clariidae	Freshwater		van As & van As (2007)
	Clarias gariepinus (Burchell, 1822)	Zimbabwe: Manyame catchment	Clariidae	Freshwater		Madanire-Moyo & Barson (2010)
	Clarias gariepinus (Burchell, 1822)	South Africa: Flag Boshielo Dam & Nwanedi-Luphephe Dam, Olifants and Limpopo River, Limpopo	Clariidae	Freshwater		Madanire-Moyo et al. (2010, 2012)
	Clarias gariepinus (Burchell, 1822)	Egypt: Nile River tributaries	Clariidae	Freshwater		Hassanin et al. (2010)
	Clarias gariepinus (Burchell, 1822)	South Africa: Hout River Dam, Polokwane	Clariidae	Freshwater		Sara et al. (2014)
	Clarias gariepinus (Burchell, 1822)	South Africa: Lake Heritage, Crocodile River	Clariidae	Freshwater		Latief et al. (2023)
	***Clarias gariepinus* (Burchell, 1822)**	**Zambia: West Lunga**	**Clariidae**	Freshwater	**P19K (18S); P19Ka (28S); P19Ki (COI)**	**Present study**

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
Lamproglena clariae Fryer, 1956	***Clarias gariepinus* (Burchell, 1822)**	**South Africa: Phongolo River and Nyamiti Pan**	**Clariidae**	Freshwater	**P20R (18S); P20Ra (28S)**	**Present study**
	***Clarias gariepinus* (Burchell, 1822)**	**South Africa: Olifants River**	**Clariidae**	Freshwater	**P23R (18S); P23Ra (28S); P24Ra (28S)**	**Present study**
	***Clarias gariepinus* (Burchell, 1822)**	**South Africa: Vaal River**	**Clariidae**	Freshwater	**P25Ka (28S); P26Ka (28S); P27Ka (28S)**	**Present study**
	***Clarias gariepinus* (Burchell, 1822)**	**South Africa: Barberspan, Harts River**	**Clariidae**	Freshwater	**P28K (18S); P28Ka (28S); P28Ki (COI)**	**Present study**
Lamproglena cleopatra Humes, 1957	**TH:** *Labeo forskalii* Rüppell, 1835	**TLOC:** Nile River	Cyprinidae	Freshwater		Humes (1957); Fryer (1968)
	Labeo molybdinus du Plessis, 1963; *Labeo rosae* Steindachner, 1894	South Africa: Nwanedi-Luphephe Dam, Flag Boshielo Dam	Cyprinidae	Freshwater		Kunutu et al. (2018)
	Labeo rosae Steindachner, 1894; *Labeo ruddi* Boulenger, 1907	Zimbabwe: River Bubye	Cyprinidae	Freshwater		Kunutu et al. (2018)
Lamproglena cornuta Fryer, 1965	**TH:** *Heterobranchus bidorsalis* Geoffroy Saint-Hilaire, 1809 *Clarias gariepinus* (Burchell, 1822)	**TLOC:** Nile River	Clariidae	Freshwater		Fryer (1965a, 1968)
Lamproglena elongata Capart, 1956	**TH:** *Citharinus citharus* (Geoffroy Saint-Hilaire, 1809)	**TLOC:** Niger Basin, Mali	Citharinidae	Freshwater		Capart (1956)
		Senegal: Dakar		Brackish		Capart (1956)
		Lake Albert; Niger River; Nile River		Freshwater		Fryer (1968)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
Lamproglena hemprichii von Nordmann, 1832 **Syn**. *Lamproglena aubentoni* Dollfus, 1960	**TH:** *Hydrocynus forskahlii* (Cuvier, 1819)	**TLOC:** Red Sea, Egypt	Alestidae	Brackish; Freshwater		von Nordmann (1832); Piasecki (1993)
	Hydrocynus vittatus (Castelnau, 1861)	Zambia: Lake Bangweulu	Alestidae	Freshwater		Fryer (1959, 1968)
	Hydrocynus brevis (Günther, 1864)	Mali: Diafarabé, Niger River	Alestidae	Freshwater		Dollfus (1960)
	Hydrocynus vittatus (Castelnau, 1861)	Zambezi River	Alestidae	Freshwater		Fryer (1960)
	Hydrocynus sp.; *Hydrocynus forskahlii* (Cuvier, 1819)	Democratic Republic of the Congo: Crique de Banana estuary; Kalemie, Lake Albert; Lake Tanganyika	Alestidae	Brackish; Freshwater		Fryer (1965b, 1968)
		Congo River; Niger River; Nile River; Volta River; Zambezi River		Freshwater		Fryer (1968)
	Brycinus nurse (Rüppell, 1832)	Nigeria: Galma River	Alestidae			Shotter (1977)
	Hydrocynus vittatus (Castelnau, 1861)	Zimbabwe: Kasesse Bay, Lake Kariba	Alestidae	Freshwater		Douëllou & Erlwanger (1994)
	Hydrocynus vittatus (Castelnau, 1861)	Zimbabwe: Sanyati Basin, Lake Kariba	Alestidae	Freshwater	OP277526 (18S); OP277527 (28S)	Mabika et al. (2016, 2019, 2022)
Lamproglena hepseti van As & van As, 2007	**TH:** *Hepsetus odoe* (Bloch, 1794)	**TLOC:** Okavango River (Shakawe), Botswana	Hepsetidae	Freshwater		van As & van As (2007)
	Hepsetus odoe (Bloch, 1794)	Botswana: Okavango River & Delta (Nxamaseri; Seronga; Duba; Boro River)	Hepsetidae	Freshwater		van As & van As (2007)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	***Hepsetus cuvieri* (Castelnau, 1861)**	**Botswana: Okavango River**	**Hepsetidae**	**Freshwater**	**P42R (18S); P42Ra (28S); P42Rb (COI)**	**Present study**
Lamproglena hoi Dippenaar, Luus-Powell & Roux, 2001	**TH:** *Labeobarbus marequensis* (Smith, 1841); *Labeobarbus polylepis* (Boulenger, 1907)	**TLOC:** Spekboom River, Mpumalanga, South Africa	Cyprinidae	Freshwater		Dippenaar et al. (2001)
	Labeobarbus polylepis (Boulenger, 1907)	South Africa: Assegaai river; Elands River; Komati River; Phongolo River	Cyprinidae	Freshwater		Austin & Avenant-Oldewage (2009)
Lamproglena lichiae von Nordmann, 1832	**TH:** *Scomberoides lysan* (Forsskål, 1775)	**TLOC:** Red Sea, Egypt	Carangidae	Brackish; Freshwater		von Nordmann (1832); Piasecki (1993)
Lamproglena monodi Capart, 1944 **syn**. *Lamproglena nyasae* Fryer, 1956	**TH:** *Serranochromis thumbergi* (Castelnau, 1861)	**TLOC:** Katanga, Democratic Republic of the Congo	Cichlidae	Freshwater		Capart (1944)
	Haplochromis eduardii Regan, 1921; *Haplochromis macrops* (Boulenger, 1911); *Haplochromis nubilus* (Boulenger, 1906); *Haplochromis serridens* Regan, 1925; *Pseudocrenilabrus philander* (Weber, 1897); *Thoracochromis fasciatus* (Perugia, 1892)	Democratic Republic of the Congo: Kafubu River; Lake Edward; Lake Kibunga; Rutshuru River; Uele River	Cichlidae	Freshwater		Capart (1944)
	Docimodus spp.; *Haplochromis* spp.; *Lethrinops* spp.; *Petrotilapia* spp.; *Pseudotropheus* spp.; *Tilapia* spp.	Lake Malawi	Cichlidae	Freshwater		Fryer (1956, 1968)
	Sarotherodon galilaeus (Linnaeus, 1758)	Mali: Niger River	Cichlidae	Freshwater		Capart (1956)
	Coptodon zillii (Gervais, 1848)	Zambia: Lake Bangweulu	Cichlidae	Freshwater		Fryer (1959, 1968)
	Oreochromis macrochir (Boulenger, 1912) ; *Serranochromis* spp.	Zambia: Chilanga; Kafue River	Cichlidae	Freshwater		Fryer (1960)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
Lamproglena monodi Capart, 1944 **syn**. *Lamproglena nyasae* Fryer, 1956	*Oreochromis niloticus* (Linnaeus, 1758)	Tanzania: Malagarasi Swamps	Cichlidae	Freshwater		Fryer (1960)
	Tilapia spp.	Lake Albert	Cichlidae	Freshwater		Fryer (1960, 1968)
	Tilapia spp., *Haplochromis* spp.	Uganda: Lake Victoria and the Victoria Nile (White Nile)	Cichlidae	Freshwater		Fryer (1961, 1968)
	Oreochromis macrochir (Boulenger, 1912)*; Haplochromis* sp.; *Haplochromis nubilus* (Boulenger, 1906);	Lake Edward; Luapula River;	Cichlidae	Freshwater		Fryer (1965b, 1968)
	Haplochromis pappenheimi (Boulenger, 1914); *Haplochromis schubotzi* Boulenger, 1914; *Haplochromis serridens* Regan, 1925; *Haplochromis squamipinnis* Regan, 1921; *Hemichromis bimaculatus* Gill, 1862; *Hemichromis fasciatus* Peters, 1857; *Oreochromis macrochir* (Boulenger, 1912); *Oreochromis niloticus* (Linnaeus, 1758); *Oreochromis schwebischi* (Sauvage, 1884); *Orthochromis polyacanthus* (Boulenger, 1899); *Orthochromis stormsi* (Boulenger, 1902); *Orthochromis torrenticola* (Thys van den Audenaerde, 1963); *Pelmatochromis ocellifer* Boulenger, 1899; *Pseudocrenilabrus philander* (Weber, 1897); *Sarotherodon galilaeus* (Linnaeus, 1758); *Thoracochromis callichromus* (Poll, 1948); *Thoracochromis moeruensis* (Boulenger, 1899); *Thoracochromis schwetzi* (Poll, 1967); *Thoracochromis wingatii* (Boulenger, 1902); *Tylochromis labrodon* Regan, 1920	Angola: Cuango Cafunfo Democratic Republic of the Congo: Avakubi River; Bamania; Bosobolo; Budjala; Congo River; Fwa River; Ishango; Katanga; Kinshasha; Kisangani; Kunungu; Lake Edward; Lake Mweru; Lodja; Lukonzolwa; Lualaba River (Kasongo); Lubumbashi; Pweto; Tshuapa River; Uele River	Cichlidae	Freshwater		Fryer (1967, 1968)
		Niger River; Nile River; Zambezi River		Freshwater		Fryer (1968)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
Lamproglena monodi Capart, 1944 **syn**. *Lamproglena nyasae* Fryer, 1956	*Oreochromis niloticus* (Linnaeus, 1758)	Nigeria: Kainji Lake Research Institute	Cichlidae	Freshwater		Okaeme et al. (1988)
	Haplochromis heusinkveldi Witte & Witte-Maas, 1987; *Haplochromis hiatus* Hoogerhoud & Witte, 1981; *Haplochromis iris* Hoogerhoud & Witte, 1981; *Haplochromis macrognathus* Regan, 1922; *Haplochromis ptistes* Greenwood & Barel, 1978; *Haplochromis pyrrhocephalus* Witte & Witte-Maas, 1987; *Haplochromis teegelaari* Greenwood & Barel, 1978	Lake Victoria	Cichlidae	Freshwater		Witte & van Oijen (1990)
	Clarias gariepinus (Burchell, 1822); *Coptodon rendalli* (Boulenger, 1897); *Hepsetus odoe* (Bloch, 1794); *Oreochromis andersonii* (Castelnau, 1861); *Oreochromis macrochir (Boulenger, 1912); Serranochromis angusticeps* (Boulenger, 1907)*; Serranochromis macrocephalus* (Boulenger, 1899); *Sargochromis giardi* (Pellegrin, 1903)	Namibia: Kwando River, Caprivi	Alestidae; Clariidae; Cichlidae	Freshwater		Avenant-Oldewage & Oldewage (1993)
	Coptodon rendalli (Boulenger, 1897); *Oreochromis mortimeri* (Trewavas, 1966); *Pharyngochromis darlingi* (Boulenger, 1911); *Pseudocrenilabrus philander* (Weber, 1897); *Sargochromis codringtonii* (Boulenger, 1908); *Serranochromis macrocephalus* (Boulenger, 1899)	Zimbabwe: Lake Kariba	Cichlidae	Freshwater		Douëllou & Erlwanger (1994)
	A variety of cichlid species	Botswana: Okavango River and Delta	Cichlidae	Freshwater		van As & van As (2007)
	Haplochromis nyererei Witte-Maas & Witte, 1985; *Haplochromis pundamilia* (Seehausen & Bouton, 1998)	Makobe Island, Lake Victoria	Cichlidae	Freshwater		Maan et al. (2008)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
	Cyprinus carpio Linnaeus, 1758	Mozambique: Limpopo River and Chuáli Lagoon	Cyprinidae	Brackish; Freshwater		Boane et al. (2008)
	Coptodon zillii (Gervais, 1848); *Oreochromis niloticus* (Linnaeus, 1758); *Oreochromis aureus* (Steindachner, 1864); *Sarotherodon galilaeus* (Linnaeus, 1758)	Egypt: El- Abbassa, Giza Kalubia, and Qena fish farms; Nile River	Cichlidae	Freshwater		Ibraheem & Izawa (2000); Hassanin et al. (2010); El-Seify et al. (2013, 2018); Hassan et al. (2013); Marzouk et al. (2013); Mahmoud et al. (2014); Abdel-Gaber et al. (2017); Ibraheem et al. (2017); Malki et al. (2021)
Lamproglena monodi Capart, 1944 **syn**. *Lamproglena nyasae* Fryer, 1956	*Oreochromis niloticus* (Linnaeus, 1758)	Burkina Faso: Loumbila and Ziga reservoirs	Cichlidae	Freshwater		Sinaré et al. (2016)
	Astatoreochromis alluaudi Pellegrin, 1904; *Copadichromis cyaneus* (Trewavas, 1935); *Haplochromis* spp.; *Haplochromis chilotes* (Boulenger, 1911); *Haplochromis gigas* (Seehausen & Lippitsch, 1998); *Haplochromis luteus* (Seehausen & Bouton, 1998); *Haplochromis mbipi* (Lippitsch & Bouton, 1998); *Haplochromis melanopterus* Trewavas, 1928; *Haplochromis nyererei* Witte-Maas & Witte, 1985; *Haplochromis omnicaeruleus* (Seehausen & Bouton, 1998); *Haplochromis pundamilia* (Seehausen & Bouton, 1998); *Haplochromis rufocaudalis* (Seehausen & Bouton, 1998); *Haplochromis sauvagei* (Pfeffer, 1896); *Haplochromis vonlinnei* van Oijen & de Zeeuw, 2008	Tanzania: Kissenda and Makobe, Lake Victoria,	Cichlidae	Freshwater		Karvonen et al. (2018); Gobbin et al. (2020; 2021a, b; 2023)
	Oreochromis niloticus (Linnaeus, 1758)	Egypt: Edfina (Natural Nile	Cichlidae			Ashmawy et al. (2018)

Species	Hosts	Distribution	Host Families	Water Body	Genetic data	References
		branch), Lake Idku, Lake Mariout				
	Oreochromis niloticus (Linnaeus, 1758)	Mauritania: Bank of Senegal River, Rosso	Cichlidae			M'bareck et al. (2019)
	Haplochromis spp.	Kenya: Lake Victoria	Cichlidae	Freshwater		Outa et al. (2021)
Lamproglena monodi Capart, 1944 **syn**. *Lamproglena nyasae* Fryer, 1956	*Oreochromis niloticus* (Linnaeus, 1758)	Kenya: Kibos Fish Farm; Egypt: Sharqia Governorate and El-Minia	Cichlidae	Freshwater	ON419438–ON419452 (18S); ON419422–ON419437 (28S)	Rindoria et al. (2022)
	***Coptodon rendalli* (Boulenger, 1897); *Oreochromis andersonii* (Castelnau, 1861); *Oreochromis macrochir* (Boulenger, 1912); *Sargochromis carlottae* (Boulenger, 1905); *Serranochromis angusticeps* (Boulenger, 1907); *Pharyngochromis acuticeps* (Steindachner, 1866)**	**Botswana: Okavango River**	**Cichlidae**	**Freshwater**	**P18K (18S); P18Ka (28S); P18Kb (COI)**	**Present Study**
Lamproglena werneri Zimmermann, 1922	**TH:** *Bagrus bajad* (Forsskål, 1775)	**TLOC:** Nile River, Sudan	Bagridae	Freshwater		Zimmermann (1922); Fryer (1968)
	Auchenoglanis occidentalis (Valenciennes, 1840)	Nigeria: River Galma	Claroteidae	Freshwater		Shotter (1977)
Lamproglena wilsoni Capart, 1956	**TH:** *Clarotes laticeps* (Rüppell, 1829)	**TLOC:** Niger River, Mali	Claroteidae	Freshwater		Capart (1956); Fryer (1968)
		Senegal: Dakar		Brackish		Capart (1956)
	Chrysichthys nigrodigitatus (Lacepède, 1803)	Nigeria: River Benue	Claroteidae	Freshwater		Shotter (1977)

The necessary permits were received from Ezemvelo KZN Wildlife (KwaZulu-Natal, permit Nos. OP 1075/2017, OP 1582/2018); Department of Rural, Environmental and Agricultural Development (North West, permit no. HO 20/02/18-057 NW); the Department of Economic, Small Business Development, Tourism and Environmental Affairs (DESTEA, Free State, permit no. JM 4066/2018); the Department of Economic Development, Environmental Affairs and Tourism (Eastern Cape, permit no. CRO 20/18CR, CRO 22/18CR); CapeNature (Western Cape, permit no. CN44-31-6790); and permission for joint research in the Upper Zambezi Basin, Zambia. Host nomenclature is from FishBase (Froese & Pauly, 2023) (www.fishbase.org), and host and parasite authorities (except for the synonymy) are not included in the references.



Figure 3.1: Map showing all sampling localities in southern Africa where adult female *Lamproglena* species were collected for this study.

3.3.2. Morphological analysis

Light microscopy (LM) and scanning electron microscopy (SEM) were used to study specimens. For LM, *Lamproglena* specimens were cleared in lactic acid for 40–60 minutes and stained in lignin pink for 30 minutes to highlight appendages for examination. Photomicrographs of specimens were taken with the aid of a ZEISS Axiocam ERc 55 camera attached to the Zeiss stemi 508 stereomicroscope. Measurements of specimens were done in millimetres using Labscope Mat Ver. 2.8.4 microscope software; expressed as mean ± standard deviation (with range in parentheses).

Representative adlult females of all three *Lamproglena* species (*L. clariae*, *L. hepseti*, *L. monodi*) were selected for SEM. Specimens were dehydrated in graded ethanol series (70%, 80%, 95% and 100%) and critically point dried or immersed in a series of graded HMDS (1,1,1,3,3,3-Hexamethyldisilazane) (Merck, Germany), at five minutes per 30%, 50%, 70%, 100%, 100% concentration (to remove ethanol) and then left to dry. All dried specimens were mounted on carbon tape aluminium stubs, sputter coated in gold palladium (Emscope SC500, Quorum Technologies, Newhaven, UK or SPI-module, SPI Supplies, Pennsylvania, USA), and viewed with a JEOL Winsem JSM IT 200 or a Phenom ProX Desktop SEM (ThermoScientific, Waltham, Massachusetts, USA). Photomicrographs were taken at 5KVa. All photomicrographs used were edited using Adobe Photoshop (v.23.4.2) and Adobe Illustrator (v. 26.3.1).

3.3.3. Infestation rates

Infestation levels of parasitic copepods were calculated as prevalence, mean intensity, and mean abundance, following Bush et al. (1997).

3.3.4. Molecular analysis

Genomic DNA was extracted from whole adult females as well as egg strings. The Macherey Nagel tissue kit (Sandton, South Africa) was used for extraction following the manufacturer's protocol. The PCRBIO Rapid Extract PCR Kit (PCRBiosystems, Analytical Solutions, Randburg, South Africa) was also used for DNA extraction and adapted as follows: 20 µl of lysis buffer, 10 µl of protease containing buffer and 150 µl molecular grade water for dilution.

Partial ribosomal RNA gene regions (18S and 28S), and a mitochondrial DNA gene region (Cytochrome *c* oxidase I, COI) were used as genetic markers. Primers by Song et al. (2008) were used for amplification of partial 18S (18SF, 18SR) and 28S (28SF, 28SR) genes. Two sets of primers were used for the amplification of the partial COI gene region: LCO1490 and HCO2198 (Folmer et al., 1994), and CrustDF1 and CrustDR1 (Cheng et al., 2019); the later primer set was used for amplification of *L. clariae* only (see Table 3.2). Each Polymerase Chain Reaction (PCR) mixture of 25 µl total volume included: 1.25 µl of 10 µM of each primer

(forward and reverse), 12.5 µl DreamTaq PCR Master Mix (2X) (ThermoFischer Scientific, Waltham, Massachusetts, USA), 7 µl molecular grade water, and 3 µl extracted DNA. Thermal cycler PCR conditions followed Song et al. (2008) for partial 18S and 28S gene regions, and Hayes et al. (2021) for the partial COI gene region. Positive PCR products were verified in a 1% agarose gel. Purification and sequencing of amplicons were done in both directions by a commercial sequencing company, Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa).

Forward and reverse sequences were viewed, assembled, aligned, and edited, if necessary, with Geneious Prime v. 2022.2.2 (Biomatters, Auckland, New Zealand). Sequences newly generated from this study were compared with closely related sequences available on GenBank using the nucleotide Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Representative 18S and 28S sequences for all four species of *Lamproglena* available on GenBank were downloaded for alignment. There were no COI sequences available on GenBank or The Barcode of Life Database for *Lamproglena*, so only newly generated sequences from this study for the different *Lamproglena* species were aligned in Geneious. *Lernaea cyprinacea* Linnaeus, 1758 (Lernaeidae) was used as the outgroup for all datasets (18S, 28S, COI) (see Table 3.3).

Using the Geneious Prime software, novel sequences and selected GenBank sequences for 18S, 28S, and COI, were aligned following implemented default parameters of MAFFT v7.490 (Katoh et al., 2002; Katoh & Standley, 2013), and trimmed. Genetic divergences among all aligned specimens were generated in Geneious Prime v. 2022.2.2 and expressed as differences in the number of nucleotides and percentage identities. The Akaike Information Criterion (AIC) implemented in the jModelTest 2.1.4 (Posada, 2008; Darriba et al., 2012) estimated the GTR+I and GTR+G nucleotide substitution model for 18S and 28S, respectively. The suggested models of nucleotide evolution were applied accordingly, for the Maximum Likelihood (ML) and Bayesian Inference (BI) phylogenetic analyses. With estimated model parameters and bootstrap values of one thousand repetitions, ML analyses were run on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) using PhyML v. 3.0 (Guindon et al., 2010).

Table 3.2: List of primers used per species, their sequences, and references as well as the different materials used for respective DNA extractions. Abbreviations: Ad – adult, Es(1) – one egg string, Es(2) – two egg strings, L(2) – two larval specimens, Tk – Machery-Nagel Tissue extraction kit, Rp – Rapid PCRBio Systems extraction Kit.

Gene regions	Specimen ID	DNA extraction material	DNA extraction method	Primers	Sequences	Sources
18S	P18K, P19K, P20R, P23R, P28K, P42R	Ad Ad Ad Es(1) Ad Es(1)	Tk Tk Rp Rp Tk Rp	18SF	5′-AAG GTG TGM CCT ATC AAC T-3′	Song et al. (2008)
				18SR	5′-TTA CTT CCT CTA AAC GCT C-3′	
28S	P18Ka, P19Ka, P20Ra, P23Ra, P24Ka, P25Ka, P26Ka, P27Ka, P28Ka, P42Ra	Ad Ad Ad Es(1) Es(2) Ad Ad L(2) Ad Es(1)	Tk Tk Rp Rp Tk Tk Tk Tk Tk Rp	28SF	5′-ACA ACT GTG ATG CCC TTA G-3′	
				28SR	5′-TGG TCC GTG TTT CAA GAC G-3′	
COI	P18Kb, P42Rb	Ad Es(1)	Tk Rp	LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer et al. (1994)
				HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	
	P19Ki, P28Ki,	Ad Ad	Tk Tk	CRUSTDF1	5′-GGTCWACAAAYCATAAAGAYATTGG-3'	Cheng et al. (2019)
				CRUSTDR1	5′-TAAACYTCAGGRTGACCRAARAAYCA-3'	

Table 3.3: List of sequences included in the phylogenetic analyses. Taxa in bold fonts are sequences generated from the present study, all other sequences are GenBank sequences. *Lernaea cyprinacea* Linneaus, 1758 (in grey shade) was used as the outgroup.

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Lamproglena chinensis	*Channa argus*	Dangjiangkou, China	DQ107553	DQ107545		Song et al. (2008)
Lamproglena clariae	***Clarias gariepinus***	**West Lunga, Zambia**	**P19K**	**P19Ka**	**P19Ki**	**Present study**
Lamproglena clariae	***Clarias gariepinus***	**Phongolo River, South Africa**	**P20R**	**P20Ra**		**Present study**
Lamproglena clariae	***Clarias gariepinus***	**Nyamiti Pan, Olifants River, South Africa**	**P23R**	**P23Ra, P24Ka**		**Present study**
Lamproglena clariae	***Clarias gariepinus***	**Vaal River, South Africa**		**P25Ka, P26Ka, P27Ka**		**Present study**
Lamproglena clariae	***Clarias gariepinus***	**Barberspan, Harts River, South Africa**	**P28K**	**P28Ka**	**P28Ki**	**Present study**
Lamproglena hemprichii	*Hydrocynus vittatus*	Lake Kariba, Zimbabwe	OP277526	OP277527		Mabika et al. (2022)
Lamproglena hepseti	***Hepsetus cuvieri***	**Okavango River, Botswana**	**P42R**	**P42Ra**	**P42Rb**	**Present study**
Lamproglena monodi	*Oreochromis niloticus*	El-Minia, Egypt	ON419448	ON419432		Rindoria et al. (2022)
Lamproglena monodi	*Oreochromis niloticus*	Sharqia, Egypt	ON419450	ON419435		Rindoria et al. (2022)

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Lamproglena monodi	*Oreochromis niloticus*	Kibos Fish Farm, Kenya	ON419439	ON419422		Rindoria et al. (2022)
Lamproglena monodi	***Serranochromis angusticeps***	**Okavango River, Botswana**	**P18K**	**P18Ka**	**P18Kb**	**Present study**
Lamproglena orientalis	*Chanodichthys dabryi*	Tangxun Lake, China	DQ107549	DQ107542		Song et al. (2008)
Lamproglena orientalis	*Chanodichthys dabryi*	E-zhou farm, China	DQ107550	DQ107543		Song et al. (2008)
Lamproglena orientalis	*Squaliobarbus curriculus*	Dangjiangkou, China	DQ107552	DQ107544		Song et al. (2008)
Lernaea cyprinacea	*Cyprinus carpio,*	Dongxi Lake, China	DQ107556			Song et al. (2008)
Lernaea cyprinacea	*Opsariichthys bidens*	Dangjiangkou, China		DQ107548		Song et al. (2008)
Lernaea cyprinacea	*Chanodichthys ilishaeformis*	China			MH982220	Hua et al. (2019)

Nodal support for ML analyses was estimated at one hundred bootstrap repetitions. Bayesian Inference analyses were performed on computational resource CIPRES Science Gateway v 3.3 (Miller et al., 2010) (https://www.phylo.org/), adapting set parameters of MrBayes v. 3.2.7a. (Ronquist et al., 2012), running two independent Markov Chain Monte Carlo (MCMC) runs of four chains for ten million generations, and sampling tree topologies every one thousand generations. Burn-in parameters were set to the first twenty-five thousand generations. Phylogenetic trees for BI and ML outputs were visualised in FigTree v 1.4.4 software (Rambaut, 2012).

3.4. Results

3.4.1. Taxonomy

Order Cyclopoida Burmeister, 1834

Family Lernaeidae Cobbold, 1879

Genus *Lamproglena* von Nordmann, 1832

Parasitic copepods from this study were morphologically identified as three different African *Lamproglena* species: *Lamproglena clariae*, *L. hepseti*, and *L. monodi*. Morphological characterisation of adult female copepods was based on the general body shape, cephalothoracic shape, antennules, antennae, maxillae, maxilliped, legs, and furcal rami, following the description and notes from Marx and Avenant-Oldewage (1996) for *L. clariae*; van As and van As (2007) for *L. hepseti*; and Ibraheem et al. (2017) and Rindoria et al. (2022) for *L. monodi*. Descriptive notes are given for all three species. Host data and infestation rates (prevalence, mean intensity and mean abundance) are provided in Table 3.4, with new distribution records of *L. clariae*. Detailed measurements for representatives of all three species are provided in Table 3.5 to compare certain morphological features across the different species.

A total of 314 lamproglenids (238 *L. clariae*; 20 *L. hepseti*; 56 *L. monodi*) were collected. Of these 25 were used for SEM; five were used for dissection; seven adult females, one larva, and four egg strings were used for DNA extraction; 30 specimens will be deposited in the collections of the National Museum, Bloemfontein, South Africa (NMB); and the remainder are in the collection of the Water Research Group, Potchefstroom, South Africa. Species-specific information including host and locality is provided in Table 3.4.

Table 3.4: Host infestation data for three African *Lamproglena* species with distribution in South Africa, Zambia, and Botswana. Abbreviations: MA – Mean Abundance, MI – Mean Intensity, N(E) – number of hosts examined, N(I) – number of hosts infested, P – Prevalence, PC – number of parasites collected.

Species	Host	Date	Locality	N(E)	N(I)	PC	P	MI	MA
Lamproglena clariae	*Clarias gariepinus*	2019–2020	South Africa: Barberspan	21	17	52	80.95	3.06	2.48
		2018	South Africa: Boskop Dam, Nyamiti Pan, Phongolo River, Usuthu River	22	6	9	27.27	1.50	0.41
		2019	South Africa: Vaal River	18	14	172	77.78	12.29	9.56
		2019	Zambia: Kabompo River	2	1	2	50	2	1
Lamproglena hepseti	*Hepsetus cuvieri*	2017	Botswana: Okavango River	1	1	20	100.00	20.00	20.00
Lamproglena monodi	*Oreochromis macrochir*	2017	Botswana: Okavango River	2	2	3	100.00	1.50	1.50
	Pharyngochromis acuticeps	2017		1	1	6	100.00	6.00	6.00
	Sargochromis carlottae	2017		3	3	11	100.00	3.67	3.67
	Serranochromis angusticpes	2016		1	0	0	0.00	0.00	0.00
	Serranochromis angusticpes	2017		5	3	19	60.00	6.33	3.80
	Oreochromis andersonii	2017		6	4	8	66.67	2.00	1.33
	Coptodon rendalli	2017		7	5	9	71.43	1.80	1.29

Table 3.5: Measurements of representatives of all three *Lamproglena* von Nordmann, 1832 species observed in the current study, given as mean ± standard deviation (with range in parentheses).

Structures	***Lamproglena clariae***	***Lamproglena hepseti***	***Lamproglena monodi***
Number of specimens measured	10	5	6
Total Length	6.98 ± 0.60 (5.84–7.83)	4.17 ± 0.23 (3.71–4.36)	3.41 ± 0.38 (3.02–4.15)
Cephalothorax Length	0.68 ± 0.04 (0.63–0.77)	0.64 ± 0.08 (0.49–0.70)	0.69 ± 0.05 (0.61–0.76)
% Cephalothorax of Total Length	8.81–11.01	11.59–18.85	17.26–21.72
Cephalothorax Width	0.57 ± 0.11 (0.47–0.68)	0.85 ± 0.08 (0.75–0.96)	0.60 ± 0.07 (0.49–0.71)
Full Thorax Length	3.69 ± 0.32 (3.04–4.07)	1.49 ± 0.11 (1.28–1.57)	1.12 ± 0.14 (0.98–1.32)
Length Thoracic Seg. 1	0.49 ± 0.08 (0.34–0.63)	fused with cephalosome	fused with cephalosome
Width Thoracic Seg. 1	0.29 ± 0.02 (0.26–0.31)	fused with cephalosome	fused with cephalosome
Length Thoracic Seg. 2	0.79 ± 0.10 (0.62–0.94)	0.35 ± 0.03 (0.31–0.41)	0.25 ± 0.06 (0.18–0.33)
Width Thoracic Seg. 2	0.29 ± 0.02 (0.26–0.33)	0.58 ± 0.05 (0.53–0.66)	0.55 ± 0.06 (0.44–0.63)
Length Thoracic Seg. 3	1.05 ± 0.11 (0.87–1.23)	0.48 ± 0.05 (0.38–0.53)	0.41 ± 0.06 (0.35–0.51)
Width Thoracic Seg. 3	0.54 ± 0.02 (0.51–0.57)	0.68 ± 0.03 (0.64–0.73)	0.72 ± 0.08 (0.61–0.86)
Length Thoracic Seg. 4	0.92 ± 0.07 (0.80–0.99)	0.66 ± 0.05 (0.57–0.72)	0.46 ± 0.05 (0.39–0.54)
Width Thoracic Seg. 4	0.64 ± 0.05 (0.55–0.70)	0.71 ± 0.04 (0.65–0.75)	0.70 ± 0.07 (0.60–0.81)
Length Genital somite	0.43 ± 0.03 (0.40–0.49)	0.34 ± 0.05 (0.27–0.41)	0.33 ± 0.04 (0.26–0.40)
Width Genital somite	0.49 ± 0.03 (0.45–0.57)	0.47 ± 0.04 (0.42–0.52)	0.47 ± 0.07 (0.40–0.61)
Full Abdomen Length	2.61 ± 0.27 (2.16–3.01)	1.64 ± 0.13 (1.40–1.75)	1.16 ± 0.22 (0.96–1.59)
Length Abdomen Seg. 1	0.94 ± 0.12 (0.79–1.12)	0.63 ± 0.13 (0.39–0.75)	0.44 ± 0.09 (0.37–0.62)
Width Abdomen Seg. 1	0.35 ± 0.02 (0.33–0.38)	0.35 ± 0.03 (0.30–0.39)	0.33 ± 0.04 (0.28–0.40)
Length Abdomen Seg. 2	0.78 ± 0.08 (0.66–0.92)	0.59 ± 0.03 (0.55–0.64)	0.40 ± 0.07 (0.31–0.53)
Width Abdomen Seg. 2	0.33 ± 0.01 (0.32–0.35)	0.32 ± 0.03 (0.27–0.35)	0.30 ± 0.04 (0.26–0.37)
Length Abdomen Seg. 3	0.82 ± 0.08 (0.67–0.94)	0.42 ± 0.05 (0.36–0.48)	0.32 ± 0.07 (0.23–0.44)
Width Abdomen Seg. 3	0.31 ± 0.01 (0.29–0.33)	0.24 ± 0.02 (0.21–0.26)	0.26 ± 0.02 (0.24–0.30)
Length Caudal/ Furcal Rami	0.07 ± 0.03 (0.03–0.13)	0.07 ± 0.03 (0.04–0.12)	0.12 ± 0.03 (0.07–0.14)

***Lamproglena clariae* Fryer, 1956**

Material examined.

A total of 184 copepods were collected from the gills of *C. gariepinus.*

South Africa: Boskop Dam (26°32'44.4"S 27°07'09.7"E), Nyamiti Pan (26°53'26.0"S 32°17'52.7"E), Phongolo River (26°55'27.7"S 32°19'43.0"E), Usuthu River (26°50'55.9"S 32°16'47.5"E), Vaal River (27°23'38.5"S 26°30'44.7"E), collected by M. Truter (2018–2019); Baberspan (26°35'24.1"S 25°35'09.5"E), collected by P. P. Fikiye (2019–2020).

Zambia: Kabompo River (13°04'56.9"S 24°40'58.3"E), collected by M. Truter (2019).

Representative DNA sequences. Sequences, with the numbers of base pairs (bp), will be submitted to GenBank as follows:

18S. *Lamproglena clariae* 1,382 bp (Phongolo Floodplain); 1,327 bp (Nyamiti Pan), and 1,329 bp (Barberspan) (in South Africa); and 1,327 bp (Kabompo River, West Lunga) (Zambia).

28S. *Lamproglena clariae* 749 bp (Barberspan & Phongolo Floodplain, three sequences from Vaal River); 749 & 750 bp (Nyamiti Pan) (in South Africa); and 750 bp (Kabompo River, West Lunga) (Zambia).

COI. *Lamproglena clariae* 671 bp (Barberspan) (South Africa); and 668 bp (Kabompo River, West Lunga) (Zambia).

Descriptive notes of adult female (Figs. 3.2–3.4).

Measurements (n = 10) follow the illustration in Fig. 3.2b and values are given in Table 3.1. Total length 5.84–7.83 mm; cephalothorax length 0.63–0.77 mm and width 0.47–0.68 mm; thorax length 3.04–4.07 mm.

Body elongate (Figs. 3.2a, b), widest at centre, slightly tapering anteriorly and posteriorly. Cephalothorax, thorax, genital somite, and abdomen distinctly demarcated. Cephalothorax (Figs. 3.2b, c) quadrangular, comprising 8–10% of total body length, disc-shape nuchal organ visible dorsally (Figs. 3.3a, b). Thorax (Fig. 3.2b) 4-segmented, consists of indistinctly demarcated pedigerous somites; sometimes bears a necklace of sessile ciliates (Figs. 3.2c, d). Somites 1–4 bear a pair of biramous legs, decreasing in size and simplifying in form; somite 4 the widest. Mature eggs visible in oviduct and ovarium of third and fourth thoracic segments. Genital somite (Figs. 3.2b, e) separated from body by deep constriction; carries fifth legs and genital pore ventrally, a pair of uniseriate egg strings attached dorsally (Fig. 3.2e). Abdomen (Fig. 3.2b) consists of three distinct somites. Furcal rami (Figs. 3.2b, f) attached to third abdominal somite, with two setae extending laterally and four to five blunt setae extending terminally.

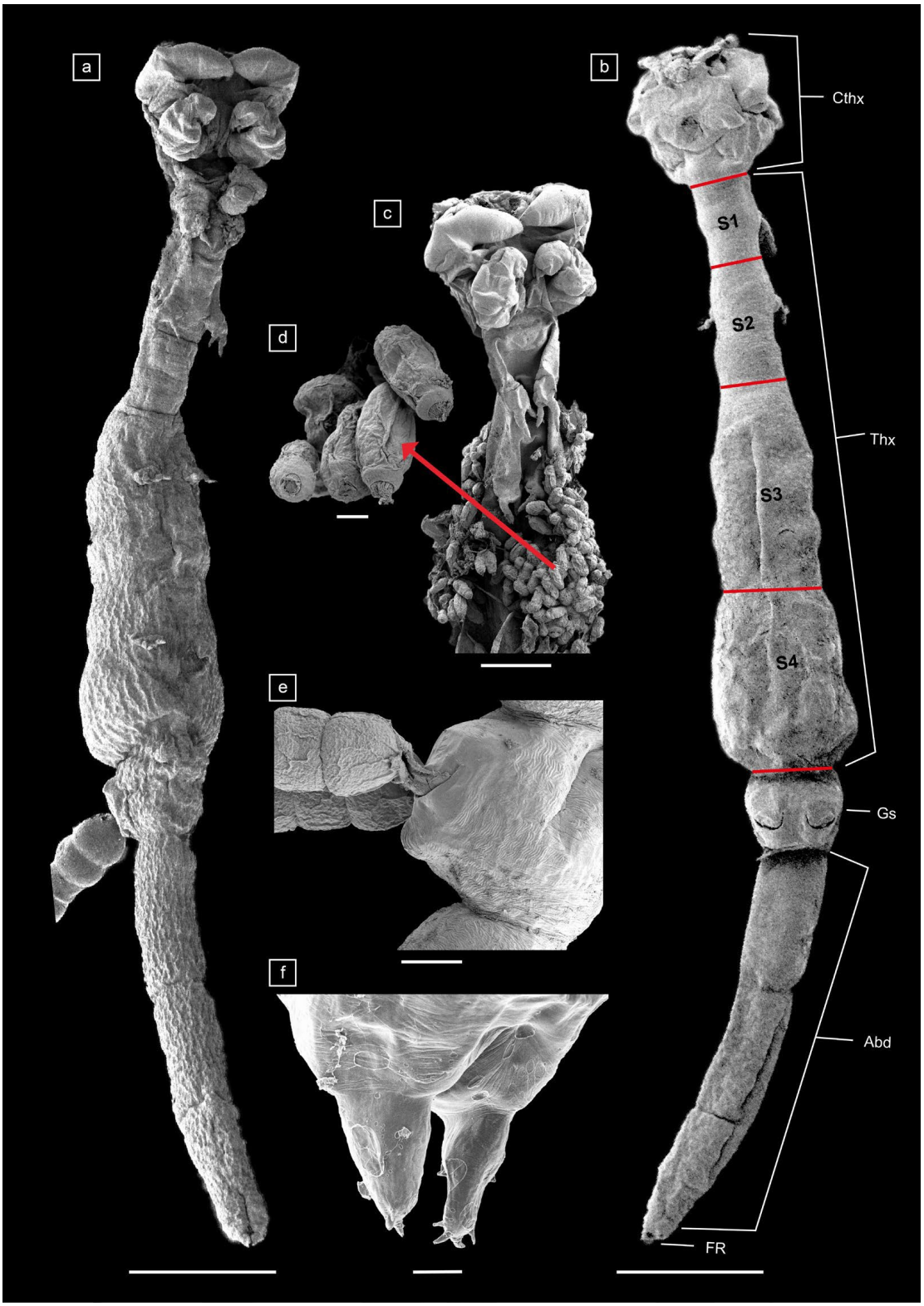


Figure 3.2: Scanning electron microscope photomicrographs of adult female *Lamproglena clariae* Fryer, 1956 specimens from South Africa: (a) full image, ventral view; (b) full image, dorsal view with labelling of body divisions; (c) anterior region—ventral view with necklace of sessile ciliates (red arrow); (d) zoomed in sessile ciliates (red arrow); (e) genital somite showing egg attachment; (f) furcal rami. Abbreviations: Abd – abdomen; Cthx – cephalothorax; FR – furcal rami; Gs – genital somite; S1–4 – thoracic segments 1–4; Thx – thorax. Scale bars: (a–b) 500 µm; (c) 200 µm; (d, f) 20 µm; (e) 100 µm.

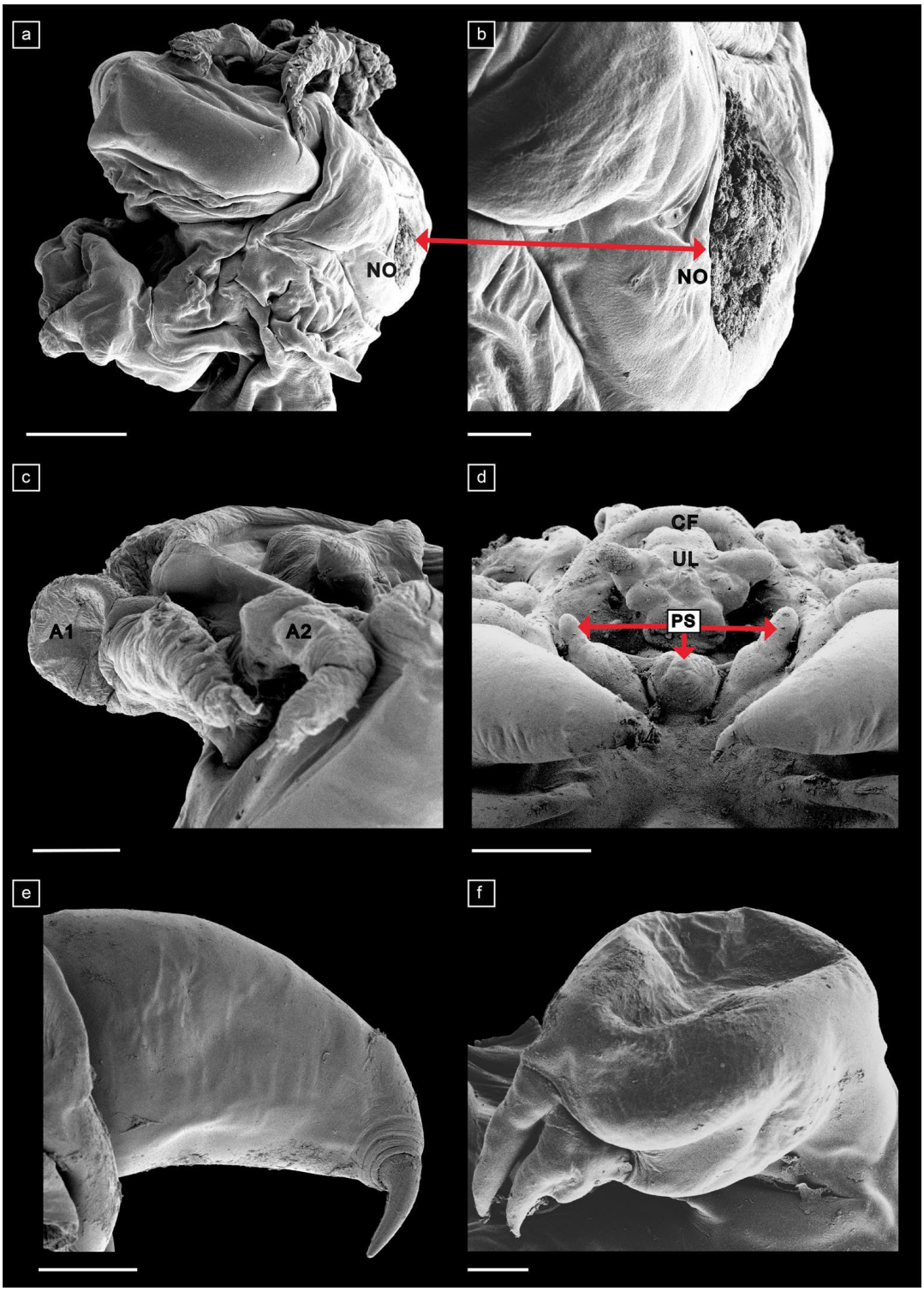


Figure 3.3: Scanning electron microscope photomicrograph of adult female *Lamproglena clariae* Fryer, 1956 specimens from South Africa showing the cephalic appendages: (a) cephalothorax, dorsolateral view highlighting the nuchal organ (red arrow) situated on the dorsal surface of the cephalothorax; (b) nuchal organ (red arrow); (c) lateral view of the cephalothorax showing the antennule and the antenna; (d) ventral view of the cephalothorax showing the chitinised framework, upper lip and three papillated structures (three red arrows) of the buccal region; (e) maxilla; (f) maxilliped. Abbreviations: NO – nuchal organ, A1 – antennule, A2 – antenna, CF – chitinised framework, UL – upper lip, PS – papillated structures. Scale bars: (a, d) 100 µm; (b, f) 20 µm; (c, e) 50 µm.

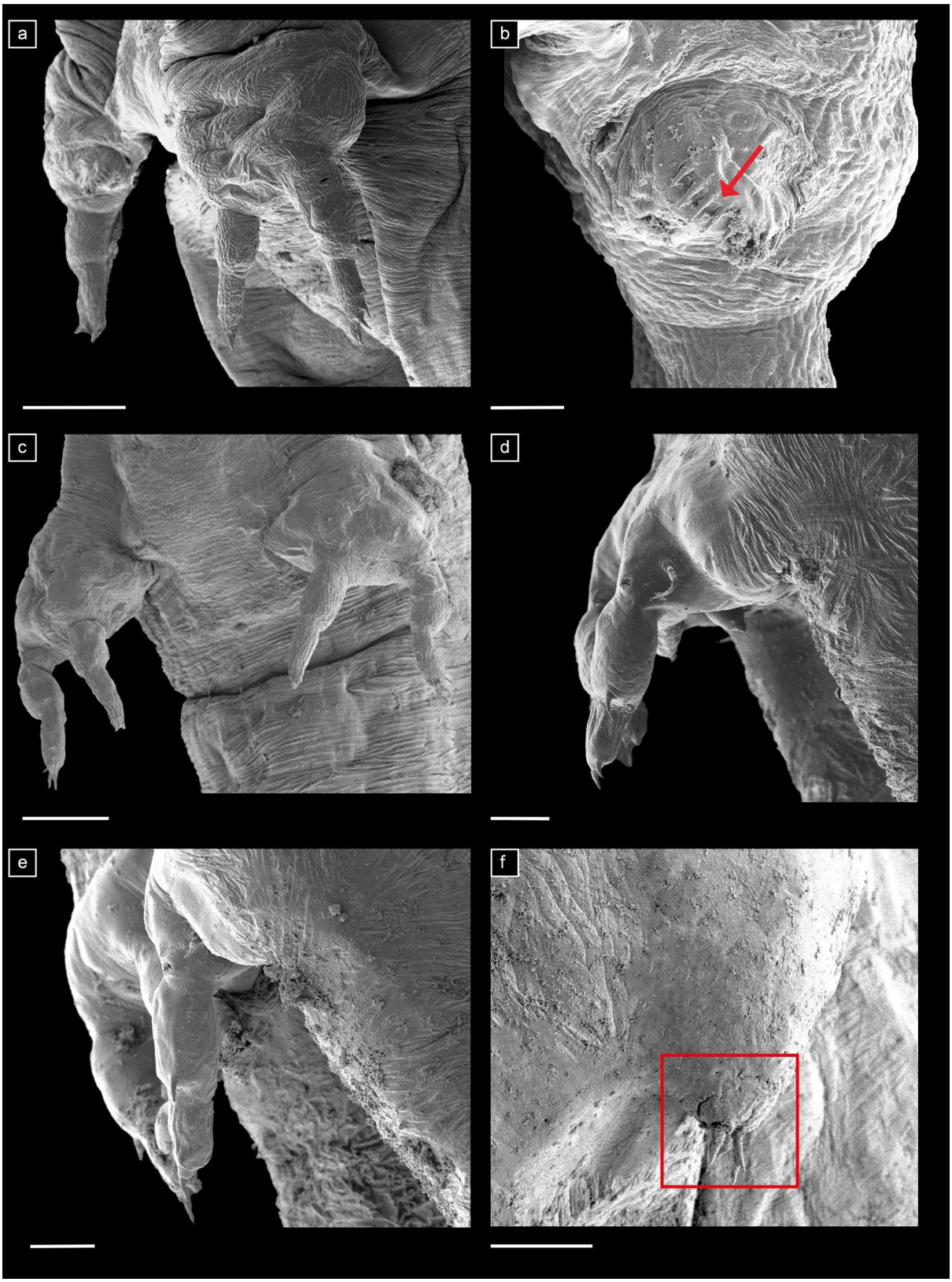


Figure 3.4: Scanning electron microscope photomicrograph of adult female *Lamproglena clariae* Fryer, 1956 specimens from South Africa showing the legs: (a) legs 1; (b) leg 1 showing the circular structure with minute denticles (red arrow); (c) legs 2; (d) leg 3; (e) leg 4 (f) leg 5, a bulging process with three short setae (red square). Scale bars: (a, c) 50 µm; (b) 10 µm; (d–f) 20 µm.

Antennule (Fig. 3.3c) uniramous, single segmented with a distal slight constriction, adorned with 16–18 setae lining the preaxial margin and terminating with one large seta and five to six small setae. Antenna (Fig. 3.3c) posterior to antennule. Buccal cavity (Fig. 3.3d) situated ventrally with chitinised framework, upper lip and three papillated structures. Maxillae (Fig. 3.3e) short and strong, consisting of two segments, terminal segment terminates in a sclerotised claw. Maxillipeds (Fig. 3.3f) 3-segmented, terminal segment with three claws.

Legs 1–4 biramous (Figs. 3.4a–e), 2-segmented, with circular pores on protopodites, varying number of spines, and setae on endo- and exopodites. Leg 1 bears a semi-circular structure with a series of minute denticles (Fig. 3.4b). Leg 5 (Fig. 3.4f) reduced, a minute papillar bulging process with 3 short setae.

***Lamproglena hepseti* van As & van As, 2007**

Material examined.

A total of 20 copepods were collected from the gills of *H. cuvieri* Okavango River, Botswana (19°27'26.8"S 22°59'55.4"E), collected by L. L. van As (2017).

Representative DNA sequences. Sequences, with the numbers of base pairs (bp), will be submitted to GenBank as follows:

18S. *Lamproglena hepseti* 1,389 bp (Okavango River, Botswana).

28S. *Lamproglena hepseti* 716 bp (Okavango River, Botswana).

COI. *Lamproglena hepseti* 700 bp (Okavango River, Botswana).

Descriptive notes of adult female (Figs. 3.5–3.7).

Measurements (n = 5) follow the illustration in Fig. 3.5b and values are given in Table 3.5. Total length 3.71–4.36 mm; cephalothorax length 0.49–0.70 mm and width 0.75–0.96 mm; thorax length 1.28–1.57 mm.

Body elongate (Fig. 3.5b), comprises distinctly segmented cephalothorax, thorax, genital somite, and abdomen. Cephalothorax broad (Figs. 3.5a, b), comprising 11.59–18.85% of total body length; ventral posterolateral protrusion with broad base extending from lateral margins of both sides; disc-shape nuchal organ visible dorsally (Figs. 3.6a, b). Thorax (Figs. 3.5b, c) consists of four pedigerous somites increasing in size posteriorly; somite 1 fused with cephalosome. Mature eggs in oviduct, visible through body wall of third and fourth pedigerous somites. Genital somite distinctly demarcated from thorax and abdomen by deep constriction (Fig. 3.5b); bears fifth leg; a pair of uniseriate egg strings each attached dorsolaterally (Fig. 3.5b). Abdomen (Fig. 3.5b) 3-segmented, tapering posteriorly. Furcal rami (Figs. 3.5b, d) conical, fused with third abdominal somite, tapering to sharp point with 2–4 minute sharply pointed setae extending laterally.

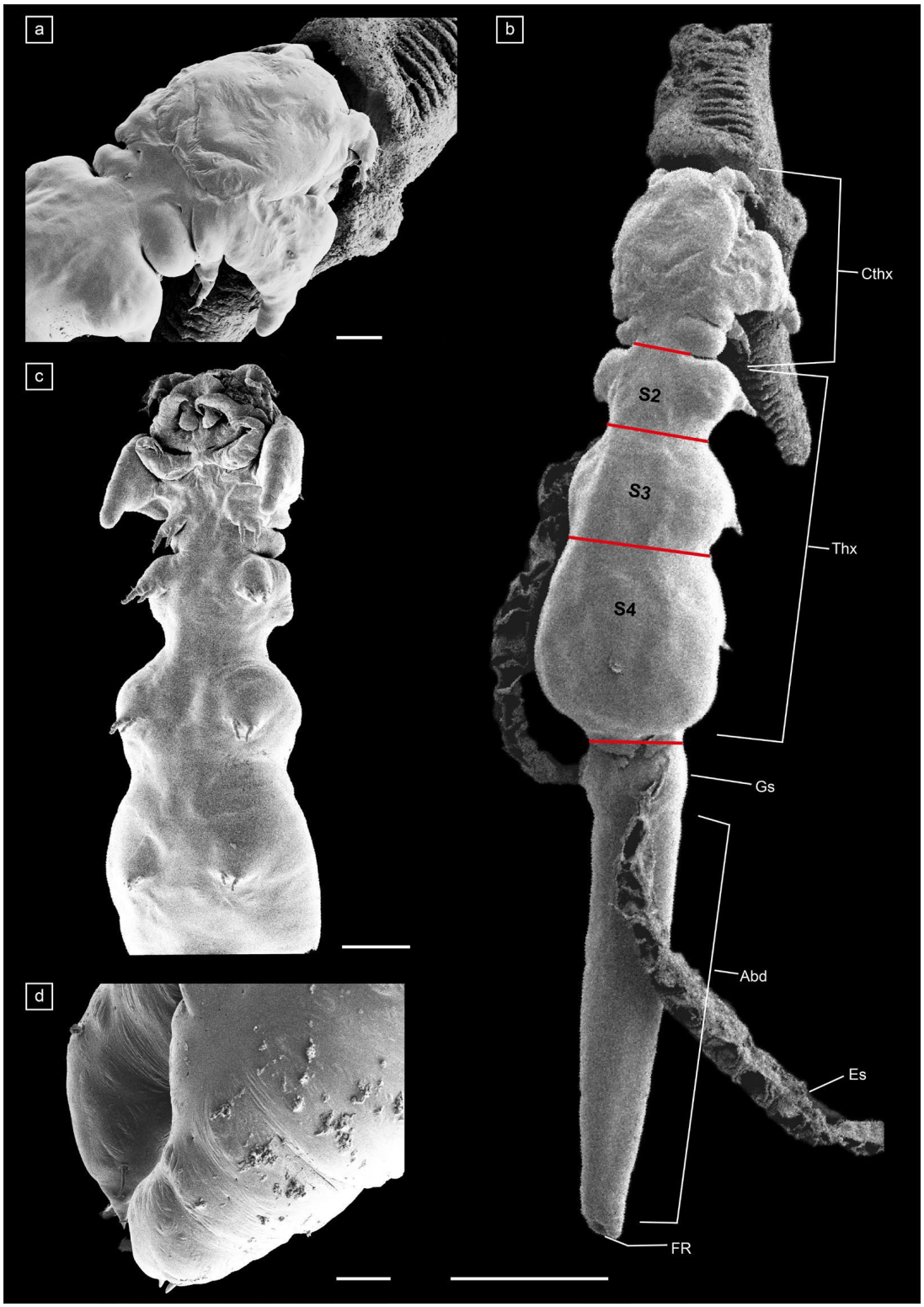


Figure 3.5: Scanning electron microscope photomicrographs of adult female *Lamproglena hepseti* van As & van As, 2007 specimens from Botswana: (a) Cephalothorax, dorsal view showing attachment to gill filament; (b) dorsal view of adult female with already hatched/spent egg strings attached to a gill filament, with labelling of body divisions; (c) cephalothorax and thorax, ventral view; (d) furcal rami. Abbreviations: Abd – abdomen; Cthx – cephalothorax; Es – egg string; FR – furcal rami; Gs – genital somite; S2–4 – thoracic segments 2–4; Thx – thorax. Scale bars: (a) 100 µm; (b) 500 µm; (c) 200 µm; (d) 20 µm.

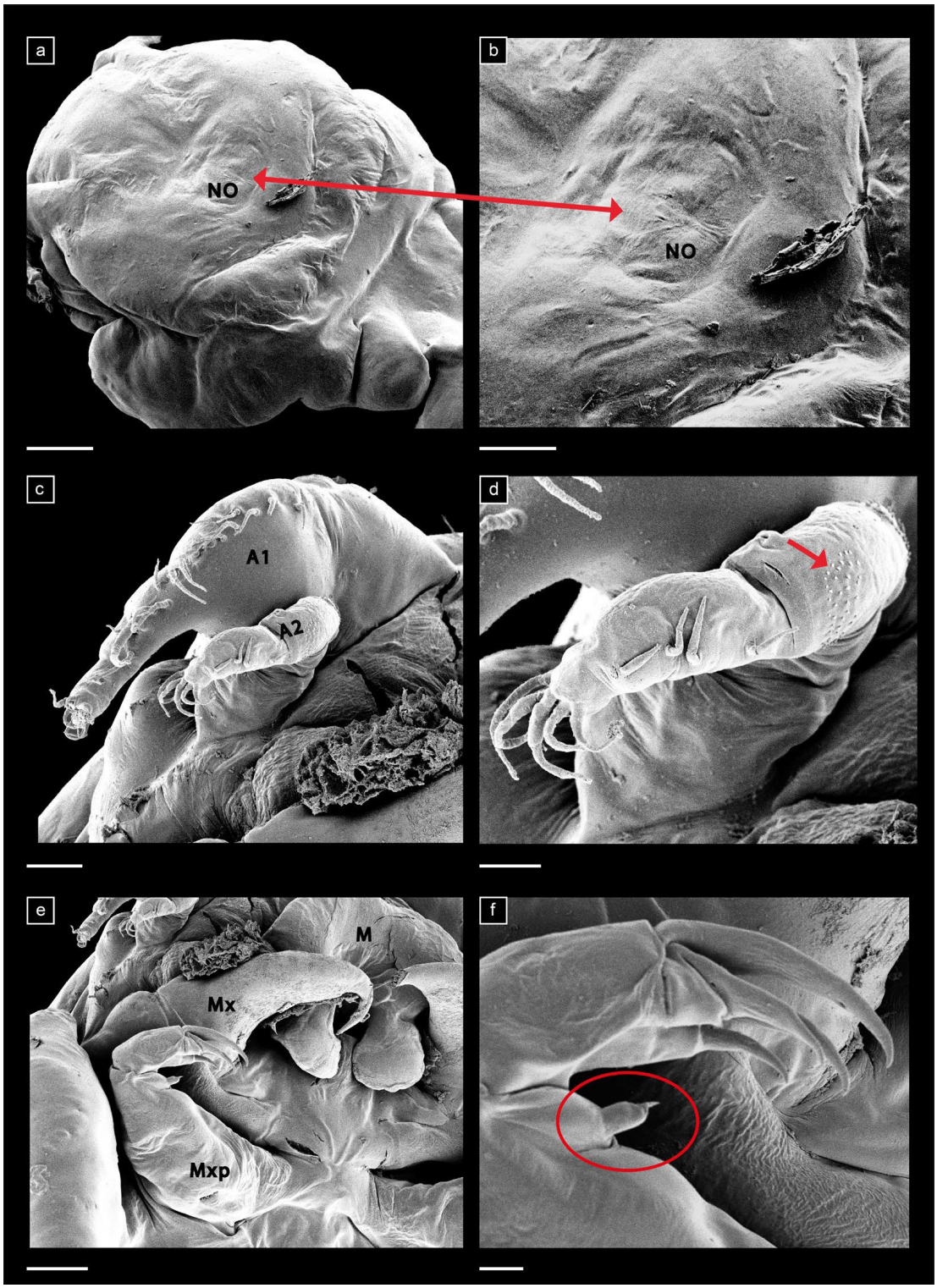


Figure 3.6: Scanning electron microscope photomicrograph of adult female *Lamproglena hepseti* van As & van As, 2007 specimens from Botswana showing the cephalic appendages: (a) cephalothorax, dorsal view, highlighting the nuchal organ (red arrow); (b) nuchal organ (red arrow); (c) cephalothorax showing the antennule and the antenna; (d) antenna showing scabrous area on third segment (red arrow); (e) ventral view of the cephalothorax showing the mouth region, maxilla, and maxilliped; (f) maxilliped highlighting single basal seta (red circle). Abbreviations: A1 – antennule; A2 – antenna; M – mouth region; Mx – maxilla; Mxp – maxilliped; NO – nuchal organ. Scale bars: (a) 100 µm; (b, e) 50 µm; (c) 20 µm; (d, f) 10 µm.

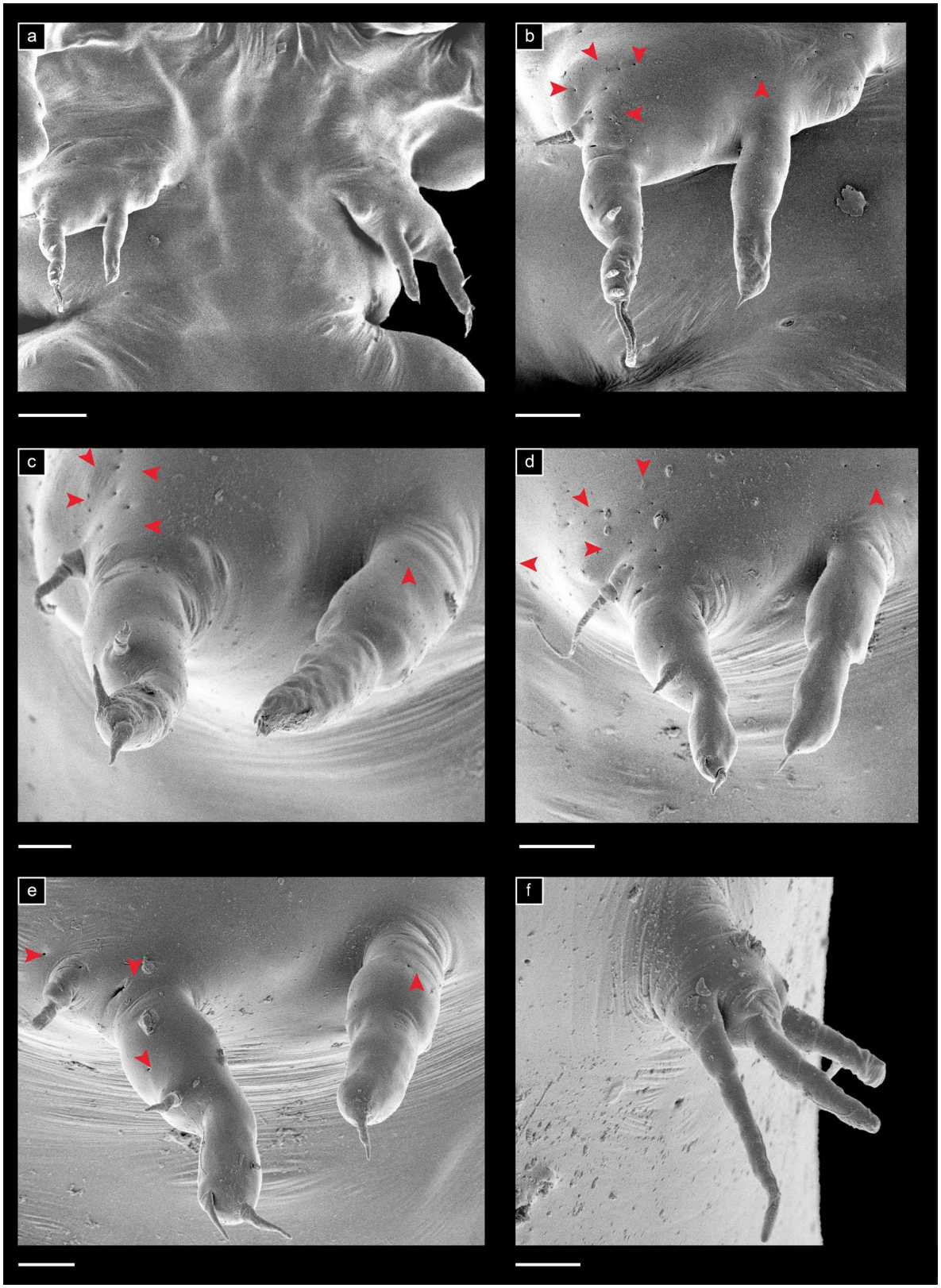


Figure 3.7: Scanning electron microscope photomicrograph of adult female *Lamproglena hepseti* van As & van As, 2007 specimens from Botswana showing the legs, and pores highlighted with red arrowheads: (a) legs 1; (b) leg 1 zoomed in (c) leg 2; (d) leg 3; (e) leg 4 (f) leg 5, three long setae of unequal length. Scale bars: (a) 50 µm; (b, d) 20 µm; (c, e) 10 µm; (f) 5 µm.

Antennule (Fig. 3.6c) uniramous, 4-segmented; basal segment broad; segments 2–4 progressively smaller; ventral margin with row of 16–18 setae of varying sizes; distal segment terminates in 4–6 setae. Antennae (Figs. 3.6c, d) uniramous, 4-segmented; basal segment unadorned; third segment with distinct scabrous areas, without setae; second segment with 3–4 ventral setae; distal segment terminating in 5 setae. Buccal region characterised by broad, horseshoe-shaped, sucker-like labrum, situated ventrally, beneath the tip of maxillae (Fig. 3.6e). Maxillae 2-segmented, hook-shaped, tapering from broad base to sharp claw (Fig. 3.6e). Maxilliped (Figs. 3.6e, f), 2-segmented; broad base tapering towards second segment with a single spine at distal margin; second segment directed inward, terminates in 4 claws.

Legs 1 to 4 similar (Figs. 3.7a–e), biramous, adorned with spines and setae; Leg 5 (Fig. 3.7f) a minute process, with 3 long terminal setae.

***Lamproglena monodi* Capart, 1944**

Material examined.

A total of 56 copepods were collected from the gills of several cichlids (see Table 3.4.).

Okavango River, Botswana (19°27'26.8"S 22°59'55.4"E), collected by L. L. van As (2016–2017).

Representative DNA sequences. Sequences, with the numbers of base pairs (bp), will be submitted to GenBank as follows:

18S: *Lamproglena monodi* 1,376 bp (Okavango River, Botswana).

28S: *Lamproglena monodi* 719 bp (Okavango River, Botswana).

COI: *Lamproglena monodi* 647 bp (Okavango River, Botswana).

Descriptive notes of adult female (Figs. 3.8–3.10).

Measurements (n = 6) follow the illustrations in Figs. 3.8b–c and values are given in Table 3.5. Total length 3.02–4.15 mm; cephalothorax length 0.61–0.76 mm and width 0.49–0.71 mm; thorax length 0.98–1.32 mm.

Body elongate (Figs. 3.8a–c), comprises cephalothorax, thorax, genital somite, and abdomen; segmentation distinct. Cephalothorax broad (Figs. 3.8b, 3.9a), comprising 17.26–21.72% of total body length; ventral posterolateral protrusion with broad base extending from lateral margins of both sides and disc-shape nuchal organ present dorsally (Figs. 9b, c). Thorax (Fig. 3.8b) consists of four pedigerous somites, increasing in size posteriorly; somite 1 fused with cephalosome. Mature eggs in oviduct, visible in body wall of third and fourth pedigerous somites. Genital somite (Fig. 3.8b) distinctly demarcated from the thorax and abdomen by deep constriction, bearing fifth leg (Fig. 3.10e); pair of uniseriate egg string each

extending dorsolaterally from openings of the posterior margin (Fig. 3.8a). Abdomen (Fig. 3.8b) 3- segmented, tapering posteriorly. Furcal rami (Figs. 3.8b, 3.10f) attached with the third abdominal somite, terminally bifurcated with one long laterally extending spine and 4–5 minute setae.

Antennules (Fig. 3.9a) 2-segmented, basal segment the largest and more flattened with a series of 14–18 short setae on its pre-apical margin; terminal segment small, one-tenth of the basal segment, terminates in numerous setae. Antennae (Fig. 3.9e) smaller and more elongate than antennule, situated behind antennules. Buccal apparatus between bases of maxillae. Maxillae (Figs. 3.9a, d, e) long and strong, 1-segmented and terminated with a hole that extends the sclerotized claw; base of maxilla with numerous minute pores (Fig. 3.9d). Maxilliped (Fig. 3.9f), possesses one seta on basal, terminal segment with three chitinised claws of equal sizes.

Legs 1–4 biramous (Figs. 3.10a–d), 2-segmented, with circular openings on protopodites, and spines and setae on exo- and endopodites; Leg 5 on genital somite as of three unequal setae (Fig. 3.10e).

Remarks.

Lamproglena clariae is characterised by an elongate body form. The maxilliped terminates in three claws; the somites of the thoracic region are pedigerous, bearing legs 1–4 but not distinctly demarcated; and a neck region is formed by the fusion of the first and second thoracic segments. Leg 1 bears a semi-circular structure with a series of minute denticles and leg 5 is a minute process with three small setae.

Lamproglena hepseti can be defined by the huge posteriolateral ventral extension, of the cephalothorax. The maxilliped terminates in four claws, the first thoracic segment is fused with cephalosome, and leg 5 bears three long setae.

Lamproglena monodi has the first thoracic segment fused with the cephalosome. The maxilliped terminates in three claws, the furcal rami bear one long laterally extending spine and 4–5 terminal minute setae, and leg 5 is a minute process with three small setae of equal length.

The body shape of *L. clariae* is narrower and 1.5–2 times longer in total body length than *L. hepseti* and *L. monodi*. In *L. hepseti* and *L. monodi*, the lateral margin of the cephalothorax is extended, forming a huge ventral posteriolateral extension with a broad base. *Lamproglena clariae* has the shortest cephalothoracic length to body length ratio (8.81–11.01%), compared to *L. hepseti* (11.59–18.85%), and *L. monodi* (17.26–21.72%); and the cephalothorax of *L. hepseti* is the broadest in cephalothoracic width out of the three species. A “necklace” of sessile ciliates was also found on the anterior part of some *L. clariae* specimens (Figs. 3.2c, d).

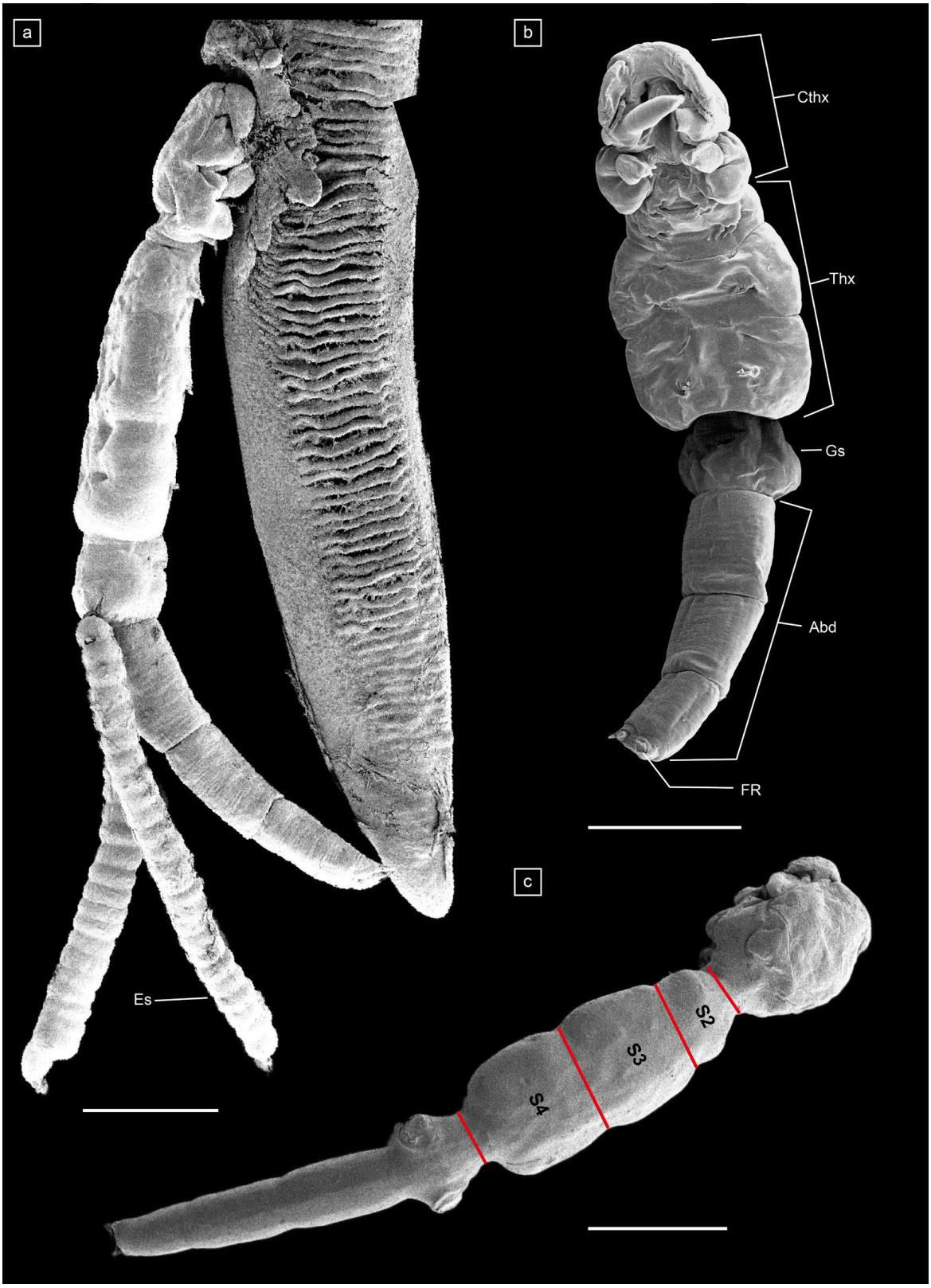


Figure 3.8: Scanning electron microscope photomicrographs of adult female *Lamproglena monodi* Capart, 1944 specimens from Botswana: (a) lateral view of an adult female with a pair of uniseriate egg strings attached to a gill filament; (b) full image, ventral view with labelling of body divisions; (c) full image, dorsolateral view. Abbreviations: Abd – abdomen; Cthx – cephalothorax; Es – egg string; FR – furcal rami; Gs – genital somite; S2–4 – thoracic segments 2–4; Thx – thorax. Scale bars: (a–c) 500 µm

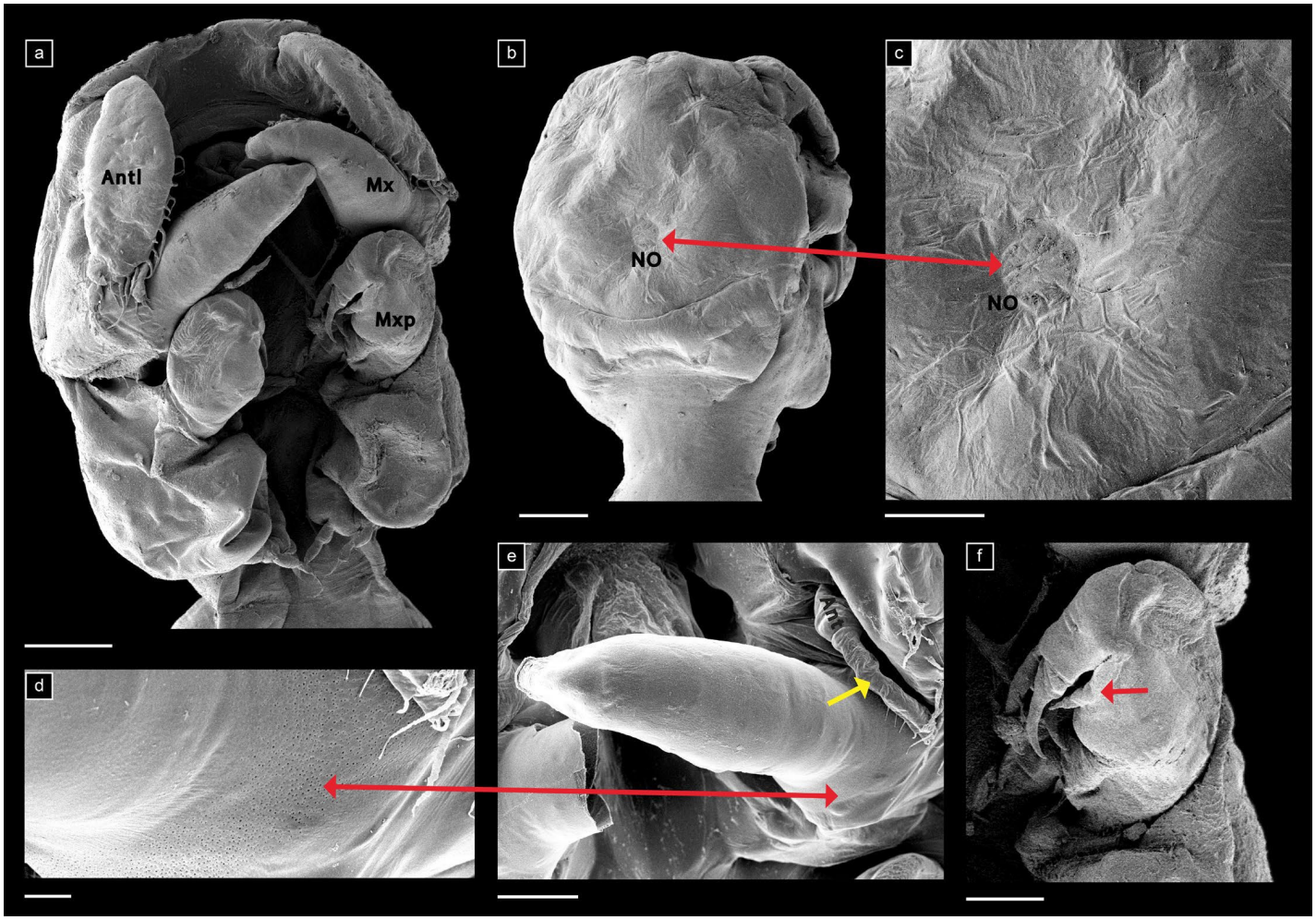


Figure 3.9: Scanning electron microscope photomicrograph of adult female *Lamproglena monodi* Capart, 1944 specimens from Botswana showing the cephalic appendages: (a) cephalothorax, ventral view, showing the antennule, maxilla and maxilliped; (b) cephalothorax, dorsal view, highlighting the nuchal organ (red arrow); (c) nuchal organ (red arrow); (d) numerous minute pores at the base of the maxilla (red arrow); (e) maxilla with numerous minute pores (red arrow), and antenna (yellow arrow); (f) maxilliped highlighting single basal seta (red arrow). Abbreviations: Antl – antennule; Mx – maxilla; Mxp – maxilliped; NO – nuchal organ. Scale bars: (a–b) 100 µm; (c, e, f) 50 µm; (d) 10 µm.

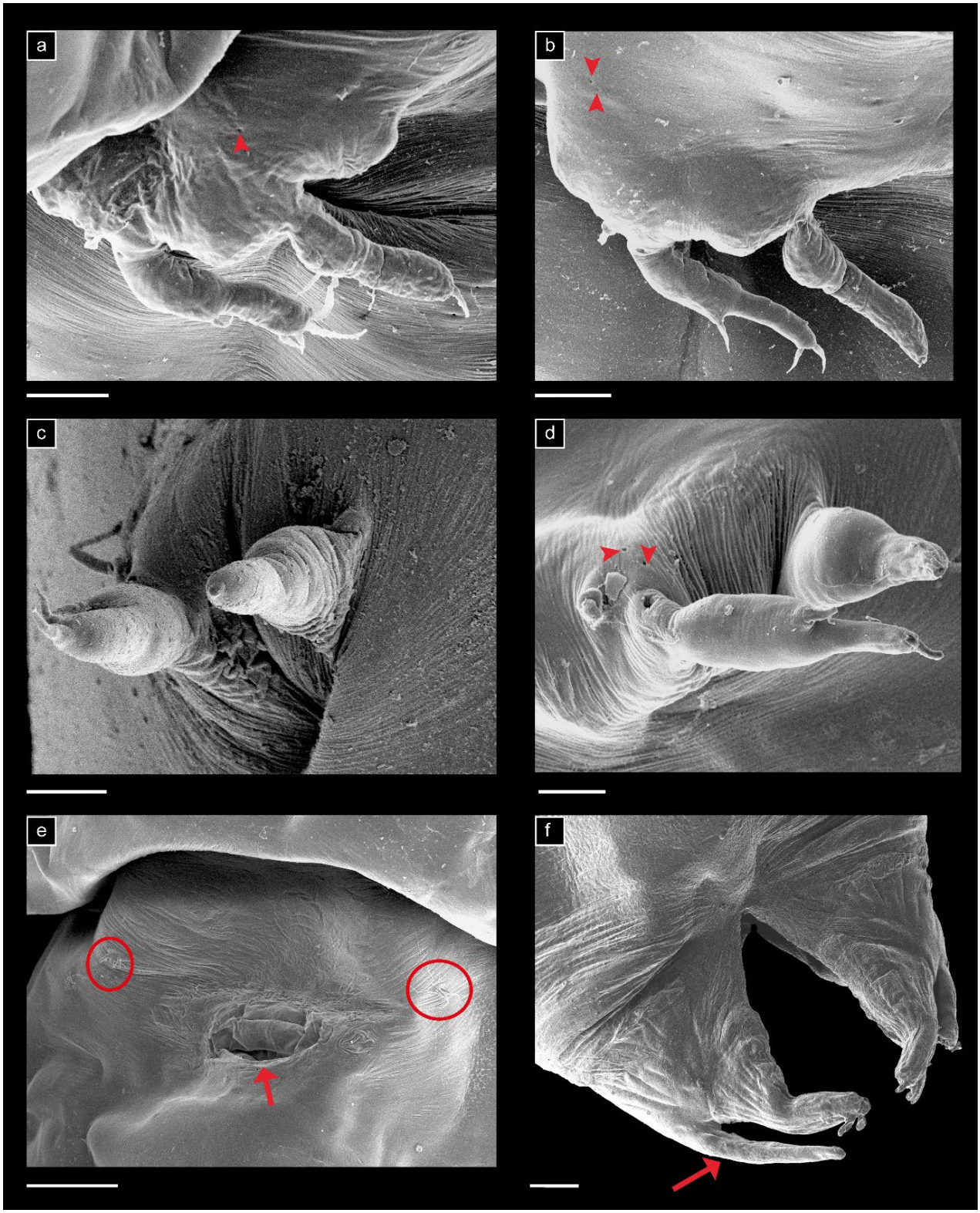


Figure 3.10: Scanning electron microscope photomicrograph of adult female *Lamproglena monodi* Capart, 1944 specimens from Botswana showing legs (pores highlighted with red arrowheads), genital pore, and furcal rami: (a) leg 1; (b) leg 2; (c) leg 3; (d) leg 4 (e) genital somite bearing leg 5 (red circles) and the genital pore (red arrow); (f) furcal rami showing highlighting the long lateral spine (red arrow). Scale bars: (a, b, f) 20 µm; (c–d) 10 µm; (e) 50 µm.

The nuchal organs of all three species are of varying sizes. Depending on the positioning and folding of the specimens during microscopy, nuchal organs may not be observed (pers. observation). The antennules of the three species differ in size and number of segments. *Lamproglena monodi* has two segments that are dorsoventrally flattened, while the antennule of *L. clariae* is single segmented with a slight constriction towards its distal end, having a more elongate appearance. *Lamproglena hepseti*, on the other hand has four segments with its basal segment broad and tapering towards the terminal segment. The antennae of *L. hepseti* have scabrous regions on the third segments not observed in *L. clariae* and *L. monodi.* The terminal segment of maxilliped ends in three claws for *L. monodi* and *L. clariae* but *L. hepseti* has four claws. A single basal seta was observed in maxillipeds of *L. hepseti* and *L. monodi* but not in *L. clariae.*

The thorax of all three species comprises four pedigerous somites and a genital somite bearing the fifth leg. In *L. hepseti* and *L. monodi*, the first thoracic segment bears leg 1 and is fused with the cephalosome, the rest are three distinctly demarcated free pedigerous somites bearing legs 2–4. In *L. clariae,* the first thoracic segment is not fused but rather forms a "neck region" together with the second segment. All thoracic segments, except the genital somite, are indistinctly demarcated. While the fifth legs of *L. hepseti* have three long setae, *L. clariae* is characterised by three short setae, and *L. monodi* has three short unequal setae. The furcal rami of *L. monodi* has a very long spine extending mid-laterally, this spine is absent in the other two species, which have short spines on the lateral margins.

3.4.2. Molecular analysis

A total of 20 novel sequences were generated from this study (six sequences for 18S, 10 sequences for 28S, and four sequences for COI). Tree topologies for Bayesian Inference and Maximum Likelihood analyses were similar for both 18S and 28S. No COI phylogenetic trees were constructed as there are no other COI sequences available to compare with for this genus.

Phylogenetic alignments of 18S sequences resulted in a length of 1,393 bp. All the newly generated *L. clariae* sequences from South Africa were 100% identical, and 99.77% (3 bp) similar to the newly generated sequence from Zambia. Novel *L. monodi* sequences had 99.85% (2 bp) similarity to *L. monodi* sequences from Kenya and Egypt. *Lamproglena hepseti* sequences from this study was most similar to all *L. monodi* (99.50–99.78%; 3–7 bp) and *L. hemprichii* (99.71%; 4 bp) than to *L. clariae* (98.48–98.87%; 15–21 bp). Within the Asian *Lamproglena* population, *L. orientalis* was most similar to the African *Lamproglena* species (97.90–98.91%) than to the Asian *L. chinensis* (97.74–98.04%) (see Table 3.6). The phylogenetic trees constructed (Fig. 3.11) yielded three clades for the African species: a *L. clariae* clade, a *L. monodi* clade, and a clade with *L. hepseti* and *L. hemprichii*.

Phylogenetic alignment of partial 28S sequences yielded an alignment length of 882 bp. Similar to the 18S alignment, all the newly generated 28S *L. clariae* sequences from South Africa were 100% identical, and 99.47% (4 bp) similar to the sequence from Zambia. Newly generated *L. monodi* specimens had a 97.49–97.56% (18 bp) similarity to *L. monodi* sequences from Kenya and Egypt. *Lamproglena hepseti* was most similar to *L. monodi* (85.50–85.89%; 102–104 bp) and *L. hemprichii* (85.04%; 105 bp) than to *L. clariae* (81.28–81.39%; 137–138 bp). Within the Asian *Lamproglena* population, *L. chinensis* was most similar to the African *Lamproglena* species (73.73–75.53%) than the Asian *L. orientalis* (69.99–72.85%) (see Table 3.7). The phylogenetic trees constructed (Fig. 3.12) yielded two large clades for the African species: a *L. clariae* clade and another clade with *L. hepseti, L. hemprichii* and *L. monodi*; and all *L. monodi* sequences (Botswana, Egypt and Kenya) clustered in a sister clade.

As mentioned earlier, there were no COI sequences for *Lamproglena* available to compare with; therefore, no phylogenetic trees were constructed. A phylogenetic alignment was, however, done for all newly generated *Lamproglena* sequences, resulting in an alignment length of 700 bp. Novel COI *L. clariae* sequences from South Africa and Zambia differed by 102 bases (Table 3.8) and only five nucleotides translated for different amino acids (missense mutations) (see Table 3.9). Compared with the other newly generated *Lamproglena* sequences, *L. clariae* differed from *L. hepseti* and *L. monodi* with a range of 134–167 bases (see Table 3.8).

Table 3.6: Genetic divergences of aligned 18S sequences used in the *Lamproglena* alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal), represented as GenBank/Sequence ID, Taxon and Country (site localities for the present study only). Sequences from the present study are in boldface (in a grey shade). *Lernaea cyprinacea* (DQ107556) was used as the outgroup. Abbreviations: BB – Broken Bridge, BP – Barberspan, BT – Botswana, CN – China, EG – Egypt, KE – Kenya, NP – Nyamiti Pan, OK – Okavango River, PH – Phongolo River, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZW – Zimbabwe.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	**P18K *Lamproglena monodi* OK-BT**		**3**	**12**	**15**	**15**	**15**	**2**	**2**	**2**	**2**	**19**	**15**	**15**	**27**	**34**
2	**P42R *Lamproglena hepseti* OK-BT**	**99.78**		**15**	**21**	**18**	**18**	**4**	**7**	**7**	**7**	**22**	**18**	**18**	**33**	**40**
3	**P19K *Lamproglena clariae* WL-ZM**	**99.1**	**98.87**		**3**	**3**	**3**	**14**	**14**	**14**	**14**	**26**	**25**	**25**	**29**	**36**
4	**P20R *Lamproglena clariae* PH-ZA**	**98.91**	**98.48**	**99.77**		**0**	**0**	**19**	**19**	**19**	**19**	**29**	**27**	**27**	**31**	**40**
5	**P23R *Lamproglena clariae* NP-ZA**	**98.87**	**98.64**	**99.77**	**100**		**0**	**17**	**17**	**17**	**17**	**26**	**25**	**25**	**30**	**39**
6	**P28K *Lamproglena clariae* BP-ZA**	**98.87**	**98.65**	**99.77**	**100**	**100**		**17**	**17**	**17**	**17**	**26**	**25**	**25**	**30**	**39**
7	OP277526 *Lamproglena hemprichii* ZW	**99.85**	**99.71**	**98.95**	**98.63**	**98.72**	**98.72**		5	5	5	20	16	16	31	38
8	ON419450 *Lamproglena monodi* EG	**99.85**	**99.5**	**98.95**	**98.63**	**98.72**	**98.72**	99.64		0	0	20	17	17	31	38
9	ON419448 *Lamproglena monodi* EG	**99.85**	**99.5**	**98.95**	**98.63**	**98.72**	**98.72**	99.64	100		0	20	17	17	31	38
10	ON419439 *Lamproglena monodi* KE	**99.85**	**99.5**	**98.95**	**98.63**	**98.72**	**98.72**	99.64	100	100		20	17	17	31	38
11	DQ107552 *Lamproglena orientalis* CN	**98.62**	**98.42**	**98.04**	**97.9**	**98.04**	**98.05**	98.56	98.56	98.56	98.56		4	4	37	43
12	DQ107550 *Lamproglena orientalis* CN	**98.91**	**98.7**	**98.12**	**98.05**	**98.12**	**98.12**	98.85	98.78	98.78	98.78	99.71		0	34	41
13	DQ107549 *Lamproglena orientalis C*N	**98.91**	**98.7**	**98.12**	**98.05**	**98.12**	**98.12**	98.85	98.78	98.78	98.78	99.71	100		34	41
14	DQ107553 *Lamproglena chinensis* CN	**98.04**	**97.63**	**97.82**	**97.76**	**97.74**	**97.75**	97.77	97.77	97.77	97.77	97.34	97.56	97.56		40
15	DQ107556 *Lernaea cyprinacea* CN	**97.53**	**97.12**	**97.29**	**97.11**	**97.07**	**97.07**	97.27	97.27	97.27	97.27	96.91	97.05	97.05	97.13	

Table 3.7: Genetic divergences of aligned 28S sequences used in the *Lamproglena* alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal), represented as GenBank/Sequence ID, Taxon and Country (site localities for the present study only). Sequences from the present study are in boldface in a grey shade. *Lernaea cyprinacea* (DQ107556) was used as the outgroup. Abbreviations: BB – Broken Bridge, BP – Barberspan, BT – Botswana, CN – China, EG – Egypt, KE – Kenya, NP – Nyamiti Pan, OK – Okavango River, PH – Phongolo River, VR – Vaal River, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZW – Zimbabwe.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	**P18Ka *Lamproglena monodi* OK-BT**		**102**	**147**	**145**	**145**	**145**	**145**	**145**	**145**	**145**	**48**	**18**	**18**	**18**	**233**	**240**	**241**	**174**	**211**
2	**P42Ra *Lamproglena hepseti* OK-BT**	**85.89**		**138**	**137**	**137**	**137**	**137**	**137**	**137**	**137**	**105**	**104**	**104**	**104**	**216**	**218**	**218**	**177**	**195**
3	**P19Ka *Lamproglena clariae* WL-ZM**	**80.35**	**81.28**		**4**	**4**	**4**	**4**	**4**	**4**	**4**	**154**	**146**	**146**	**146**	**236**	**249**	**247**	**191**	**213**
4	**P20Ra *Lamproglena clariae* PH-ZA**	**80.56**	**81.39**	**99.47**		**0**	**0**	**0**	**0**	**0**	**0**	**152**	**144**	**144**	**144**	**237**	**250**	**248**	**189**	**212**
5	**P23Ra *Lamproglena clariae* NP-ZA**	**80.56**	**81.39**	**99.47**	**100**		**0**	**0**	**0**	**0**	**0**	**152**	**144**	**144**	**144**	**237**	**250**	**248**	**189**	**212**
6	**P24Ka *Lamproglena clariae* NP-ZA**	**80.56**	**81.39**	**99.47**	**100**	**100**		**0**	**0**	**0**	**0**	**152**	**144**	**144**	**144**	**237**	**250**	**248**	**189**	**212**
7	**P25Ka *Lamproglena clariae* VR-ZA**	**80.56**	**81.39**	**99.47**	**100**	**100**	**100**		**0**	**0**	**0**	**152**	**144**	**144**	**144**	**237**	**250**	**248**	**189**	**212**
8	**P26Ka *Lamproglena clariae* VR-ZA**	**80.56**	**81.39**	**99.47**	**100**	**100**	**100**	**100**		**0**	**0**	**152**	**144**	**144**	**144**	**237**	**250**	**248**	**189**	**212**
9	**P27Ka *Lamproglena clariae* VR-ZA**	**80.56**	**81.39**	**99.47**	**100**	**100**	**100**	**100**	**100**		**0**	**152**	**144**	**144**	**144**	**237**	**250**	**248**	**189**	**212**
10	**P28K *Lamproglena clariae* BP-ZA**	**80.56**	**81.39**	**99.47**	**100**	**100**	**100**	**100**	**100**	**100**		**152**	**144**	**144**	**144**	**237**	**250**	**248**	**189**	**212**
11	OP277527 *Lamproglena hemprichii* ZW	**93.19**	**85.04**	**78.93**	**79.15**	**79.15**	**79.15**	**79.15**	**79.15**	**79.15**	**79.15**		55	55	55	242	249	250	176	214
12	ON419432 *Lamproglena monodi* EG	**97.56**	**85.5**	**80.4**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	92.3		1	0	231	239	240	176	211
13	ON419435 *Lamproglena monodi* EG	**97.49**	**85.5**	**80.4**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	92.23	99.93		1	230	238	239	176	211
14	ON419422 *Lamproglena monodi* KE	**97.56**	**85.5**	**80.4**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	**80.62**	92.3	99.93	99.93		231	239	240	176	211
15	DQ107544 *Lamproglena orientalis* CN	**70.95**	**72.69**	**71.18**	**71.06**	**71.06**	**71.1**	**71.06**	**71.06**	**71.06**	**71.06**	69.21	71.15	71.21	71.15		52	54	243	262
16	DQ107543 *Lamproglena orientalis* CN	**70.52**	**72.85**	**70.11**	**69.99**	**69.99**	**70.02**	**69.99**	**69.99**	**69.99**	**69.99**	68.8	70.59	70.65	70.59	93.69		4	238	280
17	DQ107542 *Lamproglena orientalis* CN	**70.39**	**72.85**	**70.35**	**70.23**	**70.23**	**70.26**	**70.23**	**70.23**	**70.23**	**70.23**	68.67	70.47	70.53	70.47	93.45	99.51		238	280
18	DQ107545 *Lamproglena chinensis* CN	**75.53**	**75.28**	**73.73**	**73.93**	**73.93**	**73.93**	**73.93**	**73.93**	**73.93**	**73.93**	74.49	75.04	75.04	75.04	68.65	69.21	69.21		220
19	DQ107548 *Lernaea cyprinicea* CN	**71.52**	**73.25**	**71.71**	**71.85**	**71.85**	**71.88**	**71.85**	**71.85**	**71.85**	**71.85**	70.52	71.45	71.45	71.45	67.77	66.18	66.18	69.53	

Table 3.8: Genetic divergences of newly aligned *Lamproglena* COI sequences, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal), represented as GenBank/Sequence ID, Taxon and Country (site localities for the present study only). *Lernaea cyprinacea* (DQ107556) was used as the outgroup. Abbreviations: BP – Barberspan, BT – Botswana, CN – China, OK – Okavango River, WL – West Lunga, ZA – South Africa, ZM – Zambia.

		1	2	3	4	5
1	P19Ki *Lamproglena clariae* WL-ZM		102	138	160	191
2	P28Ki *Lamproglena clariae* BP-ZA	84.73		134	167	176
3	P18Kb *Lamproglena monodi* OK-BT	78.23	78.96		138	166
4	P42Rb *Lamproglena hepseti* OK-BT	76.05	75.11	78.67		198
5	MH982220 *Lernaea cyprinacea* CN	71.41	73.77	74.34	71.71	

Table 3.9: Positions of nucleotide substitutions that resulted in missense mutations for newly generated *Lamproglena clariae* Fryer, 1956 sequences in the partial COI gene alignment, using invertebrate mitochondrion translation. Respective nucleotide substitutions are written in bold in the three-base pairs codon and the amino acids they translated for are also given. Abbreviation: WL – West Lunga, BP – Barberspan, ZM – Zambia, ZA – South Africa.

	Sequence ID	Locality	Position in alignment	Nucleotide substitutions	Codons	Codon positions	3-letter Amino acid	Amino acid
1	P19Ki	WL-ZM	22	G	**G**GA	22–24	Gly	Glycine
	P28Ki	BP-ZA		A	**A**GA		Ser	Serine
2	P19Ki	WL-ZM	28 & 29	CC	**CC**A	28–30	Pro	Proline
	P28Ki	BP-ZA		GT	**GT**A		Val	Valine
3	P19Ki	WL-ZM	31	A	**A**CT	31–33	Thr	Threonine
	P28Ki	BP-ZA		G	**G**CT		Ala	Alanine
4	P19Ki	WL-ZM	235	G	**G**TT	235–237	Val	Valine
	P28Ki	BP-ZA		A	**A**TT		Ile	Isoleucine

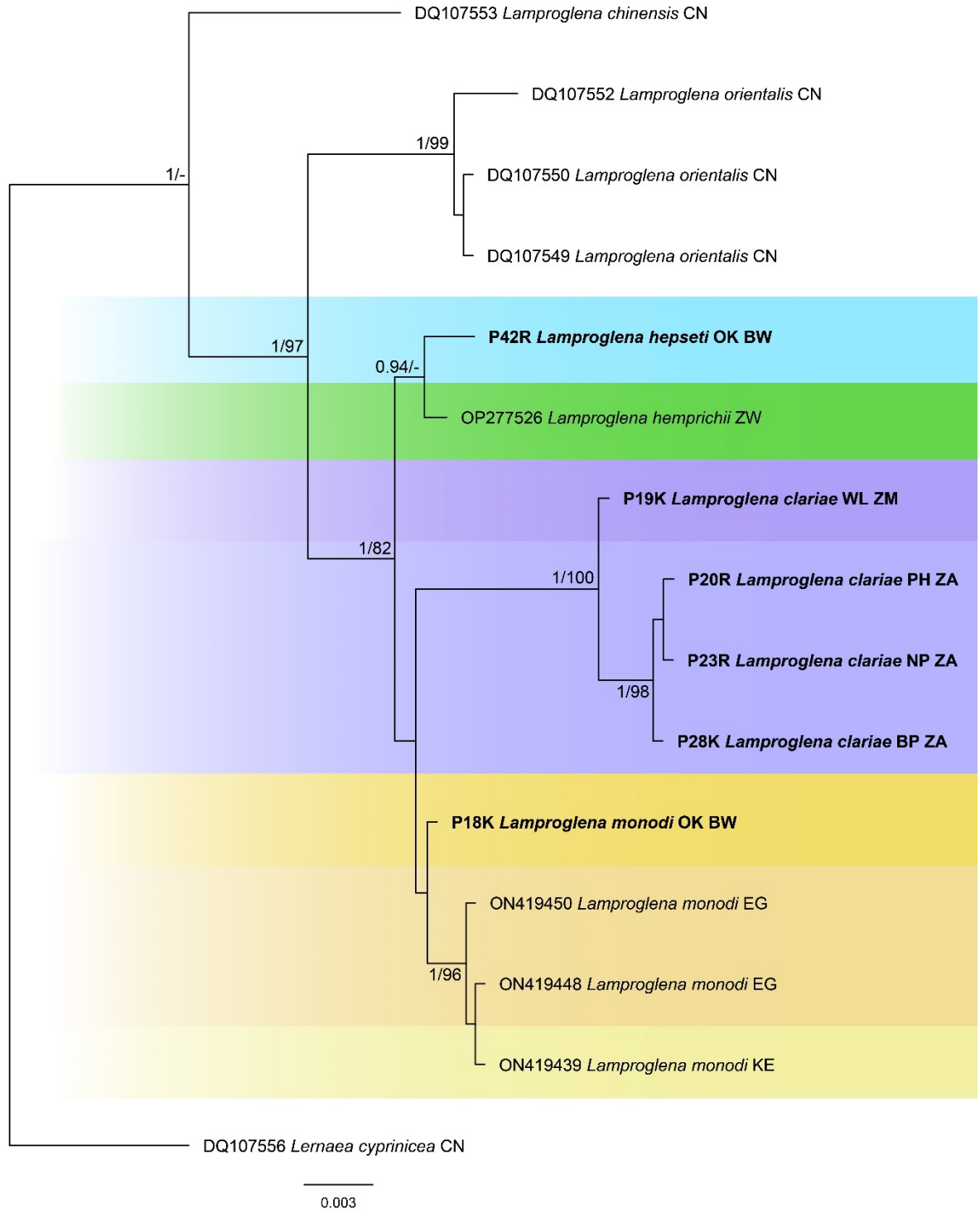


Figure 3.11: Phylogenetic relationship of *Lamproglena* species based on partial 18S rRNA gene alignments. All African species are highlighted in different coloured gradients; newly generated sequences are bold in gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lernaea cyprinacea* was used as the outgroup. Abbreviations: BP – Barberspan, BT – Botswana, CN – China, EG – Egypt, KE – Kenya, NP – Nyamiti Pan, OK – Okavango River, PH – Phongolo River, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZW – Zimbabwe.

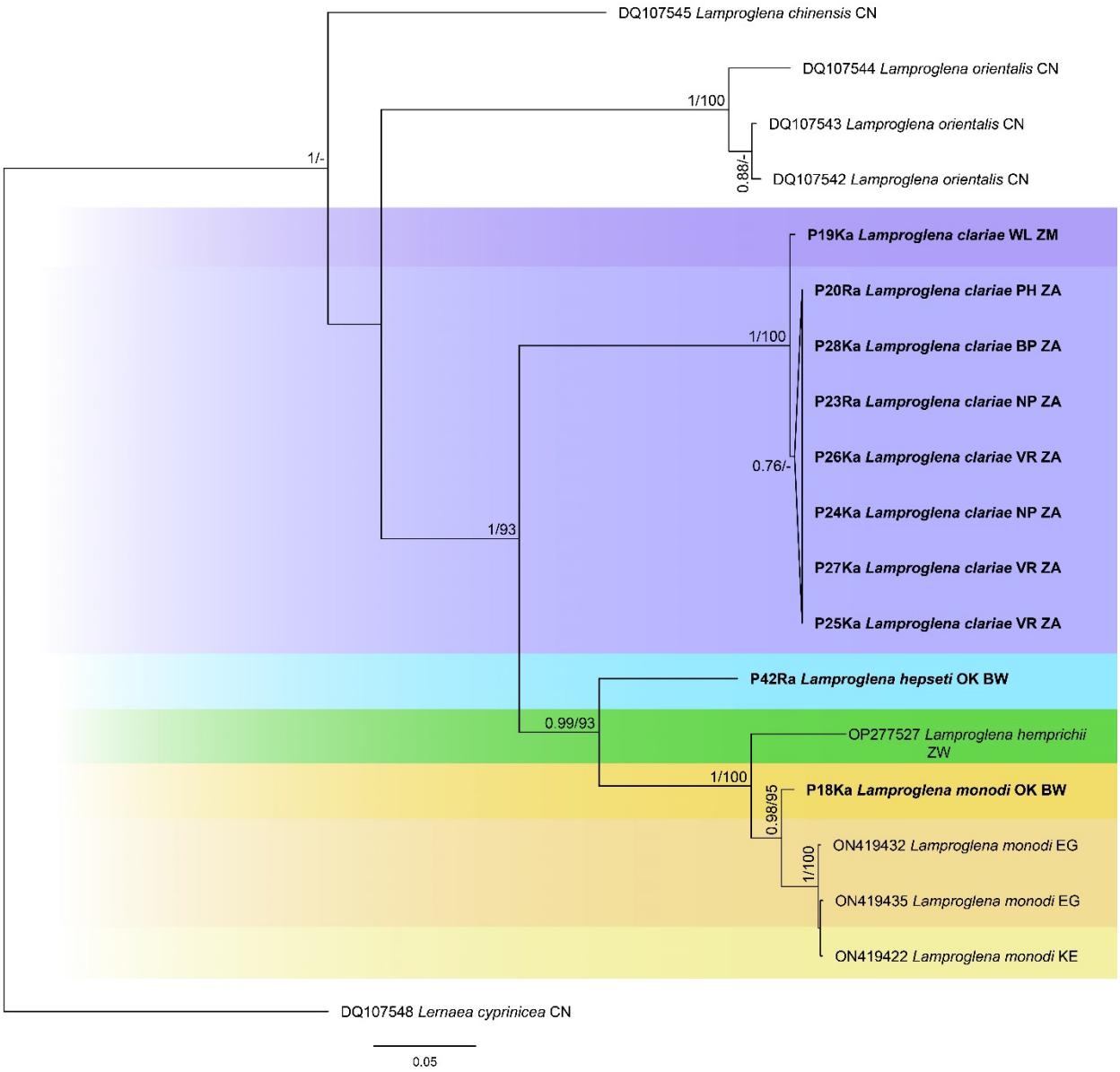


Figure 3.12: Phylogenetic relationship of *Lamproglena* species based on partial 28S rRNA gene alignments. All African species are highlighted in different coloured gradients; newly generated sequences are bold in gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lernaea cyprinacea* was used as the outgroup. Abbreviation: BP – Barberspan, BT – Botswana, CN – China, EG – Egypt, KE – Kenya, NP – Nyamiti Pan, OK – Okavango River, PH – Phongolo River, VR – Vaal River, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZW – Zimbabwe.

3.5. Discussion

Fryer (1968) observed high levels of host specificity in members of the genus *Lamproglena*. These parasites are typically found in regions where the hosts are distributed. From this study, *L. clariae* is reported from the sharptooth catfish *Clarias gariepinus* but had also been reported from other clariid host species across Africa (see checklist in Table 3.1). *Lamproglena hepseti* seems strictly associated with the characin host *Hepsetus cuvieri*, the African pike, which is endemic to Africa (van As & van As, 2007). The same is reported for *L. hemprichii*, which seems to be strictly associated with another characin host *Hydrocynus vittatus* (Castelnau, 1861), also endemic to Africa (Mabika et al., 2016; 2019; 2022). *Lamproglena monodi*, however, is distributed across several host genera in the family Cichlidae. From the current study, *L. monodi* specimens were collected from representatives of five cichlid host genera *Oreochromis* Günther, 1889, *Pharyngochromis* Greenwood, 1979, *Sargochromis* Regan, 1920, *Serranochromis* Regan, 1920, and *Coptodon* Gervais, 1853.

Among all three species examined in this study, *L. monodi* is the most widely distributed, and this corresponds to the wide distribution of its hosts. De Azevedo et al. (2012), reported *L. monodi* from the gills and body surface of two native Brazilian fish species from the familiy Cichlidae, i.e., *Astronotus ocellatus* (Agassiz, 1831) and *Cichla ocellaris* Bloch and Schneider, 1801, and on two other cichlids *Oreochromis niloticus* (Linnaeus, 1758) and *Coptodon rendalli* (Boulenger, 1897) introduced to Brazil. In their survey on the parasites of the Kwando River, Caprivi, Namibia, Avenant-Oldewage and Oldewage (1993) reported *L. monodi* mostly from cichlids and also on *C. gariepinus* (Clariidae) and *H. cuvieri* (Hepsetidae). They, however, concluded that the low infestation levels observed from *C. gariepinus* and *H. cuvieri* were possibly because they are not the preferred hosts for *L. monodi*, verifying Fryer's (1968) observation of host specificity within the genus *Lamproglena.*

All three species from the current study are adapted slightly differently for host attachment. While the entire head of *L. clariae* is usually embedded into the host's gill, only the anterior end of the cephalosome in *L. hepseti* and *L. monodi* are embedded in the gill tissue of their hosts. The huge ventral posterolateral extension of the cephalothorax in *L. hepseti* serves as a holdfast for host gill filament attachment (van As & van As, 2007), and the same is observed for *L. monodi* but not to the same extent. In *L. clariae,* this holdfast is absent and may be attributed to the way it is embedded into the host's tissue. The attachment and feeding of these parasites cause significant damage such as the proliferation of gill epithelium and necrosis of tissues (Tsotetsi et al., 2005; Shinn et al., 2023). With low infestations, these parasites may not be harmful to the hosts (Tsotetsi et al., 2005), however, in confined environments, the impact of these parasites might be intensified.

While previous studies reported infestation intensities of 1.5 and 1.9 for *L. monodi* (see Hassan et al., 2013), specimens from the current study had higher mean infestation intensities

(1.0–12.29 for *L. clariae*; 20.0 for *L. hepseti*; and 1.5–6.3 for *L. monodi* (see Table 3.4)). Although only the Okavango River were sampled for *L. hepseti* and *L. monodi*, the new locality records of *L. clariae* in the Kabompo River (Zambia), the Nyamiti Pan, Phongolo and Usuthu rivers of the Phongolo Floodplain, as well as the Boskop Dam and Barberspan of the Vaal River, show that the parasite is fast spreading across the river systems of southern Africa. Further emphasising the possibility for any of these *Lamproglena* species to become potential threats to the growing aquaculture industry in southern Africa.

Members of the genus *Lamproglena* are quite distinct morphologically compared to other copepod groups. Species from this study were accurately identified morphologically and sequenced for the first time (*L. clariae* and *L. hepseti,* at the time of analysis) in order to verify the validity of the sequences. This study adds genetic data to the very scanty database for *Lamproglena* species, including the first COI sequences for the genus.

From the current study, all African *Lamproglena* species claded with the Asian *Lamproglena* species confirming their placement in the genus *Lamproglena*. Morphologically, *L. clariae* is the most distinct compared with the other three African *Lamproglena* species with genetic data (both newly generated and from GenBank), and this was expressed in the 18S and 28S phylogenetic trees. In the 28S tree (Fig. 3.12), the *L. clariae* sequences–having a more elongated body form, claded as a sister taxon to the other species that have a relatively shorter and broader body structure. Within the *L. clariae* clade, nonetheless, there seemed to be a separation between the sequence from Zambia and the sequences from South Africa. This may imply that the Zambia sequence is more ancestral evolutionarily to the South African sequences, further suggesting the introductory pathway of the South African sequences. A similar relationship is observed between the *L. monodi* sequence from Botswana and the sequences from Egypt and Kenya.

A possible host-parasite evolutionary relationship was also observed from this study, as *L. hepseti* and *L. hemprichii*, which are host-specific to characiform fish species, also tend to cluster together (see Fig. 3.11). There is currently not enough molecular information available to provide more in-depth discussions on the relationships within the family Lernaeidae or the genus *Lamproglena*. Additional sequences and molecular studies are therefore needed to further understand the evolutionary interactions of these *Lamproglena* species. More genetic analyses linked to accurate morphological identifications are recommended.

3.6. Conclusion

The present study thus provides the first molecular characterisation of *L. clariae* and *L. hepseti* with descriptive notes, using three different genes (partial 18S, 28S, and COI), and the first partial COI sequence for *L. monodi*. This study also gives added information on the maxilliped, antennae, and antennules of *L. hepseti.* For the purpose of understanding the evolutionary relationships that exist in the genus *Lamproglena,* and for higher phylogenetic comparisons, a need for more genetic data in addition to taxonomic and distribution records is required. *Lamproglena clariae* is reported for the first time from the Kabompo River (Zambia), the Nyamiti Pan, Phongolo and Usuthu rivers of the Phongolo Floodplain, with an extension into the Vaal River tributaries (Boskop Dam and Barberspan).

3.7. References

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CHAPTER 4

New distribution records and first molecular characterisation of the invasive fish lice *Argulus japonicus* Thiele, 1900 (Branchiura) from Sub-Saharan Africa, with descriptive notes of all larval stages

4.1. Abstract

Parasites of the genus *Argulus* Müller, 1785 (Branchiura: Crustacea) are typically found on the skin, fins, and gills of their hosts. Although five *Argulus* species are known from South Africa, the Asian invasive species, *Argulus japonicus* Thiele, 1900, is the most widely encountered species in freshwater systems. As part of a larger parasitology project, from 2013–2020, four fish species were sampled from seven different sites along the Orange-Vaal River System: Barberspan, Bloemhof Dam, Gariep Dam, Krugersdrift Dam, Sand River, Vaal River, and the Vaal River Holding Dam. Following standard procedures, the fish were humanely killed, dissected, and screened for parasites. Morphological identification was done using light and scanning electron microscopy. For molecular characterisation, partial ribosomal RNA (18S and 28S), and mitochondrial DNA (COI) gene regions were used as genetic markers. A total of 16 adult females, 35 adult males, and 67 branchiuran larvae identified as *Argulus japonicus* were collected from *Clarias gariepinus* (Burchell, 1822), *Cyprinus carpio* Linnaeus, 1758*, Labeo umbratus* (Smith, 1841), and *Labeobarbus aeneus* (Burchell, 1822). This study reports the first record of *A. japonicus* from the Orange River (Gariep Dam), three new localities in the Vaal River System (Krugersdrift Dam, Sand River, and the Vaal River Holding Dam), provides descriptive notes for all the different life stages of *A. japonicus* (including the addition of a new larval stage), and the first molecular characterisation of *A. japonicus* from sub-Saharan Africa.

4.2. Introduction

Members of the genus *Argulus* Müller, 1785 (Crustacea: Branchiura), commonly called “fish lice", are primarily ectoparasitic on fish but have also been reported from frogs and salamanders (Hoffman, 1977; Wolfe et al., 2001; Poly, 2003; dos Anjos et al., 2021). These parasites are found firmly attached to the epidermis of the host’s skin and larval stages may occur on the gills. With the aid of the antennae and first maxilla in the larval forms (which develop into suckers in adult stages), fish lice can maintain a firm grip on the hosts. One

species in particular, *Argulus japonicus* Thiele, 1900, is reported globally as a major threat to aquaculture and fisheries (Paperna, 1991; Taylor et al., 2005), with reports of economic losses through massive fish kills, fish morbidities, and impaired physiological state of the fish host (Pilgrim, 1967; Avenant-Oldewage, 2001). Its high adaptability and wide host range have assisted in the spreading of this parasite, as *A. japonicus* can thrive in less-than-optimal conditions and persist with development in more favourable environmental conditions (Shafir & Oldewage, 1992).

The different developmental stages of *Argulus* species have been described by Tokioka (1936), Meehean (1940), Shimura (1981), Shafir and van As (1986), Ikuta and Makioka (1994), Rushton-Mellor and Boxshall (1994), Lutsch and Avenant-Oldewage (1995), and Møller et al. (2008). Tokioka (1936) and Meehean (1940) provided detailed information on seven developmental stages of *A. japonicus*. Since then, all other reports on larval development focused mainly on the first two morphologically very distinct larval stages. As no other descriptive records on all larval stages have been made, revisiting the larval stages, focusing on easily identifiable and distinguishing structures, could be helpful in future studies on this invasive species.

The first official record of *A. japonicus* in South Africa was in 1983 from 11 hosts sampled from the Vaal River and its tributaries (Barberspan and Bloemhof Dam) (Kruger et al., 1983). It has subsequently been reported from several other sites in the Vaal River, and the Olifants River (Mpumalanga) (Neethling & Avenant-Oldewage, 2016; Smit et al., 2017). In 2016, Neethling and Avenant-Oldewage provided a review of the research done on *A. japonicus* morphology, ecology, distribution, digestion, reproduction, and histology. However, molecular information on this species is relatively scarce. On the GenBank database, there are only three 18S *A. japonicus* sequences associated with peer-reviewed articles from China (Yang & Gu, 2021) and India (Tandel et al., 2021). For the 28S rRNA gene region, there are no published submissions, and for the mitochondrial DNA gene cytochrome *c* oxidase subunit I (COI), there are only two published articles with submissions from China, Egypt, and Syria (Wadeh et al., 2010), and India (Feroz-Khan et al., 2014). There is also no molecular data of *A. japonicus* (published or unpublished) available from sub-Saharan Africa.

The aims of this study were thus to report new locality records of *A. japonicus*, provide the first molecular data for this species from sub-Saharan Africa using 18S and 28S rRNA genes and COI gene; and also to present a potentially new *A. japonicus* larval stage, along with morphological descriptive notes of the other developmental stages, as a contribution to the already existing and reliable database.

4.3. Materials and methods

4.3.1. Sampling

As part of a larger project on freshwater fish parasites from 2013 to 2020, fish were collected along different regions of the Orange-Vaal River System, South Africa, namely: Barberspan (26°35'21.1"S 25°35'05.6"E), Bloemhof Dam (27°41'41.5"S 25°40'27.2"E), Gariep Dam (30°34'05.8"S 25°33'53.0"E), Krugersdrift Dam (28°51'49.1"S 25°57'44.9"E), Sand River (28°19'23.6"S 27°21'35.1"E), Vaal River 26°56'28.5"S 27°10'59.1"E), and the Vaal River Holding Dam (26°47'55.2"S 27°49'53.0"E) (Fig. 4.1). Fishes were caught using hook and line, electrofishing, and gill nets, and humanely killed by percussive stunning followed by pithing. This study received ethical clearance from the North-West University (NWU-00159-18-A5, NWU- 00600-19-A5) and the University of the Free State (UFS-AED2016/0127) and permits from: The Department of Rural, Environmental and Agricultural Development (NW 8065/03/2019); the Department of Economic Development, Tourism and Environmental Affairs (01/30201), and the Department of Economic, Small Business Development, Tourism and Environmental Affairs, Free State Province (JM 3293/2018 & JM4066/2018). Host and parasite authorities (except for the synonymy) are not included in the references list; host nomenclature is from FishBase (Froese & Pauly, 2023).

4.3.2. Morphological analysis

All branchiuran parasites found were removed with a fine paintbrush and fixed in 70% ethanol for morphology or 96% for molecular analysis. All larval stages (as well as the eggs) were observed at the same time from the parasite population of the fish *Labeobarbus aeneus* (Burchell, 1822), which were caught from the Vaal River Holding Dam and kept in the wet laboratory (aquarium) of the Water Research Group of the North-West University (NWU). Specimens were cleared in 100% lactic acid and stained in lignin pink (2 g lignin pink in 10 ml absolute lactic acid) for morphological studies (adapted from Humes & Gooding,1964).

For light microscopy, specimens were examined with the aid of ZEISS Stemi 508 stereo- and Zeiss AxioLab 5 compound microscopes. Photomicrographs were taken using a ZEISS Axiocam ERc 55 camera attached to the ZEISS Stemi 508 stereo microscope. The bright field and phase-contrast microscopy functions of Nikon Eclipse *Ni* microscope (Nikon Instruments, Tokyo, Japan) were also used to further study the specimens while applying the z-dimensional stacking (Z-stack) function for differential interference contrast (DIC) micrographs of different structures when necessary. With the aid of a drawing tube attached to the Nikon SMZ1500 stereo microscope, pencil drawings were made and electronically inked in Adobe Illustrator (Creative Cloud, 2022) using a Wacom Intuos 4 pro graphic tablet (PTK-640). Terminologies for the description followed Rushton-Mellor (1994). Specimens were

measured in millimetres using Labscope Mat Ver. 2.8.4 microscope software and expressed as mean ± standard deviation (with range in parenthesis).

Selected specimens for scanning electron microscopy (SEM) were analysed by adapting the protocol by Ider et al. (2014) as follows: specimens were dehydrated through a graded ethanol series for 10 minutes per concentration of ethanol (70%, 80%, 90%, 96%, 100%, 100%), passed through a series of graded HMDS (1,1,1,3,3,3-Hexamethyldisilazane) (Merck, Germany) at five minutes per 30%, 50%, 70%, 100%, 100% concentration, then allowed to dry. Dried specimens were mounted on aluminium stubs using carbon tape, sputter coated in gold/palladium (SPI-module, SPI Supplies, Pennsylvania, USA), and studied with a Phenom ProX Desktop SEM (Thermo Fisher Scientific Inc.). Photomicrographs of selected features were taken at 5Kva. Voucher specimens will be deposited in the parasite collection of the National Museum, Bloemfontein (NMB), Free State, South Africa.

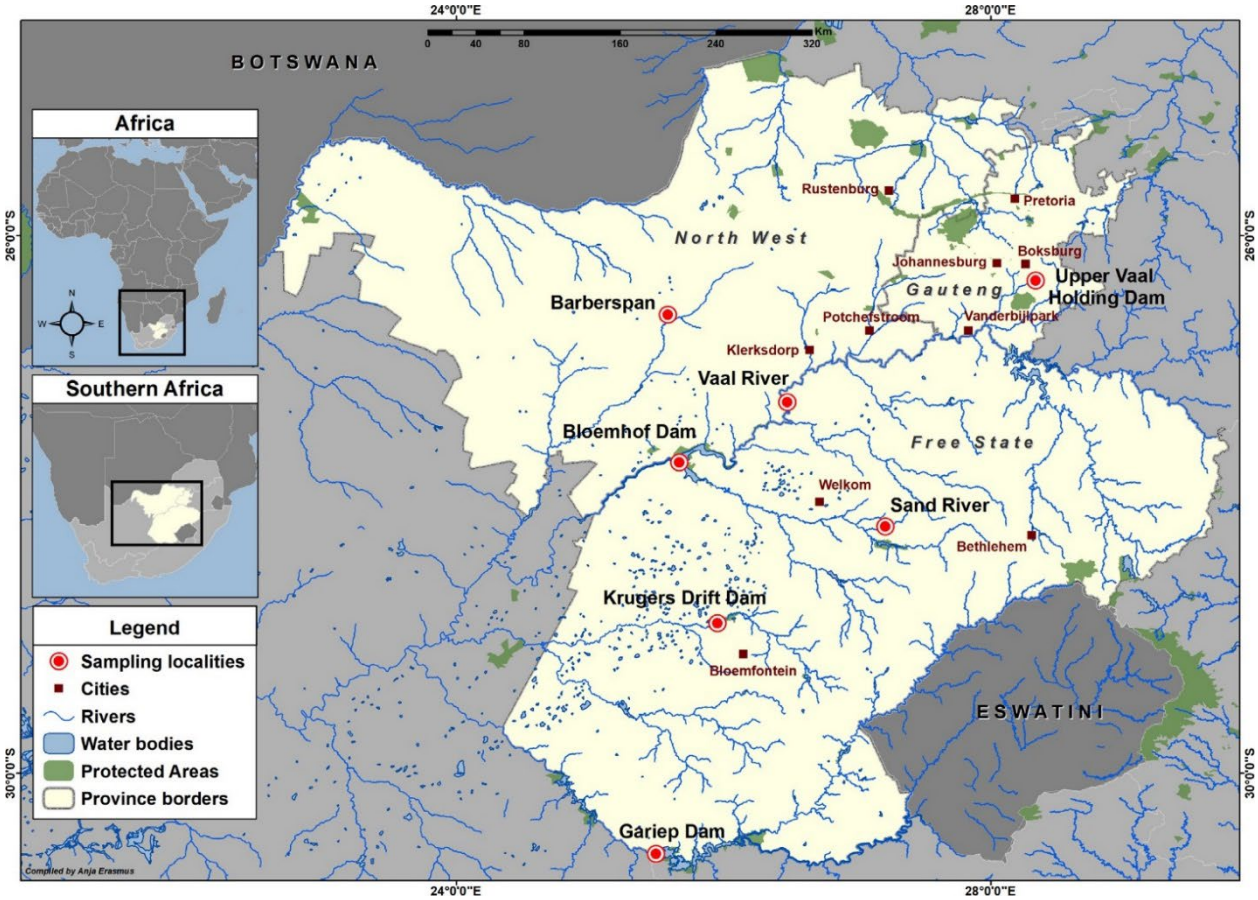


Figure 4.1. Map showing the seven sampling localities of *Argulus japonicus* Thiele, 1900 from the Orange-Vaal River System in South Africa.

4.3.3. Infestation rates

Infestation rates were expressed as prevalence (P), mean abundance (MA) and mean intensity (MI), following definitions from Bush et al. (1997).

4.3.4. Molecular analysis

Argulus genomic DNA was extracted from whole adult and larval specimens using the Macherey-Nagel tissue kit (GmbH & Co. KG, Sandton, South Africa) following the manufacturer's protocol. Two ribosomal RNA gene regions (18S & 28S) and one mitochondrial DNA gene region (cytochrome *c* oxidase subunit I) (COI) were targeted for partial gene amplification. Primers used in the DNA amplifications of 28S, 18S, and COI are listed in Table 4.1. Each Polymerase Chain Reaction (PCR) was carried out in 25 µl volumes using: 12.5 µl of DreamTaq PCR Master Mix (Thermo Fisher Scientific, South Africa), 1.25 µl of 10 µM of each primer (2.5 µl for 28S), 3 µl of DNA product (0.5 µl for 28S) and 7 µl of double distilled water. Polymerase Chain Reaction (PCR) conditions for 18S conditions followed the protocol from Neethling and Avenant-Oldewage (2020), 28S followed the protocol from Møller et al. (2008), and COI conditions followed the protocol from van As et al. (2021). PCR products were verified by 1% agarose gel electrophoresis, further purified, and sequenced in both directions by a commercial sequencing company, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. Obtained sequences were aligned, checked for ambiguity, assembled, and edited using the bioinformatics software platform, Geneious Prime v. 2022.2.2. (Biomatters, Auckland, New Zealand; Kearse et al., 2012).

Table 4.1: List of primers used for DNA amplification and sequencing (18S, 28S and COI partial genes) of *Argulus japonicus* Thiele, 1900 in the present study, with references.

Gene regions	Primers	Sequences	References
18S	18S-E	5'- CCGAATTCGTCGACAACCTGGTTGATCCTGCCA GT-3'	Littlewood & Olson (2001)
	NEM18SR	5'-GGGCGGTATCTGATCGCC-3'	Floyd et al. (2005)
28S	1274F	5'-GACCCGTCTTGAAACACG GA-3'	Markmann & Tautz (2005)
	1276R	5'-CTAGTTGCTTCGGCAGGTGAG-3'	Møller et al. (2008)
COI	LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer et al. (1994)
	HCO2198	5′TAAACTTCAGGGTGACCAAAAAATCA-3′	

Newly generated sequences (Table 4.2) were compared with *Argulus* spp. sequences available on GenBank using the nucleotide Basic Local Alignment Search Tool (BLAST) search analysis. *Argulus* sequences associated with peer-reviewed publications from GenBank were used in the phylogenetic analyses; but for the COI phylogenetic analysis, two unpublished *Argulus japonicus* sequences were included in the alignments (see Table 4.3). All published sequences with less than 700 bases were excluded from the alignments. *Chonopeltis lisikili* van As and van As, 1996 (Argulidae) was used as the outgroup for 18S and COI, and *Levisunguis subaequalis* Curran, Overstreet, Collins & Benz, 2014 (Sebekidae) was used as the outgroup for 28S.

Genetic divergences among 18S, 28S, and COI sequences were calculated following implemented default parameters of MAFFT v7.490 (Katoh et al., 2002; Katoh & Standley, 2013). Alignments for novel sequences and selected GenBank sequences were generated and trimmed. Genetic divergences among aligned sequences were calculated in Geneious Prime v. 2022.2.2 and expressed as percentage similarities and differences in the number of bases. Resultant sequences (contigs) will be submitted to GenBank.

Phylogenetic analyses for all datasets (18S, 28S, and COI) were carried out using Maximum Likelihood (ML) and Bayesian Inference (BI) criteria following an estimation of the best nucleotide substitution model for each dataset determined using the Akaike Information Criterion (AIC) implemented in the jModelTest 2.1.4 (Posada, 2008; Darriba et al., 2012). The suggested model for 18S and 28S was the general time-reversible plus gamma model (GTR+G) and for COI was the general time-reversible model incorporating invariant sites and gamma distributed among-site rate variations (GTR+I+G). The ML analyses were run using PhyML v. 3.0 (Guindon et al., 2010), on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/), which also estimated model parameters and bootstrap values (1000 repetitions). The BI analyses were executed on computational resource CIPRES Science Gateway v 3.3 (Miller et al., 2010) (https://www.phylo.org/), adapting the MrBayes v.3.2.7a. set parameters (Ronquist et al., 2012), running two independent MCMC runs of four chains for 10 million generations and sampling tree topologies every 1,000 generations. Burn-in periods were set to the first 25,000 generations. The phylogenetic trees for ML and BI outputs were visualised in FigTree v 1.4.4 software (Rambaut, 2012).

Table 4.2: List of newly generated sequences of *Argulus japonicus* Thiele, 1900 from the present study. Abbreviations: L1 – First larval stage; L2 – Second larval stage; VRHD – Vaal River Holding Dam.

Isolate	Sex/Life stage	Location	Host	Sequence lengths		
				18S	28S	COI
P03	Male	VRHD	*Labeobarbus aeneus*	925 bp	724 bp	678 bp
P04	Male	VRHD	*L. aeneus*	-	724 bp	-
P05	Female	VRHD	*L. aeneus*	933 bp	721 bp	-
P06	Female	VRHD	*L. aeneus*	-	720 bp	-
P08	L2	VRHD	*L. aeneus*	933 bp	720 bp	-
P09	Female	Vaal River	*Cyprinus carpio*	932 bp	720 bp	678 bp
P10	Male	Barberspan	*Clarias gariepinus*	930 bp	720 bp	678 bp
P11	Male	Gariep Dam	*C. gariepinus*	931 bp	724 bp	678 bp
P14	Female	Bloemhof Dam	*L. aeneus*	931 bp	723 bp	678 bp
P15	L1	VRHD	*L. aeneus*	931 bp	723 bp	678 bp
P16	L2	VRHD	*L. aeneus*	931 bp	723 bp	678 bp

Table 4.3: List of sequences included in the phylogenetic analyses. The taxa in bold are newly generated sequences, all other sequences are from the GenBank databases. *Chonopeltis lisikili* van As and van As, 1996 was used as the outgroup for 18S and COI, and *Levisunguis subaequalis* Curran, Overstreet, Collins & Benz, 2014 was used as the outgroup for 28S.

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Argulus americanus	*Amia calva*	USA	-	MN688128	-	Andres et al. (2019)
Argulus americanus	-	(Maybe USA)	-	-	NC_005935	Lavrov et al. (2004)
Argulus bicolor	*Hypanus sabinus*	USA	-	MN688129	-	Andres et al. (2019)
Argulus bengalensis	*Labeo rohita*	India	KM016969	-	-	Patra et al. (2016)
Argulus flavescens	*Acipenser oxyrinchus*	USA	-	MN688125	-	Andres et al. (2019)
Argulus foliaceus	-	-	-	EU370442	-	von Reumont et al. (2009)
Argulus foliaceus	*Carassius auratus*	India	-	-	KF723419	Feroz-Khan et al. (2014)
Argulus indicus	*Channa striata*	Indonesia	MT538304	-	-	Amriana et al. (2021)
Argulus japonicus	*Schizothorax richardsonii*	India	MW857091	-	-	Tandel et al. (2021)
Argulus japonicus	*Schizothorax richardsonii*	India	MW857090	-	-	Tandel et al. (2021)
Argulus japonicus	*Labeo rohita*	India	-	-	OM836429	Unpublished
Argulus japonicus	*Cyprinus carpio*	Japan	-	-	LC588400	Unpublished
Argulus japonicus	Egg clusters	India	-	-	KF713320	Feroz-Khan et al. (2014)
Argulus japonicus	*Carassius auratus*	India	-	-	JX025025	Feroz-Khan et al. (2014)
Argulus japonicus	*Micropterus salmoides*	China	MW866569	-	-	Yang & Gu (2021)
Argulus japonicus	***Labeobarbus aeneus***	**South Africa**	**P03; P05; P08–P011; P14–P16**	**P03–P06; P08–P011; P14–P16**	**P03; P09–P011; P14–P16**	**Present Study**
Argulus nobilis	*Lepisosteus osseus*	USA	M27187	-	-	Abele et al. (1989)
Argulus pugettensis	*Oncorhynchus clarkii*	USA	MH915670	-	-	Losee et al. (2020)

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Argulus siamensis	*Cyprinus carpio*	India	KF583879	-	-	Patra et al. (2016)
Argulus siamensis	Egg clusters	India	-	-	KF713315	Feroz-Khan et al. (2014)
Argulus siamensis	*Carassius auratus*	India	-	-	KF723415	Feroz-Khan et al. (2014)
Argulus sp.	-	USA	-	AF363322	-	Oakley & Cunningham (2002)
Argulus sp.	-	USA	-	AY210804	-	Mallat et al. (2004)
Argulus sp.	-	Mexico	-	-	MZ518075	Valdez-Moreno et al. (2021)
Chonopeltis lisikili	*Synodontis zambezensis*	South Africa	MW678625	-	MW679715	van As et al. (2021)
Levisunguis subaequalis	*Gambusia affinis*	USA	-	MN065508	-	Woodyard et al. (2019)

4.4. Results

Adult specimens were positively identified as *A. japonicus* (Fig. 4.2) based on the following characteristics: carapace dorsal ridges present; the number of supporting rods and shape of sclerites; shape and position of the respiratory areas; the shape of basal spines on the second maxillae and presence of a scabrous section on the basal plate; the presence or absence of bilobed protuberances on second swimming appendage in the male; and the shape of the peg and natatory lobe on the coxa of the fourth swimming legs in males and females, respectively.

Generally, the carapace shape is subcircular, ornamented with several backward spines at its anterior margin, has branched dorsal ridges, and extends to the basis of the 3rd pair of legs, covering the 4th pair of legs. The anterior spine of the first antenna lacks a hook; the first maxilla (sucker) comprises 49–52 supporting rods, with 5–7 sclerites per rod; the basal sclerite is elongate and decreasing in size towards the periphery. The basal plate of the second maxilla is broad with a scabrous pear-shaped section, a pair of setae, and has three relatively sharp basal spines that are widely separated. The anterior respiratory area is small and subtriangular, placed above the large reniform posterior respiratory area.

Specific characteristics for males include a posterior bilobed protrusion situated ventrally on the basis of the 2nd leg, protruding in opposite directions. The basal segment of the 3rd swimming leg is broad with a posterior lobe containing the socket, extending backwards about two-thirds the length of the segment. The 4th swimming leg of the males has a chitinous

shovel-shaped peg projecting laterally from the distal end of the basis. The basis of the 4th leg is posteriorly enlarged, and the coxa has a protruding posterior lobe (fleshy in females). Males are usually smaller compared to females with visible testes and spermathecae, respectively, in the abdominal lobes. Measurements for both adult males (n = 20) and females (n = 11) are provided in Table 4.4.

The identification of this argulid was further confirmed using the key from Rushton-Mellor (1994) for adult *Argulus* found in Africa (native and invasive) and descriptive notes by Pilgrim (1967), Nagasawa (2021), and Tandel et al. (2021). Larval stages were confirmed from descriptions by Tokioka (1936) and Meehean (1940).

Table 4.4: Measurements for male and female *Argulus japonicus* Thiele, 1900 from selected sites in the current study, from the Orange-Vaal River System in South Africa. Values are given as mean ± standard deviation (with range in parentheses). Abbreviations: %CL of TL – Percentage Carapace Length of Total Length; AbL – Abdomen Length; AbW – Abdomen Width; CL – Carapace Length; CW – Carapace Width; SDi – Sucker Diameter (inner); SDo – Sucker Diameter (outer); SiL – Sinus Length; SthL – Spermathecae Length; SthW – Spermathecae Width; TL – Total Length; TsL – Testes Length; TsW – Testes Width.

Structures	Male (n = 20)	Female (n = 11)
TL	4.13 ± 0.44 (3.40–4.76)	5.61 ± 0.94 (3.31–6.63)
CW	2.71 ± 0.41 (1.99–3.38)	3.93 ± 0.62 (2.33–4.64)
CL	3.06 ± 0.40 (2.42–3.59)	4.63 ± 0.69 (2.89–5.36)
%CL of TL	73.9 ± 3.1 (69.6–79.9)	8.30 ± 4.2 (77.6–89.3)
SDo	1.07 ± 0.11 (0.88–1.22)	1.11 ± 0.23 (0.55–1.39)
SDi	0.83 ± 0.10 (0.67–0.99)	1.04 ± 0.20 (0.57–1.26)
AbL	0.42 ± 0.09 (0.21–0.54)	0.48 ± 0.20 (0.17–0.84)
AbW	0.45 ± 0.05 (0.39–0.53)	0.69 ± 0.11 (0.44–0.81)
SiL	0.31 ± 0.04 (0.25–0.37)	0.49 ± 0.08 (0.29–0.60)
TsL/SthL	0.70 ± 0.14 (0.47–1.07)	0.13 ± 0.05 (0.08–0.21)
TsW/SthW	0.29 ± 0.05 (0.23–0.40)	0.07 ± 0.02 (0.05–0.12)

4.4.1. Taxonomy

Arguloidea Yamaguti, 1963

Argulidae Leach, 1819

Argulus Müller, 1785

***Argulus japonicus* Thiele, 1900** (Fig. 4.2)

Type host. Goldfish, *Carassius auratus* (Linnaeus, 1758).

Additional hosts from South Africa. ***Clarias gariepinus* (Burchell, 1822) [present study], *Cyprinus carpio* Linnaeus, 1758 [present study],** *Enteromius mattozi* (Guimarães, 1884), *Labeo capensis* (Smith, 1841), *Labeo rosae* Steindachner, 1894, ***Labeo umbratus* (Smith, 1841), [present study], *Labeobarbus aeneus* (Burchell, 1822) [present study],** *Labeobarbus kimberleyensis* (Gilchrist & Thompson, 1913), *Labeobarbus marequensis* (Smith, 1841), *Oncorhynchus mykiss* (Walbaum, 1792), *Oreochromis mossambicus* (Peters, 1852), *Schizothorax richardsonii* (Gray, 1832).

Type locality. Yeddo, Japan.

Additional localities from South Africa. **Barberspan [present study]**; **Bloemhof Dam [present study]**; Bronkhorstspruit River; Crocodile River (West) (Hartebeespoort Dam); **Gariep Dam [present study]**; Harts River; Incomati River; **Krugersdrift Dam [present study]**; Lydenburg Fish Hatchery; Mooi River; Olifants River; Pienaars River; **Sand River [present study]**; **Vaal River [present study]**; **Vaal River Holding Dam [present study]**.

Material examined.

A total of 159 argulids (54 adult males, 37 adult females, and 67 larvae) were examined; 20 used for SEM; five females for dissection; five adult females, five adult males and eight larvae used for DNA extraction; 10 will be deposited in the collections of the National Museum, Bloemfontein, South Africa; the remainder are in the collection of the North-West University-Water Research Group (NWU-WRG), Potchefstroom, South Africa.

South Africa. Barberspan (26°35'21.1"S 25°35'05.6"E). *Clarias gariepinus* – 15 males, one female; *Cyprinus carpio* – one male; *Labeo umbratus* – six males, collected by P. P. Fikiye (2019).

Bloemhof Dam (27°41'41.5"S 25°40'27.2"E). *Labeobarbus aeneus* – seven females, collected by L. L. van As (2019).

Gariep Dam (30°34'05.8"S 25°33'53.0"E). *C. gariepinus* – three males, one female, collected by M Truter (2018); *L. aeneus* – nine males, two females, collected by L. L. van As (2013).

Krugersdrift Dam (28°51'49.1"S 25°57'44.9"E). *C. carpio* – two males, four females, collected by L. L. van As (2012 & 2019).

Sand River (28°19'23.6"S 27°21'35.1"E). *C. gariepinus* – one female, collected by M Truter (2018).

Vaal River 26°56'28.5"S 27°10'59.1"E). *C. carpio* – four females, collected by N. J. Smit (2019).

Vaal River Holding Dam (26°47'55.2"S 27°49'53.0"E). *L. aeneus* – 15 males, 15 females, 67 larvae, collected by W. Malherbe (2019).

Representative DNA sequences (detailed information in Table 4.2). Sequences, with the numbers of base pairs (bp), will be submitted to GenBank as follows:

18S. 925–931 bp long sequences (three male specimens); 931–933 bp long sequences (three female specimens); 931–933 bp long sequences (three larvae specimens)

28S. 720–724 bp long sequences (four male specimens); 720–723 bp long sequences (four female specimens); 720–723 bp long sequences (three larvae specimens).

COI. 678 bp long sequences of (three male specimens); 678 bp long sequences (two female specimens); 678 bp long sequences (two larvae specimens).

Infestation rates. Total prevalence 58.72% (64/109), mean parasite intensity: 2.47 (158/64), mean parasite abundance 1.45 (158/109). Further details on infestation rates per host and locality are provided in Table 4.5.

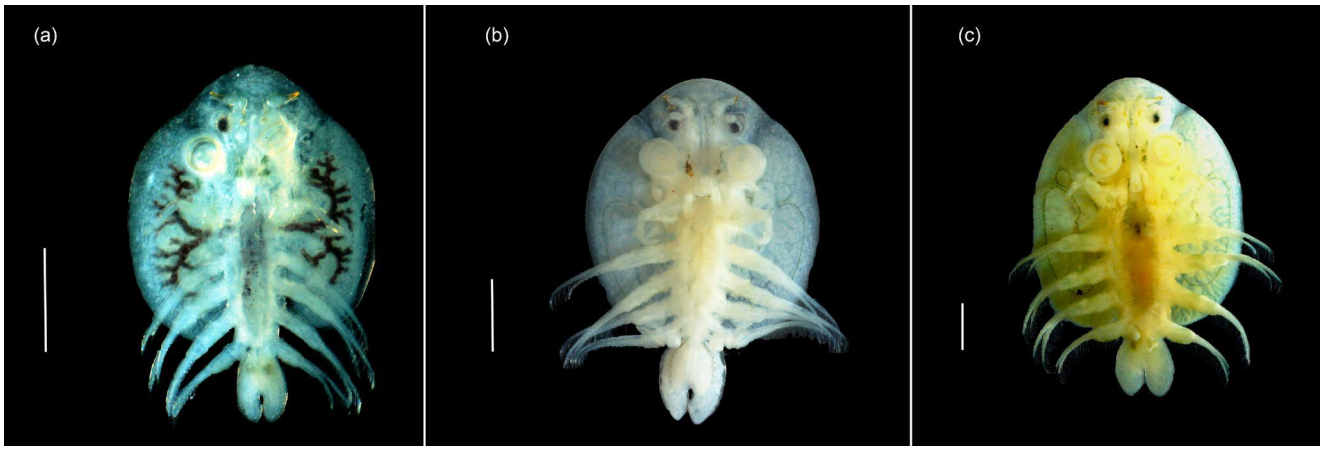


Figure 4.2. Light microscopy photomicrographs of *Argulus japonicus* Thiele, 1900 specimens collected from different localities in the current study: (a) full ventral image of female from the Vaal River Holding Dam; (b) full ventral image of male from Barberspan; (c) full ventral image of female from the Bloemhof Dam. Scale bars: (a) 0.5 mm; (b–c) 1 mm.

Table 4.5: Infestation data of *Argulus japonicus* collected from several hosts and different localities in South Africa. Abbreviations: F – Female; L – Larva; M – Male.

Locality	Host	No. Examined	No. Infested	Parasites Collected (PC)			Total PC	Prevalence	Mean Intensity	Mean Abundance
				M	F	L				
Barberspan	*Clarias gariepinus*	21	10	15	1		16	47.62	1.60	0.76
	Cyprinus carpio	2	1	1			1	50.00	1.00	0.50
	Labeo umbratus	3	2	6			6	66.67	3.00	2.00
Bloemhof Dam	*Labeobarbus aeneus*	1	1		7		7	100.00	7.00	7.00
Gariep Dam	*Clarias gariepinus*	15	6	3	1		4	40.00	0.67	0.27
	Labeobarbus aeneus	16	7	9	2		11	43.75	1.57	0.69
Krugersdrift Dam	*Cyprinus carpio*	1	1	2	4		6	100.00	6.00	6.00
Vaal River Holding Dam	*Labeobarbus aeneus*	33	33	15	15	67	97	100.00	2.94	2.94
Sand River	*Clarias gariepinus*	15	1		1		1	6.67	1.00	0.07
Vaal River	*Cyprinus carpio*	1	1		4		4	100.00	4.00	4.00
Klipdrift Dam	*Cyprinus carpio*	1	1	3	2		5	100.00	5.00	5.00
	Total	**109**	**64**	**54**	**37**	**67**	**158**	**58.72**	**2.47**	**1.45**

Table 4.6: Comparing the total lengths of all *Argulus japonicus* Thiele, 1900 larval stages from the present study with Tokioka (1936). Abbreviation: L – Larval stage.

Larval stage	Tokioka (1936) (mm)	Current study (mm)
L1	0.7–0.9	0.63–0.77
L2	0.9–1.0	0.76–0.85
New L3	-	0.82–1.27
L3	1.0–1.2	1.02–1.32
L4	1.2–1.4	1.37–1.48
L5	1.3–1.7	1.54
L6	1.6–2.0	1.91–2.69
L7	2.0–2.2	2.15–2.73

Descriptions by Tokioka (1936) and Meehean (1940) provide details on the developmental stages, and Lutsch and Avenant-Oldewage (1995) provide additional information on the newly hatched *A. japonicus* larvae. The descriptive notes (with additional measurements) from this study are summarised notes for larval stages 1–2 and 4–8; these notes are mainly for comparison of the new third larval stage proposed here.

Descriptive notes of larval stages (Figs. 4.3–4.6).

Average measurements of eight developmental stages (67 larval specimens) are compared with Tokioka's (1936) measurements in Table 4.6.

First larval stage (L1) (n = 9)**:** Total length (TL) 0.63–0.77 mm, carapace width (CW) ranging from 0.31–0.44 mm, carapace length (CL) 0.46–0.53 mm with percentage CL of TL ranging from 60.35–77.01%. Body oval-shaped dorsoventrally (Fig 4.3), convex-shaped dorsolaterally. Lateral carapace lobes short exposing thorax and legs. Pair of brown compound eyes; margin of carapace anterior to compound eyes fringed with chitinous ciliary processes. First antenna (Fig. 4.4) similar to adult stage but lacking distal segment; 4-segmented: first segment broad with anterior spine and distal hook, second segment with one anterolateral seta, third segment with one mid-terminal seta and 3–4 terminal setae. Second antenna (Fig. 4.4) situated behind first antenna, comprises two basal segments with single posterior spine and posterolateral seta per segment, large antennal palp ending in four plumose setae at distal end of second segment; third segment with small seta at distal end; fourth segment with two medially situated setae; fifth segment with terminal curved spine and 3–4 setae. The mandibular palp is unsegmented, with rows of spines on anterodistal margin and terminating in three long plumose setae; situated at the base of the proboscis. First maxilla (Fig. 4.5) is a hooked appendage (clasping organ) consisting of three segments, first segment is bulky with an anterior spine and a posterior seta; second segment has a spine on the posteroproximal margin, and a single row of very short setae situated ventrodistally; third segment with two terminal, retractable, curved hooks, one of which bears serration of three small, barbed protrusions on inner anterior margin, and the other hook smooth. Second maxilla (Fig. 4.6) 5-segmented, basal segment possesses finely bristled scales with a single spine situated posterodistally, which becomes the middle of three basal spines found in the adult. Two pairs of post-maxillary spines situated behind second maxilla, with anterior pair larger than the posterior pair. Swimming legs rudimentary.

Second larval stage (L2) (n = 10)**:** Total length 0.76–0.85 mm, carapace width ranging from 0.50–0.66 mm, carapace length 0.50–0.55 mm with percentage CL of TL ranging from 62.94%–72.15%. Parts of post-antennal palp may be observed; post-antennal spines present

(Fig. 4.4). First maxilla (Fig. 4.5) a hooked clasping appendage similar to first larval stage; distal segment of basal podomere now described as second segment of first maxilla; two strong spines with broad bases visible on ventroproximal margin of second segment; a single posterolateral spine situated at inner proximal margin of third segment; terminal claw still bears serrated protrusions. Second maxilla (Fig. 5.6) 5-segmented, similar to first larval stage; one to two sharply pointed spines and a single seta on basal segment; scanty rows of pectinate scales and one to two setae on second to fifth segment; fifth segment with two terminal claws and setae. Thorax and swimming legs ornamented with several scales; four pairs of biramous swimming legs; flagella may be present on basal segment of first pair of legs.

Third larval stage L3 (n = 8)**:** Total length 0.82–1.27 mm, carapace width ranging from 0.45–0.72 mm, carapace length 0.53–0.87 mm with percentage CL of TL ranging from 64.83–73.70%. Residual post-antennal palps absent (Fig. 4.4), antennae similar in size to second larval stage. Basal segment of first maxilla (Fig. 4.5) larger than in the second larval stage, onset of round anlage for sucker formation; two spines on second segment ventroproximal margin still visible and one spine present on posterolateral margin of third segment; serrated terminal claw with three to four processes. Paired short setae visible on basal plate of second maxilla (Fig.4.6), increased number of pectinate scales on basal segments of second maxilla, two sharply pointed basal spines present; distal end of second segment has a single elongate spine situated posterolaterally. Swimming legs same as L2; flagella visible on basal segment of first pair of legs.

Fourth larval stage L4 (n = 11)**:** Total length 1.02–1.32 mm, carapace width ranging from 0.62–0.78 mm, carapace length from 0.68–0.98 mm with percentage CL of TL ranging from 67.03–73.95%. First antenna larger than second antenna (Fig. 4.4). Enlargement of round anlage of first maxilla basal plate visible (Fig. 4.5), second segment less distinct, no spines on second segment ventroproximal margin. Second maxilla (Fig. 4.6) similar to previous stage, but with three basal spines present (third spine partially developed) on basal segment and increased number of scales on second, third, and fourth segments. Swimming legs increased in size compared to previous stage; flagella visible on basal segment of first pair of legs.

Fifth larval stage L5 (n = 4)**:** Total length 1.37–1.48 mm, carapace width ranging from 0.62–0.86 mm, and carapace length from 0.84–1.06 mm with percentage CL of TL ranging from 65.37–77.40%. Carapace extending to second pair of legs (Fig. 4.3), marginal spines on carapace reaching anterior respiratory area. Basal segment of first maxilla (Fig. 4.5) fully enlarged; visible shape of suckers well-formed, shrinkage of hook. Second maxilla (Fig. 4.6) with more pectinate scales on basal plate, spines on basal plate consistent with previous

stage. Swimming legs increasing slightly in size. Copulatory structures in males may be visible, natatory lobes of the fourth legs already visible.

Sixth larval stage L6 (n = 1)**:** Total length 1.54 mm, carapace width 1.01 mm, carapace length 1.15 mm with percentage CL of TL 74.41%. Carapace fully covers second pair of legs (Fig. 4.3), marginal spines of carapace reaching first thoracic segment. Basal segment of first maxilla (Fig. 4.5) almost a full sucker, 1–2 sclerites may be observed in supporting rods. Second maxilla (Fig. 4.6) possesses three fully formed basal spines. Swimming legs increase in size compared with previous stage.

Seventh larval stage L7 (n = 12)**:** Total length 1.91–2.69 mm, carapace width ranging from 1.17–1.76 mm, and carapace length 1.43–1.99 mm with percentage CL of TL ranging from 69.06– 78.29%. Carapace extends to third pair of legs. Marginal spines of carapace reaching anterior respiratory area; residual hook of suckers very reduced; 2–3 sclerites observed in supporting rods of sucker (Fig. 4.5). Swimming legs and thoracic segment with more spines (Fig. 4.3), copulatory structures in males visible: bilobed structure on posteroventral coxa of leg 2 visible but not completely formed; bulging process on the coxa of the third leg (which eventually becomes the socket) may be seen; anterodistal axis of basis of fourth leg possesses a round protruding process (which becomes the peg in adult males).

Eight larval stage L8 (n = 12)**:** Total length 2.15–2.73 mm, carapace width ranging from 1.33–1.73 mm, carapace length 1.56–1.95 mm with percentage CL of TL ranging from 69.95–78.32%. Carapace with more marginal spines and covers third pair of legs. Rudimentary hook mostly absent in sucker (Figs. 4.3, 4.5), 3–4 sclerites observed in supporting rods of sucker (Fig. 4.5). Structures of swimming legs become more pronounced. Testes and spermathecae can be seen in both males and females, respectively.

Remarks.

According to Tokioka (1936), seven larval stages occur upon attachment to a suitable host, with subsequent moults that bring the parasite to sexual maturity (Meehean, 1940). During this study, another larval stage not previously reported was observed. This new larval stage shares similar characteristics with Tokioka's second larval stage as it retains the two spines previously seen on the ventroproximal margin of the first maxilla's second segment, but there are no rudiments of the antennal palps, as was observed in current second larval stage specimens. While the new larval stage falls within the size range given for Tokioka's second larvae, there are two spines on the second maxilla's basal segment (of the new larval stage) that are twice the length of Tokioka's second larval stage, and the paired setae (usually only one in the second larvae) were clearly visible in this new larval stage.

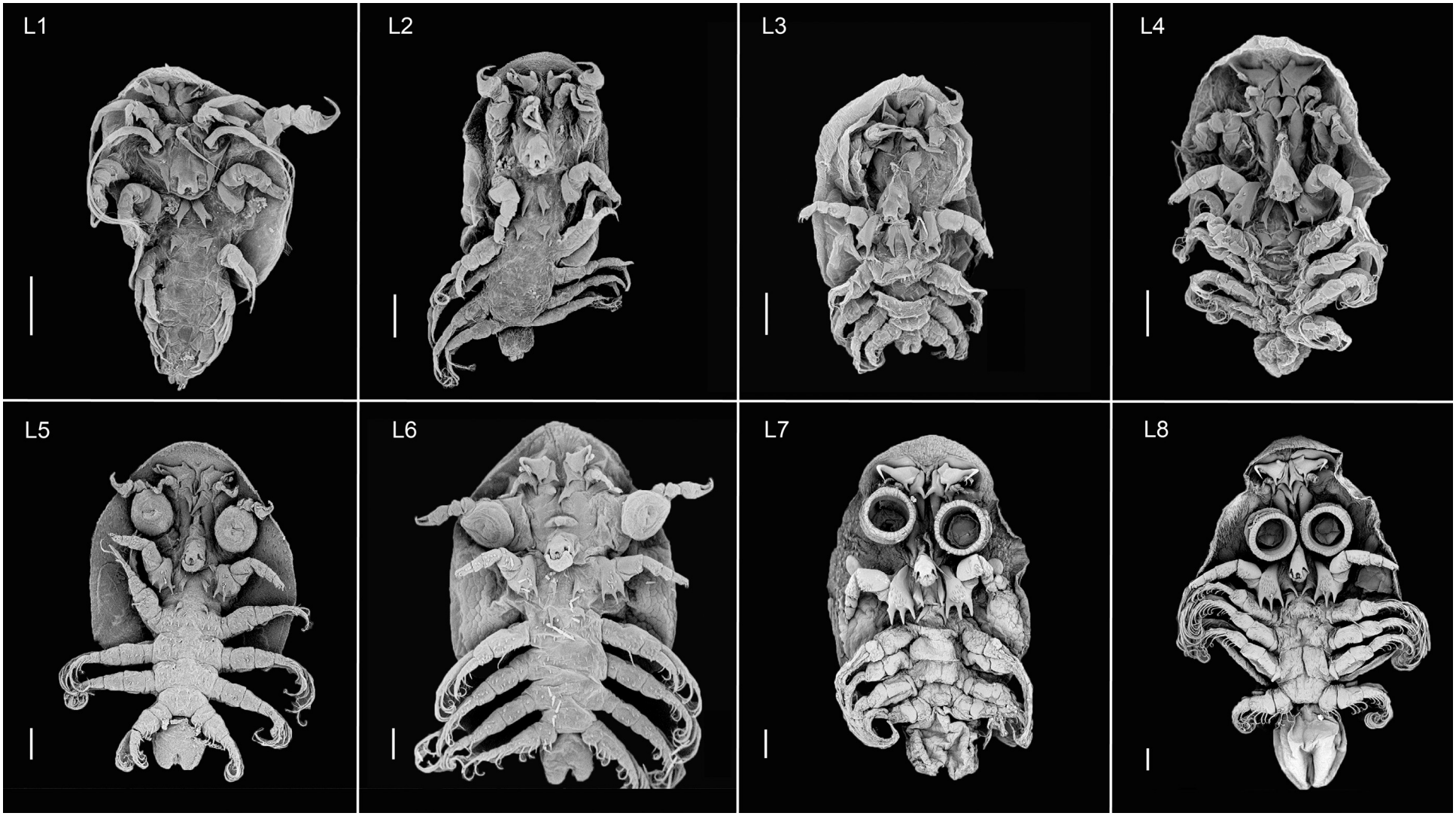


Figure 4.3. Scanning electron microscopy (SEM) images of whole *Argulus japonicus* Thiele, 1900 larval stages one (L1) to eight (L8) from the current study. Scale bars: (L1–8) 100 μm. Abbreviation: L – larval stage ontogeny.

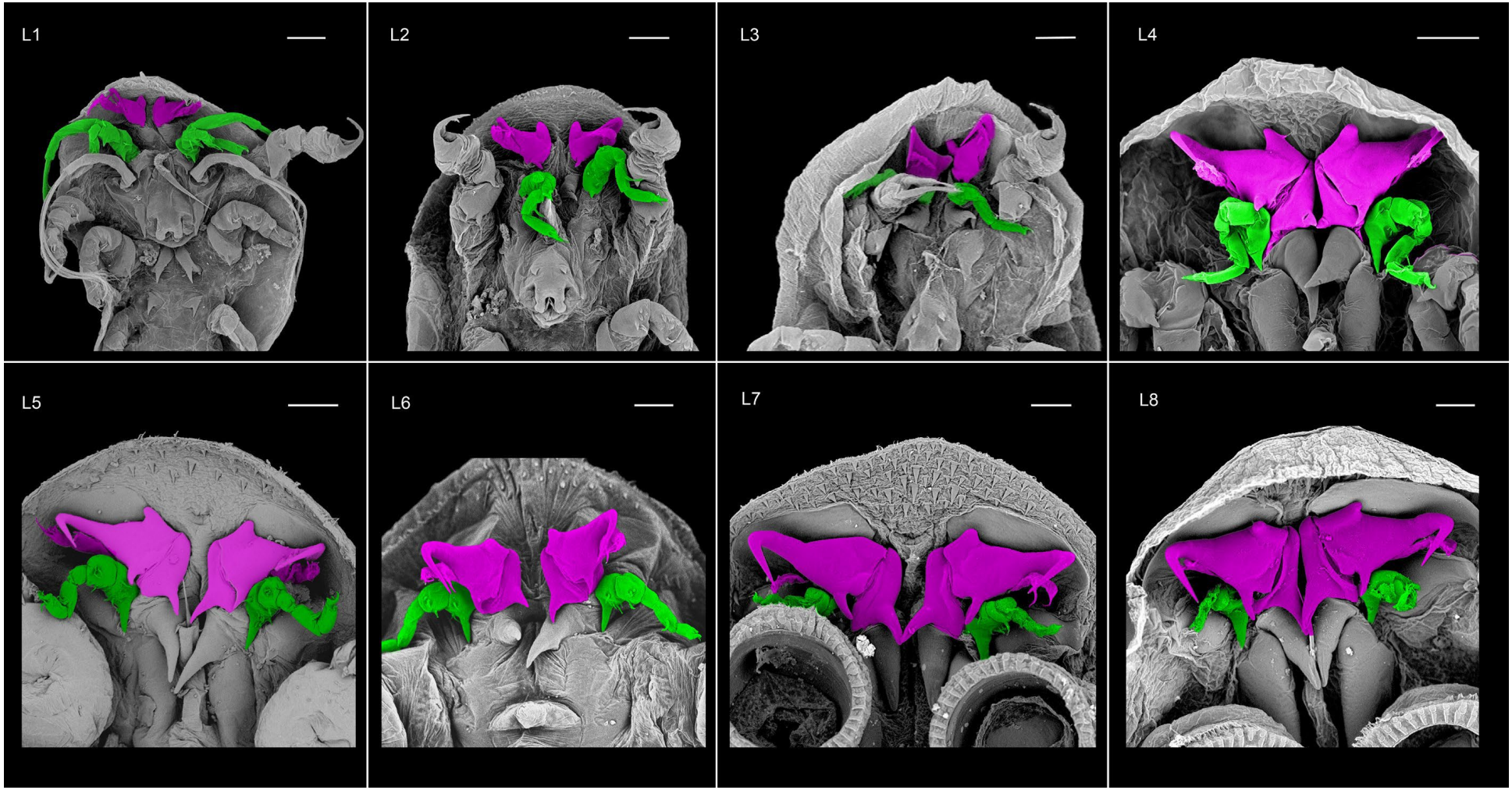


Figure 4.4. Scanning electron microscopy (SEM) images of the first antenna (pink) and second antenna (green) for all *Argulus japonicus* Thiele, 1900 larval stages from the current study. Scale bars: (L1) 80 μm; (L2–8) 50 μm. Abbreviation: L = larval stage ontogeny.

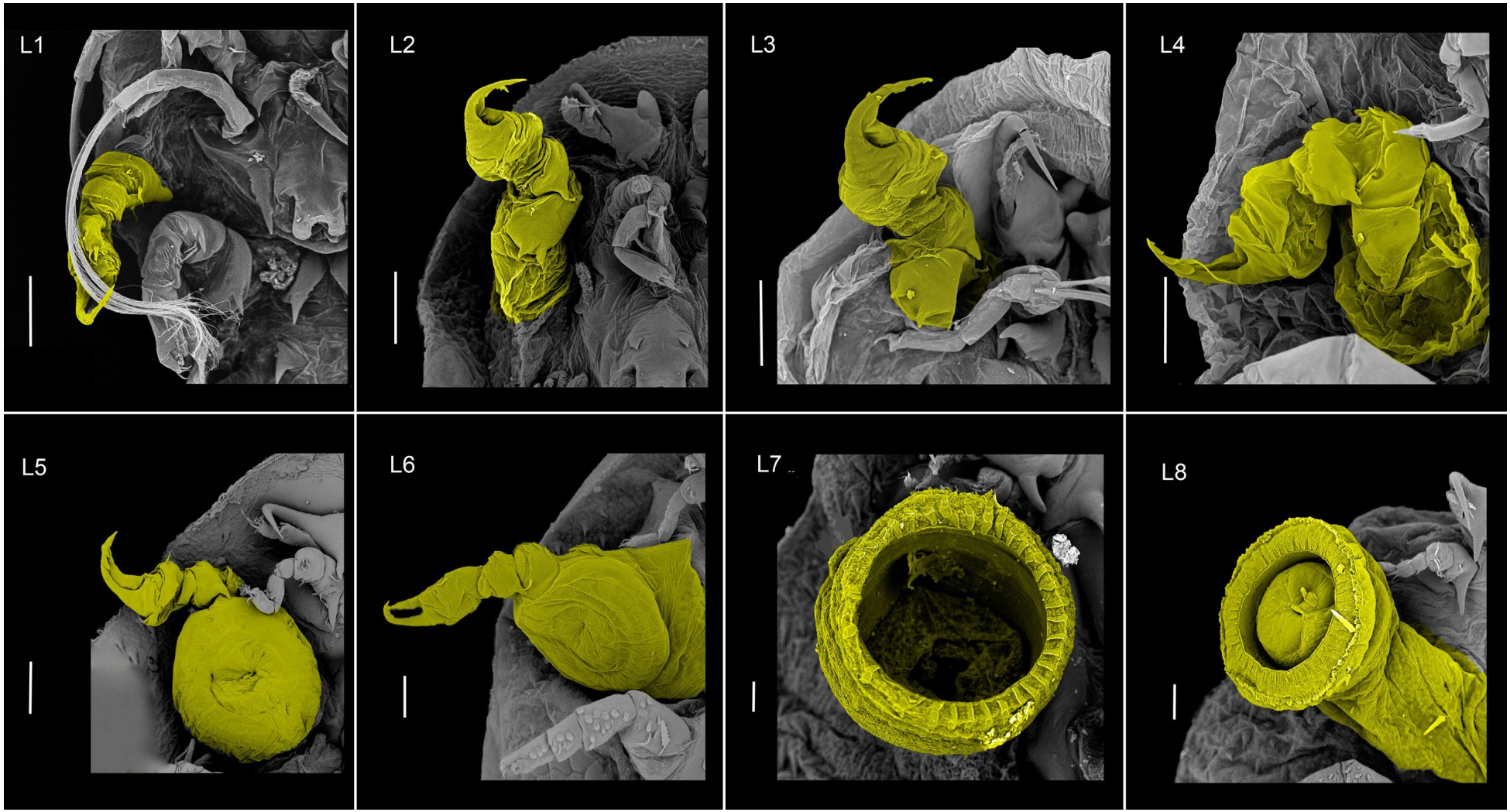


Figure 4.5. Scanning electron microscopy (SEM) images of the first maxilla (highlighted in yellow) for all *Argulus japonicus* Thiele, 1900 larval stages from the current study. Scale bars: (L1) 80 μm; (L2–8) 50 μm. Abbreviation: L = larval stage ontogeny.

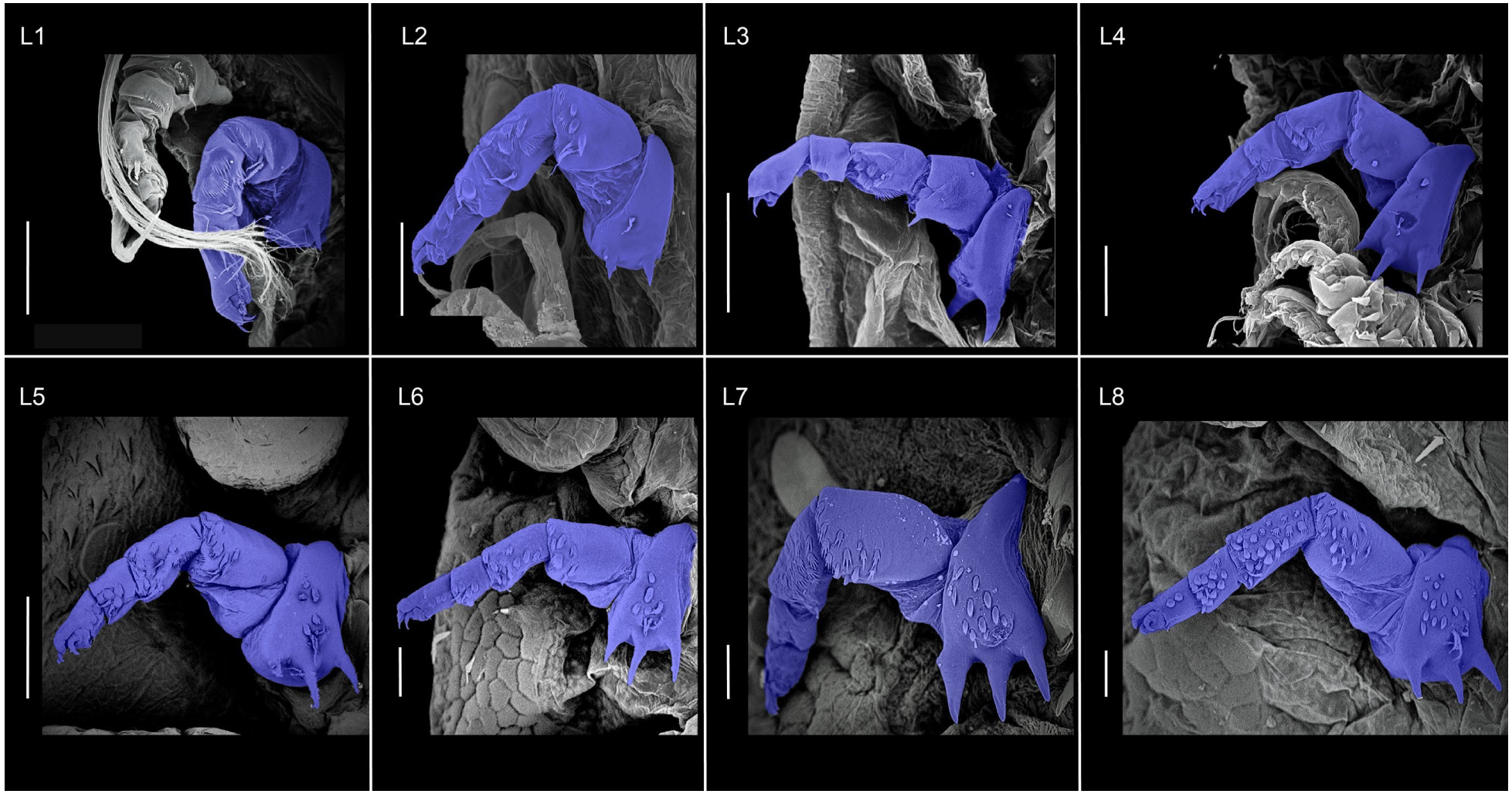


Figure 4.6. Scanning electron microscopy (SEM) images of the second maxilla (highlighted in blue) for all *Argulus japonicus* Thiele, 1900 larval stages from the current study. Scale bars: (L1–2, 4, 6–7) 50 µm; (L3 & 5) 80 µm. Abbreviation: L = larval stage ontogeny.

When compared with Tokioka's third larval stage, individuals from this new larval stage were smaller in size (see Table 4.6). Although degeneration of the first maxilla from a clasping appendage begins from this new larval stage, the round anlage for sucker formation reported on the first maxilla's basal segment in Tokioka's third larval stage is not distinctly seen. There are also three spines on the second maxilla's basal segment (one short), compared to the two observed in the new larval stage. Based on these differences, a new larval stage between Tokioka's second and third larval stages are proposed as the new third stage, bringing the total number of larval stages to eight (Figs. 4.3–4.6).

4.4.2. Molecular analysis

A total of 27 novel sequences were generated from this study (nine sequences for 18S, 11 sequences for 28S, and seven sequences for COI) (see representative DNA section above). Tree topologies for Bayesian Inference (BI) and Maximum Likelihood (ML) analyses were similar for all datasets (18S, 28S, and COI).

Phylogenetic alignments of 18S sequences resulted in a length of 1,102 bases. All the newly generated *A. japonicus* sequences (except a sequence from Barberspan that differed with a single nucleotide) were 100% identical. *Argulus japonicus* sequences from China and India had 99.69–99.84% similarity (1–3 bp) with sequences generated from this study (see Table 4.7). In the 18S phylogenetic tree (Fig. 4.7), *A. japonicus* sequences clustered in two clades: sequences from India; and newly generated sequences with a sequence from China. The sequences generated from the second larval stage (L2) formed a sister clade, while the sequence for the first larval stage (L1) formed a sister clade with the *A. japonicus* sequence from China (Fig. 4.7). Adult male and female *A. japonicus* sequences formed sister clades throughout the larger clade (Fig. 4.7).

Phylogenetic alignment of partial 28S sequences yielded an alignment length of 873 bases. In the 28S alignment, all the newly generated *A. japonicus* sequences were 100% identical but there are no other published *A. japonicus* sequences to compare with. When compared with other *Argulus* sequences in the alignment, newly generated *A. japonicus* sequences were most similar to *A. foliaceus* specimens (97.08–97.10%; 21 bp) (see Table 4.8). From the phylogenetic tree constructed (Fig. 4.8), newly generated sequences clustered in a separate clade from other *Argulus* sequences. Similar to the 18S tree, adult male and female sequences formed sister clades throughout the larger *A. japonicus* clade, and the larval stages also clade together (L1 and L2), with one L2 sequence forming a sister clade with a sequence for a female of the same host and locality (see Fig. 4.8).

Table 4.7: Genetic divergences among aligned 18S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (site localities are included for sequences from the present study). Sequences from the present study in boldface and grey shade. *Chonopeltis lisikili* (MW678625) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; CN – China; F – Female; GD – Gariep Dam; ID – Indonesia; IN – India; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	**P03 *Argulus japonicus* (M) VRHD ZA**		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**1**	**1**	**93**	**29**	**23**	**24**	**19**	**153**
2	**P05 *Argulus japonicus* (F) VRHD ZA**	**100**		**0**	**0**	**0**	**1**	**0**	**0**	**1**	**2**	**1**	**1**	**96**	**29**	**24**	**24**	**19**	**155**
3	**P08 *Argulus japonicus* (L2) VRHD ZA**	**100**	**100**		**0**	**0**	**1**	**0**	**0**	**1**	**2**	**1**	**1**	**96**	**29**	**24**	**24**	**19**	**155**
4	**P09 *Argulus japonicus* (F) VR ZA**	**100**	**100**	**100**		**0**	**0**	**0**	**0**	**1**	**2**	**1**	**1**	**95**	**29**	**23**	**24**	**19**	**155**
5	**P10 *Argulus japonicus* (M) BP ZA**	**100**	**100**	**100**	**100**		**1**	**0**	**0**	**1**	**2**	**1**	**1**	**96**	**29**	**24**	**24**	**19**	**155**
6	**P11 *Argulus japonicus* (M) GD ZA**	**100**	**99.9**	**99.9**	**100**	**99.9**		**1**	**1**	**2**	**3**	**1**	**1**	**97**	**29**	**25**	**24**	**19**	**155**
7	**P14 *Argulus japonicus* (F) BhD ZA**	**100**	**100**	**100**	**100**	**100**	**99.9**		**0**	**1**	**2**	**1**	**1**	**96**	**29**	**24**	**24**	**19**	**155**
8	**P15 *Argulus japonicus* (L1) VRHD ZA**	**100**	**100**	**100**	**100**	**100**	**99.9**	**100**		**1**	**2**	**1**	**1**	**95**	**29**	**23**	**24**	**19**	**155**
9	**P16 *Argulus japonicus* (L2) VRHD ZA**	**100**	**99.9**	**99.9**	**99.9**	**99.9**	**99.79**	**99.9**	**99.9**		**3**	**1**	**1**	**96**	**29**	**24**	**24**	**19**	**155**
10	MW866569 *Argulus japonicus* CN	**99.79**	**99.79**	**99.79**	**99.79**	**99.79**	**99.69**	**99.79**	**99.79**	**99.69**		3	3	98	31	26	26	21	157
11	MW857090 *Argulus japonicus* IN	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	99.53		2	86	11	14	7	20	83
12	MW857091 *Argulus japonicus* IN	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	99.53	99.68		86	11	14	7	20	83
13	KM016969 *Argulus bengalensis* IN	**90.37**	**90.48**	**90.52**	**90.42**	**90.52**	**90.42**	**90.52**	**90.38**	**90.33**	90.23	86.95	87.09		110	75	102	75	230
14	MT538304 *Argulus indicus* ID	**96.89**	**96.92**	**96.92**	**96.94**	**96.92**	**96.92**	**96.92**	**96.92**	**96.94**	96.73	98.27	98.29	88.68		41	39	46	169
15	M27187 *Argulus nobilis* USA	**97.56**	**97.56**	**97.57**	**97.62**	**97.57**	**97.47**	**97.57**	**97.61**	**97.52**	97.34	97.80	97.82	92.63	95.68		32	41	169
16	MH915670 *Argulus pugettensis* USA	**97.38**	**97.41**	**97.41**	**97.42**	**97.41**	**97.41**	**97.41**	**97.41**	**97.42**	97.21	98.90	98.91	89.33	95.82	96.57		42	168
17	KF583879 *Argulus siamensis* IN	**97.77**	**97.79**	**97.79**	**97.81**	**97.79**	**97.79**	**97.79**	**97.79**	**97.81**	97.58	96.94	96.97	91.40	94.70	95.27	95.16		165
18	MW678625 *Chonopeltis lisikili* ZA	**85.09**	**85.07**	**85.06**	**85.10**	**85.07**	**85.07**	**85.07**	**85.07**	**85.10**	84.91	87.70	87.83	78.39	83.54	83.77	83.38	82.47	

Table 4.8: Genetic divergences among aligned 28S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (site localities are included for sequences from the present study). Sequences from the present study in boldface and grey shade. *Levisunguis subaequalis* (MN065508) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; CN – China; F – Female; GD – Gariep Dam; ID – Indonesia; IN – India; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	**P03 *Argulus japonicus* (M) VRHD ZA**		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
2	**P11 *Argulus japonicus* (M) GD ZA**	**100**		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
3	**P14 *Argulus japonicus* (F) BhD ZA**	**100**	**100**		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
4	**P15 *Argulus japonicus* (L1) VRHD ZA**	**100**	**100**	**100**		**0**	**0**	**0**	**0**	**0**	**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
5	**P16 *Argulus japonicus* (L2) VRHD ZA**	**100**	**100**	**100**	**100**		**0**	**0**	**0**	**0**	**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
6	**P05 *Argulus japonicus* (F) VRHD ZA**	**100**	**100**	**100**	**100**	**100**		**0**	**0**	**0**	**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
7	**P06 *Argulus japonicus* (F) VRHD ZA**	**100**	**100**	**100**	**100**	**100**	**100**		**0**	**0**	**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
8	**P08 *Argulus japonicus* (L2) VRHD ZA**	**100**	**100**	**100**	**100**	**100**	**100**	**100**		**0**	**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
9	**P10 *Argulus japonicus* (M) BP ZA**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**		**0**	**23**	**68**	**29**	**21**	**32**	**29**	**274**
10	**P09 *Argulus japonicus* (F) VR ZA**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**		**23**	**68**	**29**	**21**	**32**	**29**	**274**
11	MN688128 *Argulus americanus* USA	**96.48**	**96.48**	**96.48**	**96.48**	**96.48**	**96.48**	**96.48**	**96.48**	**96.48**	**96.48**		71	43	27	44	43	274
12	MN688129 *Argulus bicolor* USA	**89.74**	**89.74**	**89.74**	**89.74**	**89.74**	**89.74**	**89.74**	**89.74**	**89.74**	**89.74**	89.27		64	72	65	64	274
13	MN688125 *Argulus flavescens* USA	**95.96**	**95.96**	**95.96**	**95.96**	**95.96**	**95.96**	**95.96**	**95.96**	**95.96**	**95.96**	93.43	90.26		44	1	0	278
14	EU370442 *Argulus foliaceus**	**97.1**	**97.1**	**97.1**	**97.1**	**97.1**	**97.09**	**97.09**	**97.09**	**97.09**	**97.08**	95.87	89.14	93.86		47	44	276
15	AF363322 *Argulus* sp. USA	**95.59**	**95.58**	**95.58**	**95.58**	**95.58**	**95.57**	**95.57**	**95.57**	**95.57**	**95.57**	93.27	90.11	99.86	93.52		3	281
16	AY210804 *Argulus* sp. USA	**96.01**	**96.01**	**96.01**	**96.01**	**96.01**	**95.99**	**95.99**	**95.99**	**95.99**	**95.98**	93.43	90.26	100	93.95	99.58		278
17	MN065508 *Levisunguis subaequalis* USA	**68.58**	**68.54**	**68.54**	**68.54**	**68.54**	**68.43**	**68.43**	**68.43**	**68.43**	**68.4**	65.71	65.66	67.75	68.35	67.7	68.12	

Table 4.9: Genetic divergences among aligned COI sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (site localities are included for sequences from the present study). Sequences from the present study in boldface and grey shade. *Chonopeltis lisikili* (MW679715) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; E – Egg; F – Female; GD – Gariep Dam; IN – India; JP – Japan; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	10	11	12	13	14	15	16	17
1	**P03 *Argulus japonicus* (M) VRHD ZA**		**11**	**11**	**11**	**11**	**0**	**0**	**147**	**113**	**96**	**163**	**140**	**174**	**165**	**163**	**148**	**154**
2	**P09 *Argulus japonicus* (F) VR ZA**	**98.38**		**0**	**0**	**0**	**11**	**11**	**151**	**116**	**97**	**168**	**143**	**178**	**170**	**168**	**146**	**154**
3	**P10 *Argulus japonicus* (M) BP ZA**	**98.38**	**100**		**0**	**0**	**11**	**11**	**151**	**116**	**97**	**168**	**143**	**178**	**170**	**168**	**146**	**154**
4	**P11 *Argulus japonicus* (M) GD ZA**	**98.39**	**100**	**100**		**0**	**11**	**11**	**151**	**116**	**97**	**168**	**143**	**178**	**170**	**168**	**146**	**154**
5	**P14 *Argulus japonicus* (F) BhD ZA**	**98.39**	**100**	**100**	**100**		**11**	**11**	**151**	**116**	**97**	**168**	**143**	**178**	**170**	**168**	**146**	**154**
6	**P15 *Argulus japonicus* (L1) VRHD ZA**	**100**	**98.38**	**98.38**	**98.39**	**98.39**		**0**	**147**	**113**	**96**	**163**	**140**	**174**	**165**	**163**	**148**	**154**
7	**P16 *Argulus japonicus* (L2) VRHD ZA**	**100**	**98.38**	**98.38**	**98.39**	**98.39**	**100**		**147**	**113**	**96**	**163**	**140**	**174**	**165**	**163**	**148**	**154**
8	JX025025 *Argulus japonicus* IN	**76.81**	**76.18**	**76.18**	**76.18**	**76.18**	**76.81**	**76.81**		149	163	8	40	31	16	16	148	153
9	LC588400 *Argulus japonicus* JP	**83.46**	**82.97**	**82.97**	**82.99**	**82.99**	**83.43**	**83.46**	76.5		27	170	146	174	173	171	155	163
10	OM836429 *Argulus japonicus* IN	**85.94**	**85.76**	**85.76**	**85.78**	**85.78**	**85.92**	**85.94**	74.29	96.05		184	161	186	185	183	173	178
11	KF713320 *Argulus japonicus* (E) IN	**75.6**	**74.85**	**74.85**	**74.85**	**74.85**	**75.6**	**75.6**	98.74	74.55	72.46		51	32	10	10	151	171
12	NC_005935 *Argulus americanus* USA	**79.5**	**79**	**79**	**79.03**	**79.03**	**79.47**	**79.5**	93.69	78.62	76.43	92.37		82	60	58	130	141
13	KF723419 *Argulus foliaceus* IN	**73.95**	**73.35**	**73.35**	**73.35**	**73.35**	**73.95**	**73.95**	95.11	73.95	72.16	95.11	87.72		25	25	157	183
14	KF723415 *Argulus siamensis* IN	**75.34**	**74.59**	**74.59**	**74.59**	**74.59**	**75.34**	**75.34**	97.48	74.14	72.35	98.48	91.03	96.19		0	155	180
15	KF723315 *Argulus siamensis* (E) IN	**75.34**	**74.58**	**74.58**	**74.58**	**74.58**	**75.34**	**75.34**	97.47	74.13	72.31	98.46	91.23	96.14	100		151	176
16	MZ518075 *Argulus* sp. USA*	**76.17**	**76.49**	**76.49**	**76.49**	**76.49**	**76.17**	**76.17**	75.94	75.04	72.14	75.65	79.07	74.72	75.04	75.37		124
17	MW679715 *Chonopeltis lisikili* ZA	**77.53**	**77.46**	**77.46**	**77.49**	**77.49**	**77.49**	**77.53**	75.95	76.21	74.01	74.48	79.43	72.68	73.17	73.45	80.12	

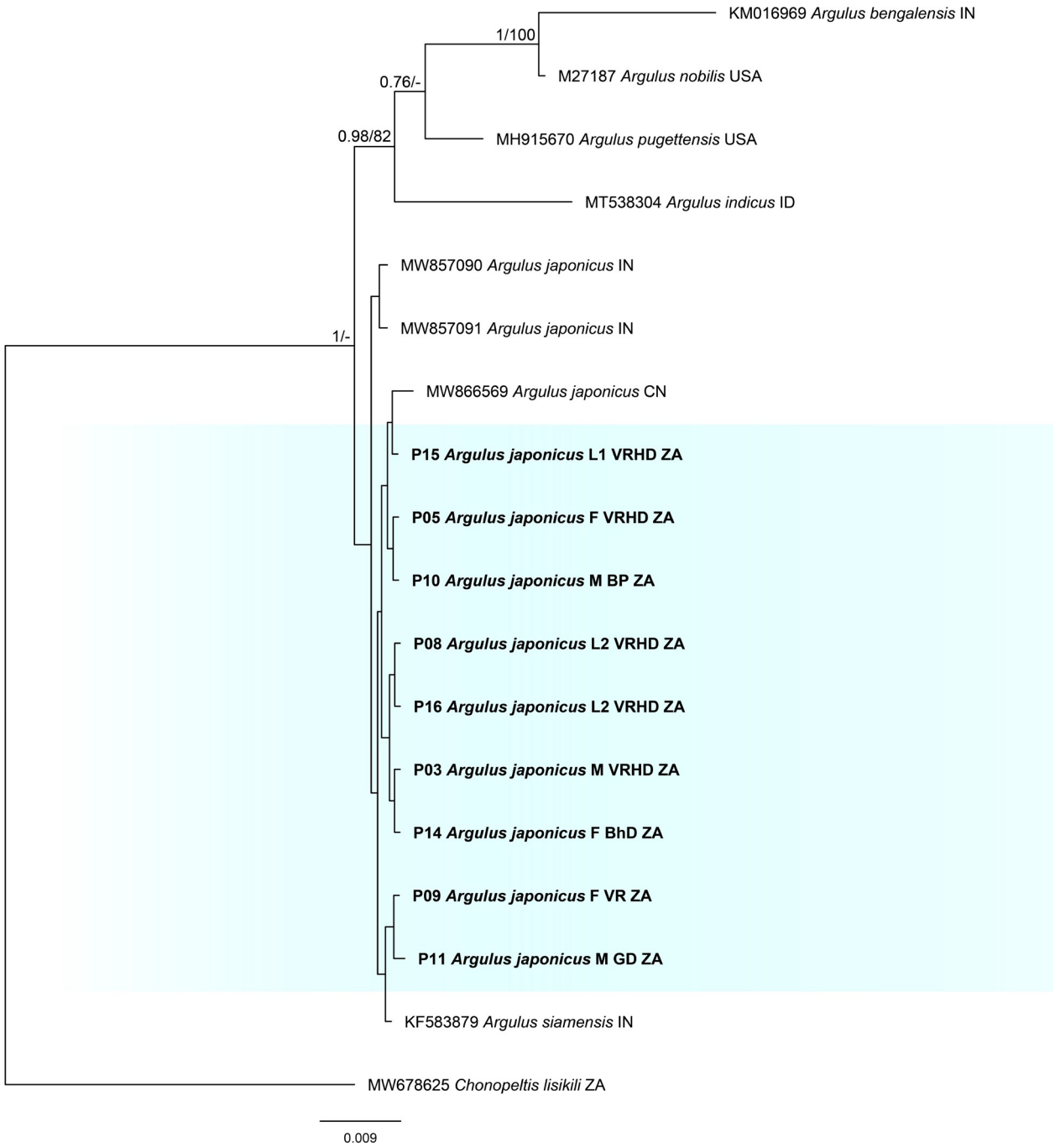


Figure 4.7: Phylogenetic tree of *Argulus* species based on partial 18S rRNA gene alignments. Newly generated sequences are bold in a gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Chonopeltis lisikili* (MW678625) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; CN – China; F – Female; GD – Gariep Dam; ID – Indonesia; IN – India; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

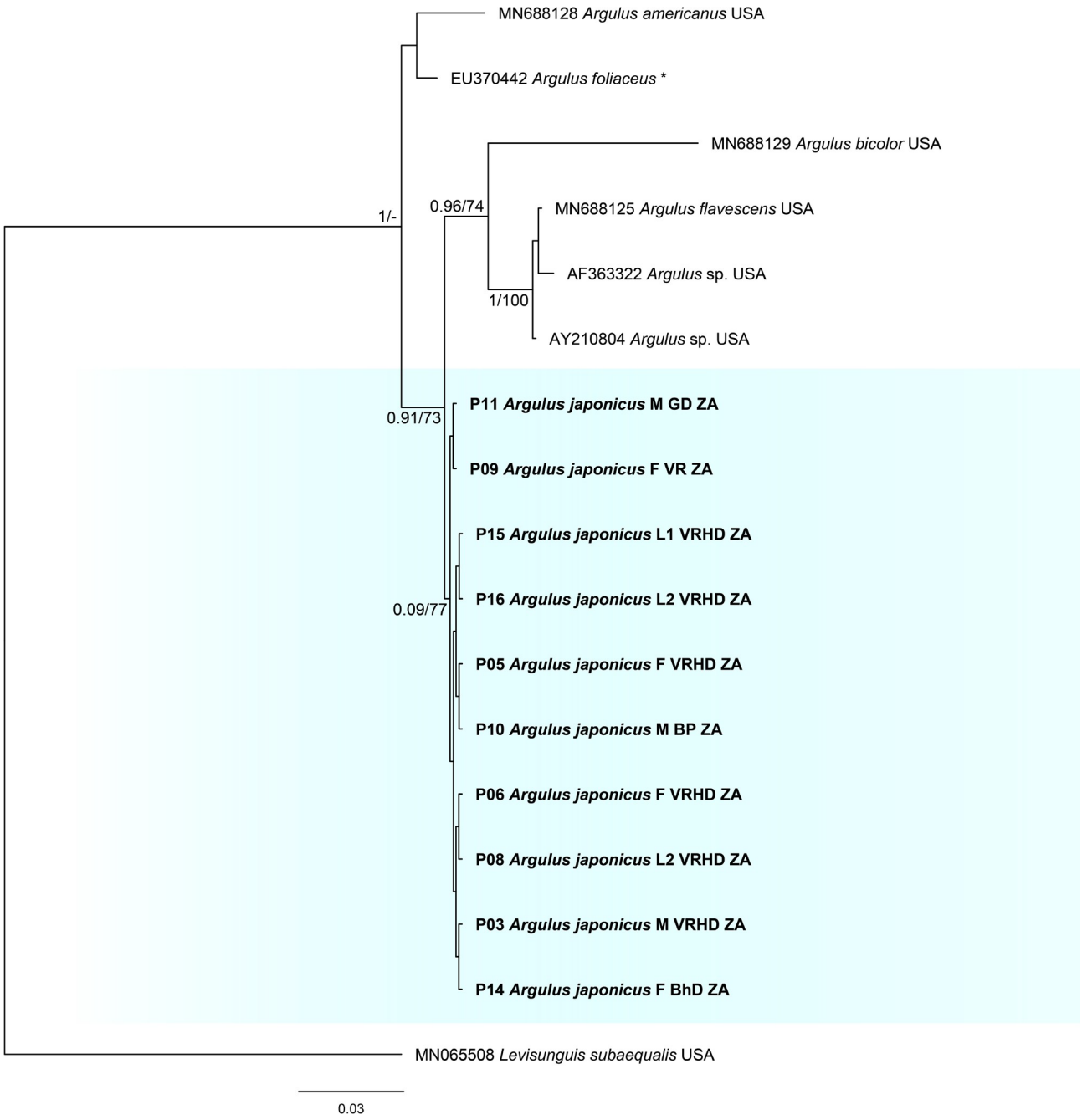


Figure 4.8: Phylogenetic tree of *Argulus* species based on partial 28S rRNA gene alignments. Newly generated sequences are bold in a gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Levisunguis subaequalis* (MN065508) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; CN – China; F – Female; GD – Gariep Dam; ID – Indonesia; IN – India; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

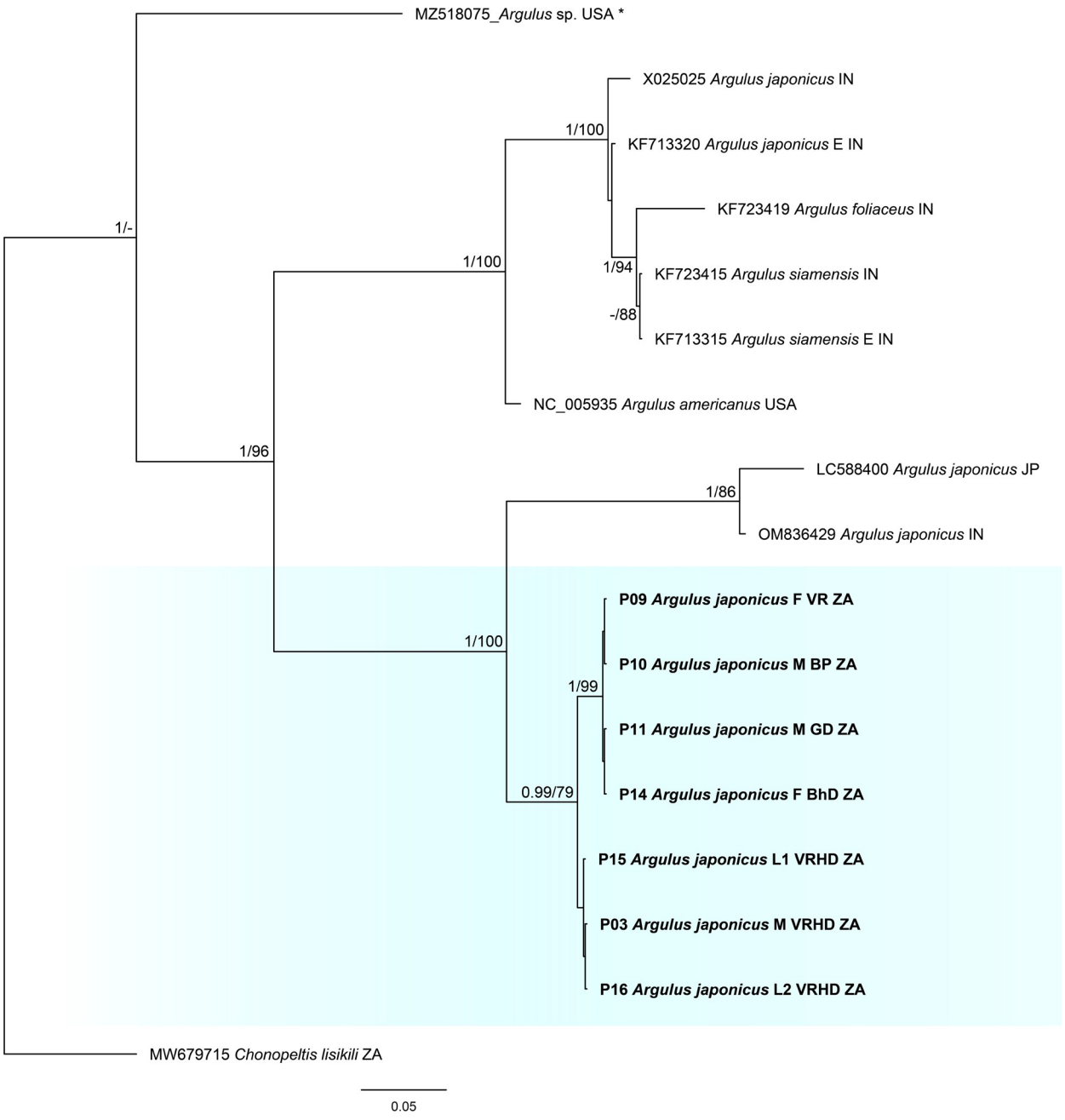


Figure 4.9: Phylogenetic tree of *Argulus* species based on partial COI mtDNA gene alignments. Newly generated sequences are bold in a gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Chonopeltis lisikili* (MW679715) was used as the outgroup. Abbreviations: BhD – Bloemhof Dam; BP – Barberspan; CN – China; E – Egg; F – Female; GD – Gariep Dam; IN – India; JP – Japan; L – Larva; M – Male; USA – The United States of America; VR – Vaal River; VRHD – Vaal River Holding Dam; ZA – South Africa.

For the COI analysis, the phylogenetic alignment of partial COI sequences yielded an alignment length of 683 bases. While some of the newly generated sequences were 100% identical, the rest differed with 11 nucleotides, all of which translated for the same amino acids as the alignment. There was no observed pattern for percentage similarity or base pair differences among males, females or larvae (see Table 4.9). Novel sequences were most similar to *A. japonicus* from India (74.85–75.60%) (96–97 bp) (see Table 4.9). The phylogenetic tree constructed yielded two *A. japonicus* clades: one for GenBank sequences from Japan and India, the second for newly generated sequences (see Fig. 4.9). As seen with the 18S and 28S analyses, adult male and female sequences formed sister clades. Sequences from the Vaal River Holding Dam (larvae and male) clustered in a separate sister clade from the sequences from the other localities (see Fig. 4.9).

4.5. Discussion

Kruger et al. (1983) first recorded *A. japonicus* from the Vaal River System in 1983 but suggested that *A. japonicus* may have been introduced much earlier into the Vaal System, following the introduction of the common carp and goldfish in the 1960s (see also, Smit et al., 2017). After Kruger et al. (1983), there have been more reports of this invasive parasite along the Vaal River System, occurring in at least nine native host species (see Smit et al., 2017), but there have been no reports from the Orange River. From the current study, *A. japonicus* was reported from the Krugersdrift Dam (Modder River) and the Sand River (tributaries of the Vaal River), and from the Vaal River Holding Dam. This is the first report of *A. japonicus* from these three sites in the Vaal River System, which suggests that this invasive branchiuran has now been established throughout the Vaal River. The occurrence of *A. japonicus* in Lake Barberspan (Harts River) and Bloemhof Dam (Vaal River) are confirmations of this invasive parasite's distribution in South Africa. The current study also reports *A. japonicus* for the first time from the Gariep Dam in the Orange River.

As there is a transboundary movement of water from the Orange River System via the Sunday tunnel to the Eastern Cape, there is the possibility that *A. japonicus* can also be present in the Eastern Cape's Fish and Sunday River Systems (see Truter et al., 2023). The translocation and introduction of fish species into non-native environments create opportunities for co-introduction of their parasites (Smit et al., 2017). This distribution and translocation of parasites is mostly linked to the introduction of fish hosts, either via aquaculture importation, trade as food or ornamental fishes, or sports fishing (Shafir & Oldewage, 1992; McCafferty et al., 2012); and according to Pilgrim (1967), where the host is, its parasites should be expected too.

The report of *A. japonicus* collected from four different hosts (Clariidae and Cyprinidae) and 11 localities support the categorisation of this species as a generalist parasite with wide spreading distribution (Walker et al., 2004; Nagasawa et al., 2009; Alsarakibi et al., 2014). Numerous studies have been conducted on different aspects of the *A. japonicus* developmental stages, but these seem to focus on egg hatching and early larval development stages (Shafir & As, 1986; Lutsch & Avenant-Oldewage, 1995; Tam & Avenant-Oldewage, 2006; Møller et al., 2008; Tam & Avenant-Oldewage, 2009). The updated larval descriptive notes provided in this study focus on changes and development of the cephalic appendages: in the first and second antennae, first and second maxillae, modification of attachment appendages and structures across the developmental stages, as well as the increase in size and function of swimming legs. All larval stages were observed simultaneously from a single population during the current study. Selected cephalic appendages with observable changes across the parasite's eight larval stages are provided in a pictorial guide for classification and coloured to highlight specific development per respective structure (Figs. 4.3–4.6).

The argulids from the current study were morphologically and genetically identified as *A. japonicus*. There were, however, slight intraspecific differences in the size and shape of carapaces observed across the different localities (Fig. 4.2). Additionally, all specimens observed from the Vaal River Holding Dam had visible diverticula (Fig. 4.2a) compared to those from the other localities (Fig. 4.2b–c). According to Tam and Avenant-Oldewage, (2006), the diverticula originate laterally from the anterior midgut into the carapace. The lumen of the anterior midgut and diverticula is lined with cilia and the epithelium contains numerous melanopores (Tam & Avenant-Oldewage, 2006). This observation is not unusual for argulids, as a few authors have reported similar pigmentation from the diverticula of adult *A. japonicus* following a blood meal (Tam & Avenant-Oldewage, 2006; 2009; Walker et al., 2011), as well as in *A. foliaceus* (Yildiz & Kumantas, 2002). It is therefore possible that the parasites from the holding dam in this study may have been actively feeding—whether fish mucus or blood for the larvae and adults, respectively—during collection and fixation.

The ecology of the Vaal River Holding Dam seemed to have also played a role in the intraspecific differences observed with the holding dam population. Fish fries may have been introduced into these holding dams when water from the Vaal River was pumped into the dams. This fish population thus becomes isolated from the other natural fish populations, as well as their parasites. The parasites further replicate through several generations for as long as the fish are in captivity and possibly undergo adaptive mutation in the process, which might yield slight differences when compared to wild populations (see Wadeh et al., 2010). Interestingly, the specimens from the holding dam in the current study also showed genetic differences in the COI genes (99.38–99.39% similarity) (11 bases different) compared to specimens from the other sites.

Phylogenetic analyses of the partial 18S gene region showed that the sequences of *A. japonicus* from the present study are most similar to a sequence of *A. japonicus* from China, which was collected from the largemouth bass *Micropterus salmoides* (Lacepède 1802). This evolutionary relationship could potentially point to the introduction of the parasite from Asia; in southern Africa, *A. japonicus* is suspected to have been introduced via the imported carp and goldfish (see Smit et al. 2017). The 18S phylogenetic analyses also showed that *A. japonicus* sequences from India formed a separate clade from the rest of the *A. japonicus* sequences in the alignment. The study of Tandel et al. (2021) (India sequences) provided a morphological examination of their sampled *A. japonicus* specimens, further confirming the identity of the branchiurans found in both their study and the present study. Although, the 18S sequence of *A. siamensis* from Patra et al. (2016) differed by 19 bases from the newly generated sequences of *A. japonicus,* the *A. siamensis* sequence clustered in a sister clade with *A. japonicus* sequences from the current study (see Table 4.7 and Fig. 4.7). Patra et al. (2016) did not present a morphological assessment of their specimens, and thus it is not possible to determine if their specimen was indeed *A. siamensis* or rather a case of misidentification that may warrant further investigation. From the phylogenetic analyses, this study presents sequences of *A. japonicus* individuals in larval stages (L1 and L2) that match the sequences of adults.

Generally, the 18S and 28S analyses showed little variability compared to the COI which has a higher rate of evolution. Published COI sequences of *A. japonicus* on GenBank proved problematic (with the presence of stop codons, sequences less than 500 bases, and questionable values of percentage similarity) and were not included in the current study. Unpublished COI sequences from India and Japan were, however, used in the alignment (see Table 4.9). Newly generated adult and larval COI sequences formed a clade with sequences from India and Japan, confirming the identity of the larval and adult sequences as *A. japonicus.* Similar studies of linking sequences of *Argulus* eggs to adults have been done by Feroz-Khan et al. (2014). The clustering into a separate sister clade observed in the adult male and larval sequences from the Vaal River Holding Dam (Fig. 4.9) (separate from all the other newly generated sequences) suggests that the holding dam specimens are probably a different population from the other specimens from this study. Whether this is a result of single or multiple events of colonisation is unknown at this point. The phylogenetic analysis of the COI gene region corroborates the morphological observations from this study.

The sequences generated from this study are the first to be generated from sub-Saharan Africa. As the specimens from this study have been morphologically characterised as *A. japonicus,* the sequences generated from this study can thus be presented as true data for ribosomal RNA (18S and 28S) and mitochondrial DNA (COI) sequences of *A. japonicus* in South Africa. This notable addition will enable more integrative and further evolutionary studies within this species and genus. The current study also provides a basis for understanding the

possible events of invasion of this parasite in Africa. Furthermore, the successful linking of the genetic data for larval and adult stages is a significant addition to the diversity studies that involve environmental DNA, which is an emerging tool for revealing hidden aquatic biodiversity (Simmons et al., 2020).

4.6. Conclusion

This study provides the first record of *A. japonicus* from the Orange River System, identifies an additional larval stage, and highlights a refinement in the early developmental stages of this parasite. As the material from which DNA was extracted in this study was morphologically examined, this study provides the first integrative report of *A. japonicus* from sub-Saharan Africa with 27 newly generated sequences including larval and adult parasite stages. This significant addition will enable further evolutionary studies on this invasive parasite species. It is impossible to state at this point if a single or multiple colonisation event resulted in the invasion of *A. japonicus* in South Africa, therefore, more integrative studies across South Africa is recommended.

4.7. References

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CHAPTER 5

Molecular characterisation of *Lernaea cyprinacea* Linnaeus, 1758 (Lernaeidae: Cyclopoida) from South Africa with new host and locality records

5.1. Abstract

Lernaea cyprinacea, commonly known as anchor worm, is a copepod parasite that infests the skin, fins, gills, and oral cavities of various fish species. Originally described in Europe from the common carp (*Cyprinus carpio* Linnaeus, 1758), this parasite has been reported worldwide. Anchor worm infestations have detrimental effects on fish health, including the development of haemorrhagic ulcers, necrosis of gill tissues, respiratory difficulties, and increased susceptibility to secondary infections. Such infestations have caused significant morbidity and mortality, particularly in fish farming. The introduction of *L. cyprinacea* as an invasive species to different regions, including South Africa, has been attributed to the movement of aquarium species. Genetic studies on *L. cyprinacea* are limited, with only two previous studies incorporating DNA barcoding from Asia and Australia. Reports from other countries suggest high morphological variability in *L. cyprinacea,* but no such observations have been made in South Africa. Although *L. cyprinacea* has been reported in major river systems in South Africa, there are no published records of infestation in the co-introduced host *C. carpio*. Thus, this study aimed to molecularly characterise *L. cyprinacea* from multiple localities in South Africa and assess the morphological variability among individuals. A total of 79 fish specimens belonging to the families Cichlidae and Cyprinidae were collected from six localities in South Africa and examined for *L. cyprinacea* infestation. Parasites were identified using light and scanning electron microscopy techniques, while genetic analysis utilised partial 18S, 28S, and COI genes as molecular markers. This study presents the first report of *L. cyprinacea* infestation in South Africa on its type host, *C. carpio*, and identifies a new host, the smallmouth yellowfish, *Labeobarbus aeneus* (Burchell, 1822), within the Orange-Vaal River System. *Lernaea cyprinacea* was also reported for the first time from the Incomati System, as well as additional sites in the Phongolo Floodplain and the Vaal River. This study further provides valuable insights into the molecular and morphological characteristics of *L. cyprinacea* in South Africa, highlights the epizoic associations of filamentous algae, sessile ciliates, and diatoms with *L. cyprinacea*, as well as shedding light on anchor shapes variabilities within the South African population.

5.2. Introduction

Individuals from species of the copepod genus *Lernaea* Linnaeus, 1758 (Lernaeidae: Cyclopoida), commonly referred to as anchor worms, are widely known as external parasites of freshwater fishes (Hadfield, 2019). By use of their specialised anchors, adult females burrow into the host's body causing a pathological condition known as lernaeosis (Hadfield, 2019), triggering certain immunological responses, and increasing the host's susceptibility to opportunistic pathogens (Nikhila Reshmi et al., 2022). Once attached, adult parasites are difficult to remove due to the cephalic anchors growing inside the host tissues. Haemorrhagic ulcers at the sites of attachments, gill damage, and difficulty in breathing are some of the major reasons for severe morbidity and mortality of hosts (Putz & Bowen, 1964; Hossain et al., 2018; Hadfield, 2019). The species, *Lernaea cyprinacea* Linnaeus, 1758 was originally described from the common carp *Cyprinus carpio* Linnaeus, 1758 in Europe, it has been reported as an invasive species across several regions and continues to spread (being introduced and translocated to other fishes) worldwide (Fryer, 1968; Song et al., 2008; Stavrescu-Bedivan et al., 2014; Smit et al., 2017; Welicky et al., 2017; Chakona et al., 2019; Hua et al., 2019; Zhu et al., 2021).

In South Africa, *L. cyprinacea* was first reported in the 1980s, (van As & Basson, 1984; van As & Viljoen, 1984) although it had been recorded in Africa two decades earlier (Robinson & Avenant-Oldewage, 1996). This invasive parasite species has since been reported from 12 native fish species in South Africa (Smit et al., 2017) including the endangered rocky kurper, *Sandelia bainsii* Castelnau, 1861 (see Chakona et al., 2019), and across major river systems in South Africa (see Smit et al., 2017; Weyl et al., 2020).

Lernaea cyprinacea has very low host specificity and can utilise fish, tadpoles, and salamanders as hosts (Kabata, 1979; Hossain et al., 2018; Salinas et al., 2019). Sites of host infestation for *L. cyprinacea* include the body surface, fins, gills, buccal cavities, pharyngeal cavity, and anus (Al-Hamed & Hermiz, 1973). The developmental stages of this copepod comprise three free-living nauplius larval stages after the eggs hatch, and five parasitic copepodite stages before becoming mature adults (see Hossain et al., 2018). Although the copepodites of *L. cyprinacea* are parasitic on the gills of fish, only adult female copepods are found embedded into host tissues (Hossain et al., 2018).

The embedded female mimics its host's immune response to evade the host's immune system (Nikhila Reshmi et al., 2022). Points of attachment are characterised by redness, swelling and ulceration. Koyuncu and Dönmez (2006) reported *L. cyprinacea* as the cause of mortality observed in an ornamental fish farm in Mersin, Turkey. The parasites were collected from the skin and anus of their hosts, and infested fishes showed signs such as swimming disorders, rubbing against surfaces and the edges of the aquaria, as well as general weakness and fatigue.

The anchors, also known as the holdfast or arms, are a characteristic feature of members in the genus, *Lernaea*. Several authors have reported variability in the shape of the anchors of *L. cyprinacea* across several localities and from different hosts (Fryer, 1968; Kabata, 1979; McCredden, 2016; Hua et al., 2019). Fryer (1968) suggested that the variability in anchor processes observed correlated with the sites of attachment on the host. The morphological fluidity of the anchors may have led to the description of new species at the time: *Lernaea carassii* Tidd, 1933; *Lernaea elegans* Leigh-Sharpe, 1925; *Lernaea esocina* Hermann, 1783; *Lernaea ranae* Stunkard & Cable, 1931; *Lernaea tentaculis* Linnaeus, 1746; *Lernaea tentaculis quatuor* Linnaeus, 1746; *Lernaeocera cyprinacea* (Linnaeus, 1746); and *Lernaeocera gasterostei* Brühl, 1860, which have now been synonymised with *L. cyprinacea* (see Walter & Boxshall, 2023). Recently, Hua et al. (2019) and Zhu et al. (2021) used an integrative approach of morphology and genetics to study *L. cyprinacea.* Hua et al. (2019) provided detailed measurements of the body length, as well as anchor lengths and widths of specimens from China, in addition to molecularly characterising the species using fragments of the 18S, 28S, and COI gene regions. Zhu et al. (2021) reported on specimens from Australia and provided detailed drawings of the anchors and the pregenital prominence, which is also very variable in *L. cyprinacea* (see Fryer, 1968), and presented molecular data for fragments of the 18S, 28S, and COI gene regions. In their study, Hua et al. (2019) reported *Lernaea. cruciata* (Le Sueur, 1824) and *L. cyprinacea* were molecularly identical, thus *L. cruciata* is another junior synonym for *L. cyprinacea.*

In South Africa, the first molecular study on *L. cyprinacea* was carried out by Welicky et al. (2017), using fragments of the 18S ribosomal RNA genes (KY435939; 932 bases). Two years later, Chakona et al. (2019) also molecularly analysed this parasite using the 18S gene (MN243682), although this sequence was fairly short (392 bases). There has, therefore, been no extensive molecular analysis of *L. cyprinacea* from South Africa, hence the aim of the current study to molecularly characterise *L. cyprinacea* specimens collected from several regions in South Africa using fragments of two ribosomal RNA genes (18S and 28S) and one mitochondrial DNA gene (COI), document variability in the lengths and shapes of the anchor processes, and report new distributions and host records in South Africa for future studies on this invasive parasite.

5.3. Materials and methods

5.3.1. Sampling

Sampling was done was done between 2021 and 2022 at six different sites in South Africa: Barberspan Bird Sanctuary, Harts River (26°35'24.1"S 25°35'09.5"E); Klipdrif Dam (26°36'36.8"S 27°18'25.9"E); Nyamiti Pan, Phongolo Floodplain in the Ndumo Game Reserve (26°53'26.0"S 32°17'52.7"E); Shokwe Pan, Ndumo Game Reserve (26°52'17.5"S 32°12'57.6"E); the lower Sabie River, Incomati System, Kruger National Park (25°09'45.5"S 31°57'04.3"E); and Takwasa, Vaal River (26°52'02.7"S 27°17'36.0"E) (Fig. 5.1). A total of 26 fish specimens belonging to two families (Cichlidae and Cyprinidae) were caught using cast and seine nets and examined for parasites or lesions. Parasitised fishes (with visible parasite attachments and/or lesions) were transported in buckets with aerated water from the sampling site(s) and taken to the designated field laboratory sites or in aerated tanks back to the North-West University (Ethics number: NWU-00600-19-A5). Fishes that were not parasitised were counted and released at the site of capture. The body surface of fishes was screened, and any copepods observed were carefully removed using tweezers and dissection needles and preserved in 70% ethanol for further morphological and molecular analysis.

The necessary permits were received from Ezemvelo KZN Wildlife (KwaZulu-Natal, permit Nos. OP 1075/2017, OP 1582/2018); Department of Rural, Environmental and Agricultural Development (North West, permit no. HO 20/02/18-057 NW); the Department of Rural, Environmental and Agricultural Development (NW 8065/03/2019) and the Department of Economic Development, Tourism and Environmental Affairs (01/30201). Fishes were identified with the aid of Skelton (2001), host nomenclature follows FishBase (Froese & Pauly, 2023); host and parasite authorities are not included in the reference list.

5.3.2. Morphological analysis

Morphological analyses were carried out using light microscopy (LM) and scanning electron microscopy (SEM) techniques. Examination of parasite macro-morphology was done with the aid of the Zeiss Stemi 508 stereomicroscope. Photomicrographs were captured with the aid of a ZEISS Axiocam ERc 55 camera attached to the stereomicroscope. The length of the whole body, length and width of anchor arms, and length of the processes of *Lernaea* species were made in millimetres using the Labscope Mat Ver. 2.8.4 software of the stereomicroscope, following Hua et al. (2019). Additional parameters measured in this study were the ventral arm width and the posterior dorsal process width of the anchors (Fig. 5.2).

Specimens for SEM were dehydrated through a graded alcohol series at ten minutes per 80%, 90%, 96%, 100%, 100% ethanol concentration and then through a graded Hexamethyldisilizane (HMDS) series at five minutes per concentration of 30%, 50%, 70% 90%, 100% 100%. Specimens were left to dry. Dried specimens were mounted on stubs with

carbon tapes, sputter coated with gold/palladium (EMSCOPE SC500 Oxford Instruments Inc., USA) and observed at 5kVa with the JSM JOEL-IT 200 electron microscope. Photomicrographs were edited and electronically inked with Photoshop 2023 and Adobe Illustrator 2023, respectively. Specimen identification and terminologies followed Zhu et al. (2021).

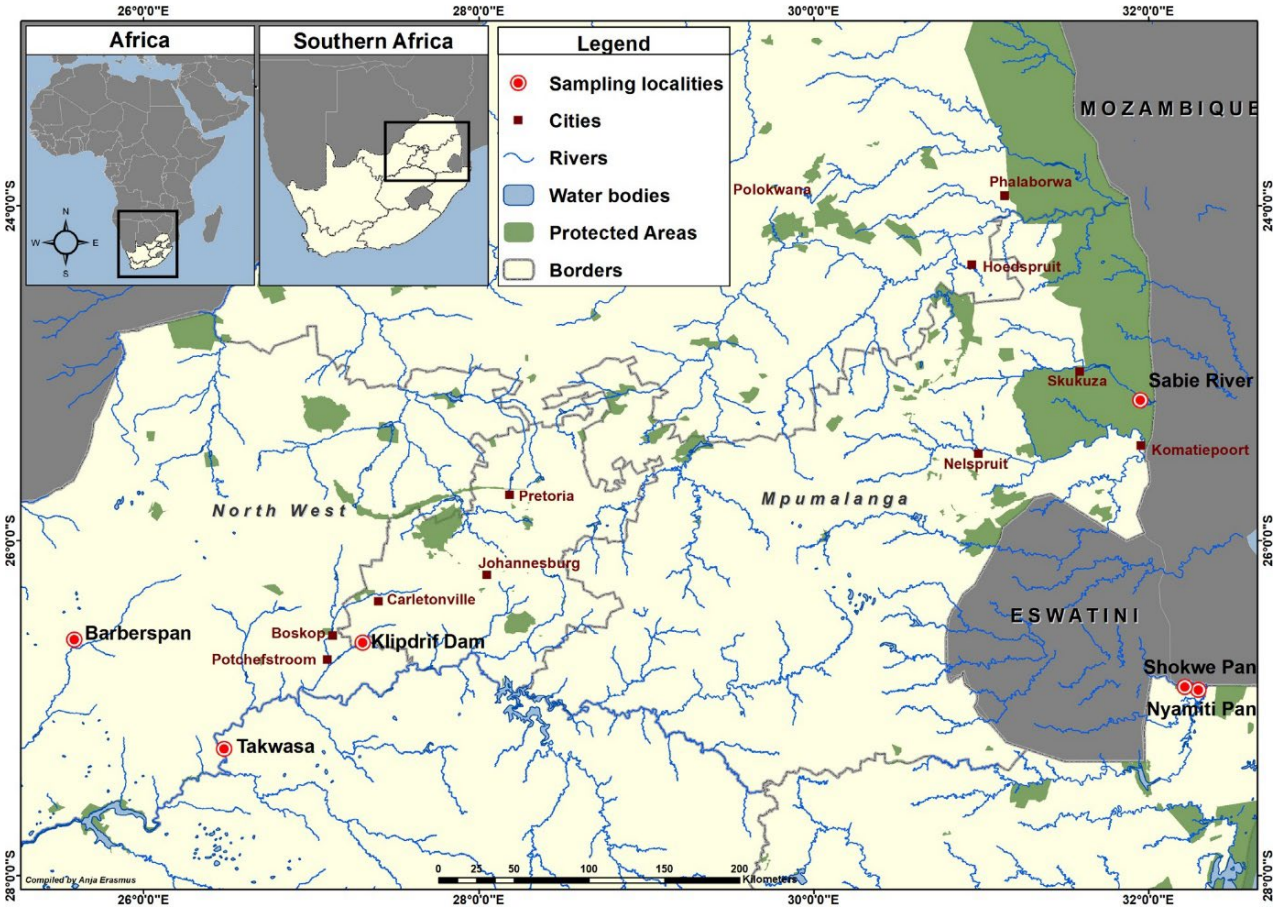


Figure 5.1: Map of the six different localities in South Africa where *Lernaea cyprinacea* Linnaeus, 1758 were collected from 2021–2022.

5.3.3. Infestation rates

Epidemiological indices (prevalence, mean intensity, and mean abundance) were calculated according to Bush et al. (1997).

5.3.4. Molecular analysis

One egg string each from 13 parasite individuals was selected for genomic DNA extraction following the manufacturer's protocol for the Machery Nagel tissue extraction kit. Fragments of two ribosomal RNA genes (18S and 28S) as well as fragments of one mitochondrial DNA

gene (COI) were used as molecular markers in this study. Primers used for amplification of 18S (18SF and 18SR) and 28S (28SF and 28SR) were designed by Song et al. (2008) (Table 5.1). For amplification of the partial COI gene region, the LerCOIF and LerCOIR designed by Hua et al. (2019) were used (primer names were given by the authors in the present study as only forward and reverse sequences were provided in Hua et al. (2019)). After the extraction from the Nyamiti site failed to amplify using the primers by Hua et al. (2019), a different primer set, CrustDF1 and CrustDR1, designed by Cheng et al. (2019) was used (see Table 5.1).

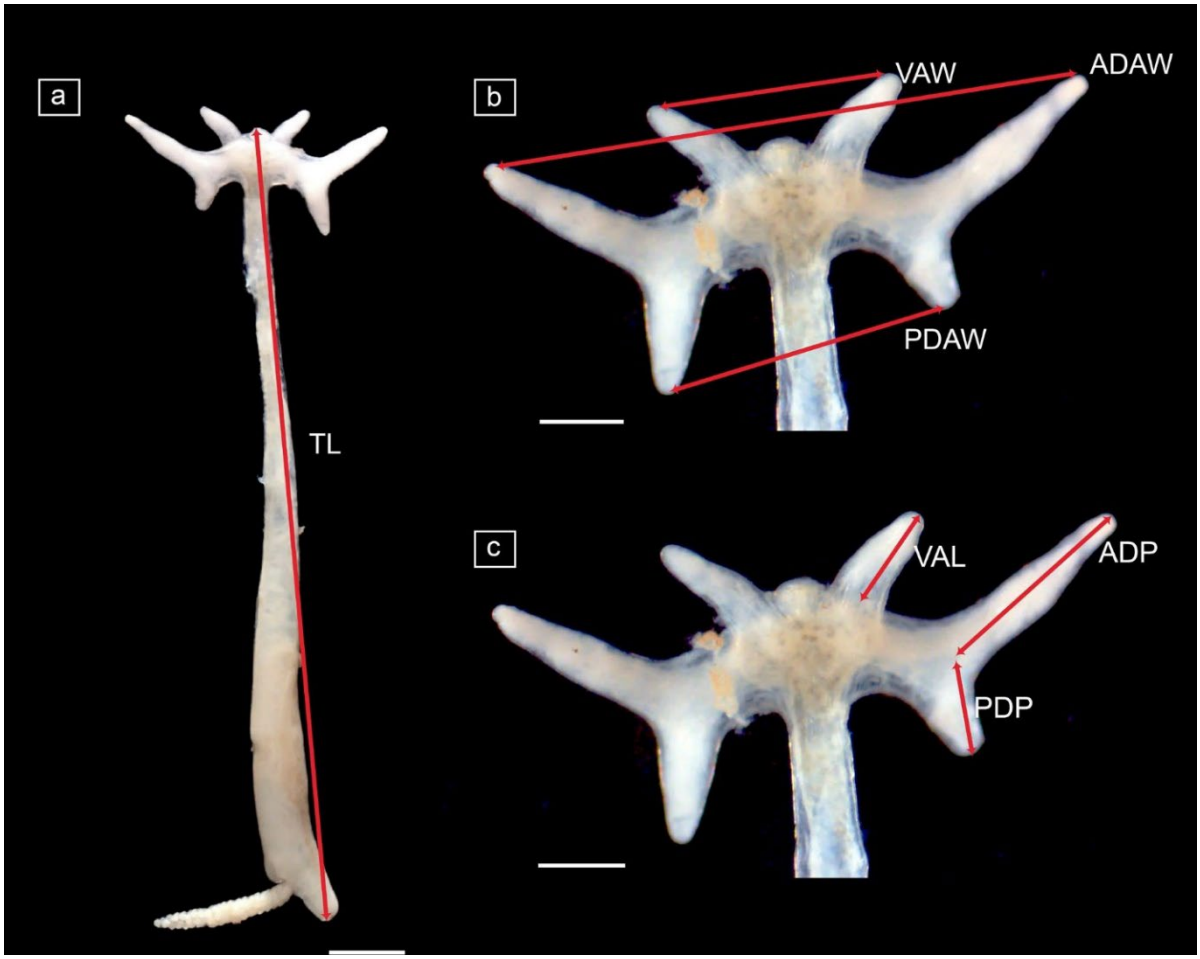


Figure 5.2: Figure showing the different measurements used for *Lernaea cyprinacea* Linnaeus, 1758. (a) Total length from the tip of the head to the tip of the abdomen; (b) anchor widths; (c) anchor lengths. Abbreviations: ADAW – anterior dorsal anchor width; ADP – anterior dorsal process; PDAW – posterior dorsal anchor width; PDP – posterior dorsal process; TL – total length; VAL – ventral anchor length; VAW – ventral anchor width. Scale bars: (a) 1 mm; (b–c) 0.5 mm.

Polymerase Chain Reaction (PCR) reactions were performed with volumes of 25 µl consisting of 12.5 µl of DreamTaq PCR Master Mix (2X) (ThermoFischer Scientific, Waltham, Massachusetts, USA), 1.25 µl of 10 µM of each primer (forward and reverse), 3–10 µl DNA, and final reaction volumes were made up with molecular grade water. Thermocycling conditions followed Song et al. (2008) for 18S and 28S, and Hayes et al. (2021) for COI. Polymerase Chain Reaction (PCR) products were verified with 1% agarose gel electrophoresis, and positive products were sent to Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa, for purification and sequencing in both directions.

Resultant sequences were viewed and assembled, and chromatogram-based contigs were generated and trimmed using Geneious Prime Ver 2022.2.2. Contigs were checked for similarities with other GenBank sequences using the Basic Local Alignment Search Tool (BLAST). Only sequences associated with peer-review publications from the genus *Lernaea* were utilised. *Lamproglena orientalis* Markevich, 1936 (Lernaeidae) sequences were used as the outgroup for 18S and 28S analyses. As there were no published COI data for *Lamproglena* on GenBank, *Sinergasilus polycolpus* (Markevich, 1940) (Ergasilidae) was downloaded as the outgroup for the COI analysis (see Table 5.2). Genetic divergence of sequences in the alignments was calculated in Geneious Prime Ver. 2022.2.2.

Based on Akaike's Information Criteria (AIC), the best nucleotide evolution models were selected using the jModelTest 2.1.3 (Darriba et al., 2012) as HKY+G for 18S, HKY for 28S, and GTR+ I for COI. Bayesian Inference (BI) analyses were performed with CIPRES Science Gateway v 3.3 (Miller et al., 2010) and BI trees were generated with the aid of MrBayes v. 3.2.7a (Ronquist et al., 2012), running two independent Markov Chain Monte Carlo (MCMC) runs of four chains for ten million generations and sampling tree topologies every, one thousand generations. Burn-in parameters were set to the first 25,000 generations. Maximum Likelihood (ML) analyses were run on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/); with estimated substitution parameters and bootstrap values of 1000 repetitions; trees were generated using PhyML v. 3.0 (Guindon et al., 2010). Phylogenetic trees were visualised in FigTree v 1.4.4 (Rambaut, 2012) and combined tree topologies were represented using Adobe Illustrator CC 2022.

Table 5.1: List of primers used for DNA amplification of South African *Lernaea cyprinacea* Linnaeus, 1758 specimens, with references. Abbreviations: BP – Barberspan; LSR – Lower Sabie River; NP – Nyamiti Pan; TW – Takwasa.

Gene regions	Specimen codes	Localities	Primers	Sequences	References
18S	P44K P455 P61K P64K P65K P69K P70K P72K	TW TW NP NP NP BP LSR LSR	18SF	5′-AAG GTG TGM CCT ATC AAC T-3′	Song et al. (2008)
			18SR	5′-TTA CTT CCT CTA AAC GCT C-3′	
28S	P44K* P455* P61K* P64K* P65K* P69K* P70K* P72K*	TW TW NP NP NP BP LSR LSR	28SF	5′-ACA ACT GTG ATG CCC TTA G-3′	
			28SR	5′-TGG TCC GTG TTT CAA GAC G-3′	
COI	P44k** P69K**	TW BP	LerCOIF	5′-TAG TTG GAA TTT GGG CTG GC-3′	Hua et al. (2019)
			LerCOIR	5′-ATT AGG GGC CTT GTT GGG AAG-3′	
	P64K***	NP	CrustDF1	5′-GGT CWA CAA AYC ATA AAG AYA TTG G-3′	Cheng et al. (2019)
			CrustDR1	5′-TAA ACY TCA GGR TGA CCR AAR AAY CA-3′	

* to indicate a 28S reaction using 28SF & 28SR primers;
** to indicate a COI reaction using LerCOIF & LerCOIR primers (primer names were given by the authors in the present study as only forward and reverse sequences were provided in Hua et al (2019));
*** to indicate a reaction using CrustDF1 & CrustDR1 primers.

Table 5.2: List of sequences used in the alignment with their host information, GenBank accession number, number of bases pairs (B) and references. Taxa in boldface are newly generated sequences from this study, all other sequences are from GenBank. Outgroup sequences are shaded in grey. Sequences less than 300 bases were not included in the alignment. Abbreviations: BP – Barberspan (Harts River), LSR – Lower Sabie River (Kruger National Park), NP – Nyamiti Pan (Phongolo Floodplain), TW – Takwasa, Venterskroon (Vaal River).

Taxon	Host	Host Families	Locality	GenBank Accession Numbers			Reference
				18S	28S	COI	
Lernaea cruciata	*Gambusia holbrooki*	Poeciliidae	China	MH982203	MH982215	MH982226; MH982227	Hua et al. (2019)
Lernaea cyprinacea	***Larbeobarbus aeneus***	**Cyprinidae**	**TW, South Africa**	**P44K, P45K,**	**P44Ka, P45Ka**	**P44Kh**	**Present study**
Lernaea cyprinacea	***Oreochromis mossambicus***	**Cichlidae**	**NP, South Africa**	**P61K, P64K, P65K**	**P61Ka, P64Ka, P65Ka**	**P64Ki**	**Present study**
Lernaea cyprinacea	***Labeo umbratus***	**Cyprinidae**	**BP, South Africa**	**P69K**	**P69Ka**	**P69Kh**	**Present study**
Lernaea cyprinacea	***Oreochromis mossambicus***	**Cichlidae**	**LSR, South Africa**	**P70K, P72K**	**P70Ka, P72Ka**	**-**	**Present study**
Lernaea cyprinacea	several (host not specified per sequence)	several families	Australia	MT367695	MT371347	MT371895	Zhu et al. (2021)
Lernaea cyprinacea	several (host not specified per sequence)	several families	Australia	MT367704	MT371367	MT371896	Zhu et al. (2021)
Lernaea cyprinacea	*Sandelia bainsii*	Anabantidae	South Africa	MN243682	-	-	Chakona et al. (2019)
Lernaea cyprinacea	*Odontesthes bonariensis*	Atherinopsidae	Argentina	MH198054	-	-	Waicheim et al. (2019)
Lernaea cyprinacea	*Percichthys trucha*	Percichthyidae	Argentina	MH198053	-	-	Waicheim et al. (2019)
Lernaea cyprinacea	*Oreochromis mossambicus*	Cichlidae	South Africa	KY435939	-	-	Welicky et al. (2017)
**Lernaea cyprinacea*	*N/A*	-	China	-	-	KM235194	Su et al. (2016)
Lernaea cyprinacea	*Pseudorasbora parva*	Gobionidae	Romania	-	KF751649	-	Stavrescu-Bedivan et al. (2014)
Lernaea cyprinacea	*Lepomis gibbosus*	Centrarchidae	Romania	-	KF751648	-	Stavrescu-Bedivan et al. (2014)

Taxon	Host	Host Families	Locality	GenBank Accession Numbers			Reference
				18S	28S	COI	
Lernaea cyprinacea	*Carassius auratus*	Cyprinidae	China	MH982195	MH982210	MH982221	Hua et al. (2019)
Lernaea cyprinacea	*Chanodichthys erythropterus*	Xenocyprididae	China	MH982197	MH982208	MH982220	Hua et al. (2019)
Lernaea cyprinacea	*Squaliobarbus curriculus*	Xenocyprididae	China	MH982198	-	MH982219	Hua et al. (2019)
Lernaea cyprinacea	*Cyprinus carpio*	Cyprinidae	China	MH982196	MH982204	MH982216	Hua et al. (2019)
Lernaea cyprinacea	*Odontesthes bonariensis*	Atherinopsidae	Argentina	-	KX908211	-	Soares et al. (2018)
Lernaea cyprinacea	*Hemiculter leucisculus*	Cyprinidae	China	DQ107554	DQ107546	-	Song et al. (2008)
Lernaea cyprinacea	*Chanodichthys erythropterus*	Cyprinidae	China	DQ107555	DQ107547	-	Song et al. (2008)
Lernaea cyprinacea	*Cyprinus carpio*	Cyprinidae	China	DQ107556		-	Song et al. (2008)
Lernaea cyprinacea	*Opsariichthys bidens*	Cyprinidae	China	DQ107557	DQ107548	-	Song et al. (2008)
Lernaea cyprinacea	*Plecoglossus altivelis*	Plecoglossidae	Japan	KP235363	KP235364	-	Yoshimine et al. (2015)
Lernaea cyprinacea	*Carassius auratus*	Cyprinidae	Australia	-	KY346866	-	McCredden, (2016)
Lamproglena orientalis	*Squaliobarbus curriculus*	Cyprinidae	China	DQ107552	DQ107544	-	Song et al. (2008)
Lamproglena orientalis	*Chanodichthys erythropterus (Cultrichthys erythropterus)*	Xenocyprididae	China	DQ107551	DQ107541	-	Song et al. (2008)
Lamproglena orientalis	*Chanodichthys mongolicus (Erythroculter mongolicus)*	Xenocyprididae	China	DQ107550	DQ107543	-	Song et al. (2008)
Lamproglena orientalis	*Chanodichthys dabryi (Erythroculter dabryi)*	Xenocyprididae	China	-	DQ107542	-	Song et al. (2008)
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	Cyprinidae	China	-	-	KR263117	Feng et al. (2016)

* Complete mitochondrial genome

5.4. Results

5.4.1. Taxonomy

Order Cyclopoida Burmeister, 1834
Family Lernaeidae Cobbold, 1879
Genus *Lernaea* Linnaeus, 1758 (Figs. 5.3–5.6)

Parasitic copepods collected from this study were morphologically and molecularly identified as *Lernaea cyprinacea* using the descriptions by Robinson and Avenant-Oldewage (1996) and Zhu et al. (2021). Adult females were visible on the body surface of the fish hosts, close to the operculum, and by the margins of the dorsal, pectoral, pelvic, and caudal fins (Fig. 5.3; Table 5.3). Parasite attachment areas were characterised by ulcerations on the body of their hosts. Specimens collected from this study showed no preference for a particular site of infestation on fish hosts. Some *L. cyprinacea* specimens collected in this study were covered with filamentous algae, sessile ciliates and diatoms (Figs. 5.3a, d, 5.5, 5.6).

***Lernaea cyprinacea* Linnaeus, 1758**

Material examined.

Seventy-nine *L. cyprinacea* individuals were collected from the body surface of 15 infested fish hosts. Three copepods used for SEM; one used for dissection; 13 egg strings used for extraction; 10 will be deposited in the collections of the National Museum, Bloemfontein, South Africa; the remainder are in the possession of the Water Research Group, Potchefstroom, South Africa.

Localities. Barberspan, Harts River (26°35'24.1"S 25°35'09.5"E), collected by P.P. Fikiye (2019–2020). Klipdrif Dam (26°36'36.8"S 27°18'25.9"E), collected by W. Landman (2022). Nyamiti Pan Ndumo Game Reserve, Phongolo Floodplain (26°53'26.0"S 32°17'52.7"E), Shokwe Pan, Ndumo Game Reserve, Phongolo Floodplain (26°52'17.5"S 32°12'57.6"E), and Lower part of Sabie River, Incomati System, Kruger National Park (25°09'45.5"S 31°57'04.3"E), collected by W. Malherbe (2022). Takwasa, Vaal River (26°52'02.7"S 27°17'36.0"E), collected by W. Malherbe (2022).

Representative DNA sequences. Only samples from Barberspan, Sabie River, Nyamiti Pan, and Takwasa were successfully amplified. Samples from the Shokwe sites were unsuccessful (see Table 5.2). Sequences, with the numbers of base pairs (bp), will be submitted to GenBank as follows:

18S. Eight sequences (1,272–1,398 bp) from four localities.

28S. Eight sequences (716–746 bp) from four localities.

COI. Three sequences (618–1,154 bp) from three localities.

Infestation rates: Total infestation prevalence 57.7% (15/26), mean intensity 5.27 (79/15), mean abundance 3.04 (79/26). Detailed information on infestation rates, individual sites, and hosts is provided in Table 5.3.

Table 5.3: Infestation data of *Lernaea cyprinacea* Linnaeus, 1758 parasitising three fish species from five localities in South Africa. Abbreviations: MA – Mean Abundance, MI – Mean Intensity, N(E) – number of hosts examined, N(I) – number of hosts infested, P – Prevalence, PC – number of parasites collected, SOI – Site(s) of infestation.

Sampling sites	Host	Family	SOI	N(E)	N(I)	PC	%P	MA	MI
Barberspan, Harts River	*Labeo umbratus*	Cyprinidae	Skin	3	2	4	66.7	1.3	2.0
Klipdrif Dam	*Cyprinus carpio*	Cyprinidae	Skin	1	1	1	100	1	1
Takwasa, Vaal River	*Labeobarbus aeneus*	Cyprinidae	Under operculum	13	3	3	23.1	0.2	1.0
Nyamiti Pan, Phongolo Floodplain	*Oreochromis mossambicus*	Cichlidae	Skin	4	4	50	100	12.5	12.5
Shokwe Pan, Phongolo Floodplain	*Oreochromis mossambicus*	Cichlidae	Skin	3	3	12	100	4.0	4.0
Lower Sabie River, Incomati System	*Oreochromis mossambicus*	Cichlidae	Skin	2	2	9	100	4.5	4.5

Descriptive notes of adult female (Figs. 5.2, 5.4–5.6).

Measurements (n = 20) follow the illustration in Fig. 5.2 and values are given in Table 5.4. Total length 7.15–11.73 mm.

Body elongate (Figs. 5.5, 5.6a). All specimens collected in this study had a cephalic holdfast (anchor) (Fig. 5.4) which bears the head, antennules, antennae, and mouthparts, an elongated tubular trunk (the thorax) ending in a posterior tail region (abdomen) with paired genital swellings. Paired multiseriate egg sacs present in some specimens, furcal rami retained at posterior extremity of tail region. Specimens had five pairs of swimming legs: legs 1 situated ventrally on cephalothorax, legs 2–4 located on the thorax, legs 5 are rudimentary, attached to the abdomen and covered by pregenital prominence.

Table 5.4: Measurements of body and anchoral processes of *Lernaea cyprinacea* Linnaeus, 1758 (Lcy) specimens collected from this study compared to wild and lab populations of *L. cyprinacea* and the synonymised *L. cruciata* (Lcr) specimens reported by Hua et al. (2019). All measurements for Mean ± SD and range are presented in mm.

Measured characters		Present study	Hua et al. (2019)			
			Lcy wild	Lcr wild	Lcy lab	Lcr lab
Body Length (n = 20)	Mean ± SD	**9.76 ± 1.63**	7.79 ± 2.44	7.11 ± 0.92	8.86 ± 1.71	7.10 ± 1.00
	Range	**7.15–11.73**	5.01–12.40	5.69–8.26	6.67–12.3	5.79–8.05
	%Coeff. of Variation	**16.76**	31.35	12.98	19.3	14.1
Anchor width (n = 20)	Mean ± SD	**3.39 ± 1.14**	3.31 ± 1.09	1.16 ± 0.34	2.31 ± 1.37	1.19 ± 0.29
	Range	**1.09–5.2**	1.45–6.03	0.71–2.00	0.87–6.13	0.89–1.64
	%Coeff. of Variation	**33.65**	32.91	29.11	59.24	24.22
Anterior dorsal processes length (n = 20)	Mean ± SD	**0.97 ± 0.39**	0.80 ± 0.33	0.56 ± 0.18	0.90 ± 0.39	0.48 ± 0.09
	Range	**0.25–1.58**	0.07–1.73	0.31–1.00	0.26–1.74	0.33–0.56
	%Coeff. of Variation	**40.40**	40.73	33.07	42.91	19.43
Posterior dorsal processes length (n = 19)	Mean ± SD	**0.54 ± 0.23**	0.42 ± 0.19	-	0.44 ± 0.20	-
	Range	**0.21–0.95**	0.15–0.95	-	0.21–0.96	-
	%Coeff. of Variation	**42.11**	45.68	-	44.82	-
Ventral arm length (n = 20)	Mean ± SD	**0.43 ± 0.16**	0.65 ± 0.23	0.53 ± 0.16	0.55 ± 0.21	0.49 ± 0.10
	Range	**0.21–0.85**	0.26–1.13	0.33–1.00	0.31–1.01	0.34–0.58
	%Coeff. of Variation	**36.44**	34.5	30.82	37.39	20.15
Ventral Arm width (n = 20)	Mean ± SD	**1.15 ± 0.27**	-	-	-	-
	Range	**0.74–1.95**	-	-	-	-
	%Coeff. of Variation	**23.49**	-	-	-	-
Posterior dorsal process width (n = 20)	Mean ± SD	**1.73 ± 0.37**	-	-	-	-
	Range	**1.24–2.63**	-	-	-	-
	%Coeff. of Variation	**21.10**	-	-	-	-

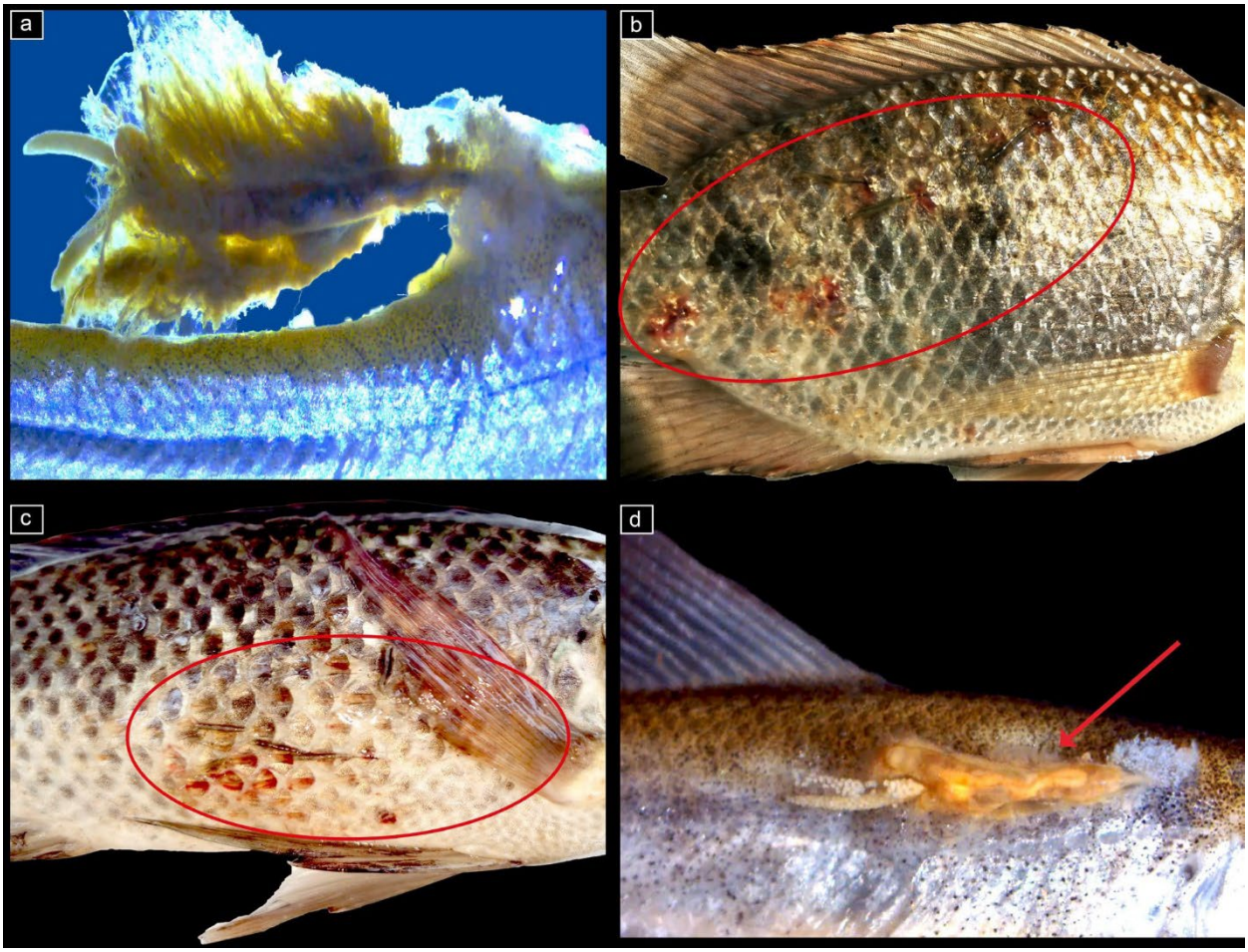


Figure 5.3: Images of the yellowfish *Labeobarbus aeneus* (a, d) and the Mozambique tilapia *Oreochromis mossambicus* (b–c) showing sites of attachment of the anchor worm, *Lernaea cyprinacea* Linnaeus, 1758. (a) *Lernaea cyprinacea* (with filamentous algae) attached to a yellowfish; (b–c) the Mozambique tilapia with lesions and *L. cyprinacea* attached on body surface (red circle); (d) *Lernaea cyprinacea* (red arrow) with sessile ciliates, attached to a yellowfish. Photo credit: Dr Hannes Erasmus (b–c).

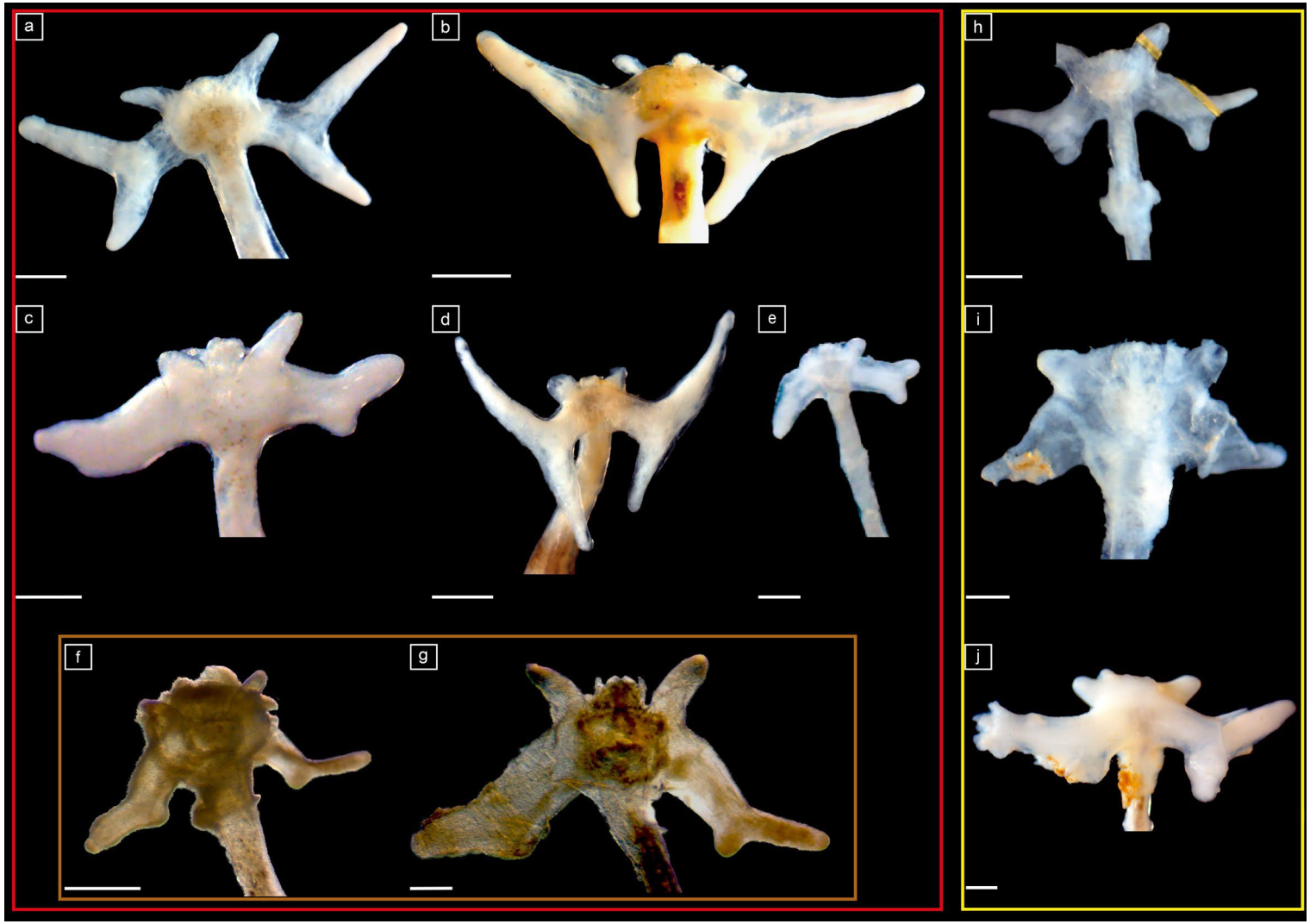


Figure 5.4: Variability in anchors shapes of *Lernaea cyprinacea* Linnaeus, 1758 specimens collected from *Oreochromis mossambicus* from Nyamiti Pan (a–g, red block) and Shokwe Pan (h–j, yellow block) Phongolo Floodplain, South Africa. The orange block shows anchors of the same specimen with a sheathed cuticular covering (f) and without a sheathed cuticular covering (g). Scale bars: (a, c, e, f, h, j) 0.5 mm; (b, d) 1 mm; (g, i) 0.2 mm.

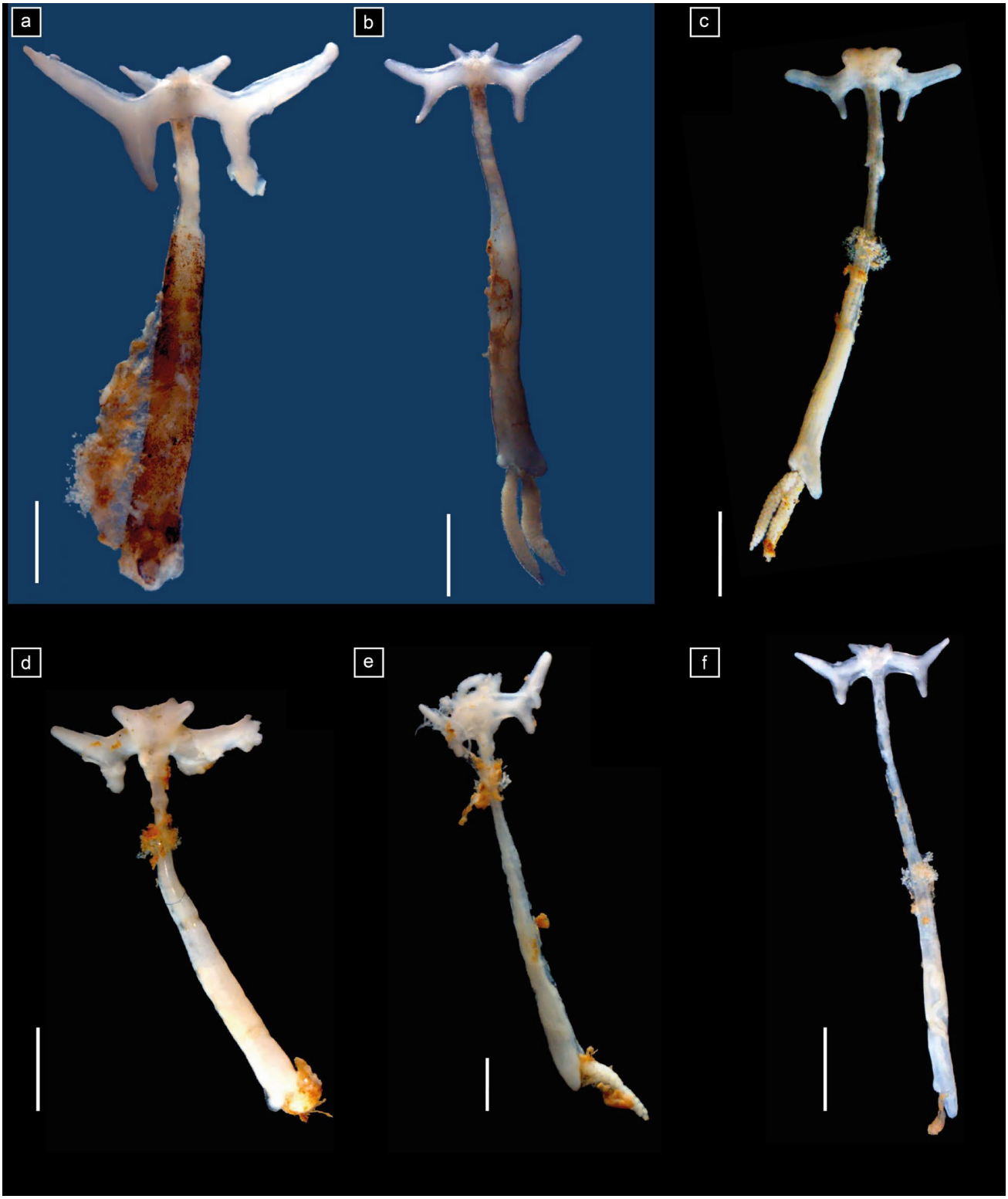


Figure 5.5: Full images of *Lernaea cyprinacea* Linnaeus, 1758 specimens of variability in sizes from the Nyamiti Pan (a–b, blue background) and Shokwe Pan (c–f, black background) Phongolo Floodplain, South Africa, showing epizoic associations with algae and sessile ciliates. Scale bars: (a–d, f) 2mm; (e) 1 mm.

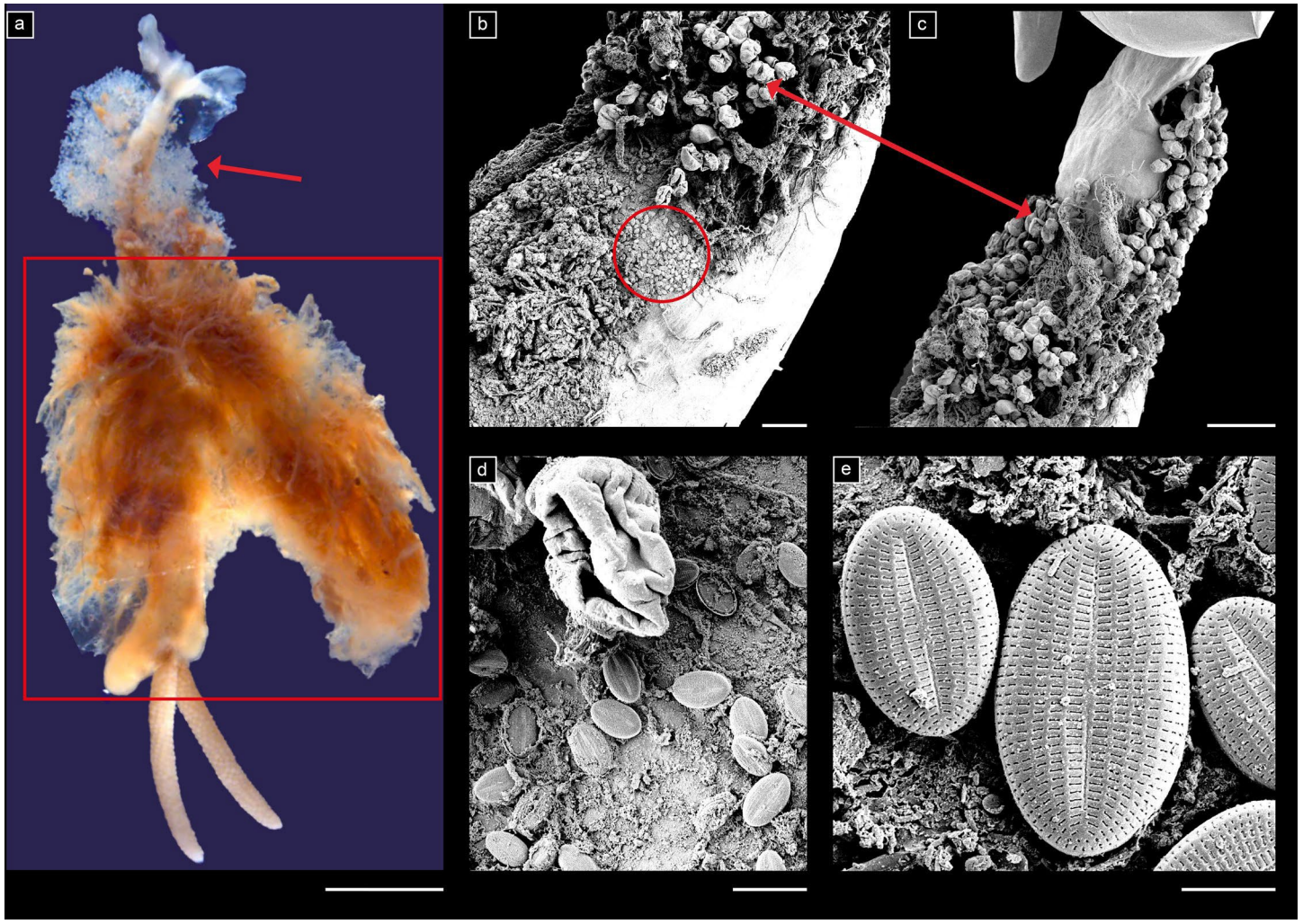


Figure 5.6: Light (a) and scanning electron microscopy (b–e) photomicrographs of *Lernaea cyprinacea* Linnaeus, 1758 with algae, and hyper symbionts. (a) full image of *L. cyprinacea* with damaged anchors, covered with sessile ciliates (red arrow) and filamentous algae (red block); (b & c) trunk of *L. cyprinacea* showing sessile ciliates (red arrows) and diatoms (red circle) and strands of filamentous algae; (d) a sessile ciliate (top) and diatoms (bottom); (e) close up of diatoms showing details of its intricate pattern. Scale bars: (a) 2 mm; (b) 100 µm; (c) 200 µm; (d) 50 µm; (e) 5 µm.

Remarks.

Four fish species from six localities in South Africa were infested with *L. cyprinacea. Oreochromis mossambicus* (Cichlidae) was the most represented fish host in this study. It was caught from two sites in the Ndumo Game Reserve of the Phongolo Floodplain (Nyamiti Pan and Shokwe Pan), and from the Sabie River, in the Incomati System, Kruger National Park. Three cyprinids were also reported from this study: the type host for *L. cyprinacea,* the common carp *C. carpio,* was reported from the Klipdrif Dam; *Labeo umbratus* (Smith, 1841) was caught from the Barberspan Bird sanctuary, an impoundment in the Harts River; and *Labeobarbus aeneus* (Burchell, 1822) from the region of the Vaal River at Takwasa, Venterskroon, North West Province. Four localities had 100% infestation prevalence, except Barberspan which had two of the three hosts infested (prevalence = 66.7%), and Takwasa with three of thirteen hosts infested (prevalence = 23.1%) (see Table 5.3).

Of the six localities reported from this study, the Nyamiti fish had the highest parasite intensity: a total of 50 *L. cyprinacea* were collected from four *O. mossambicus* individuals with mean infestation intensity of 12.5. All three *O. mossambicus* individuals from the Shokwe Pan were infested with a total of 12 parasites making it the second highest locality with a mean intensity of four. Only two fish were caught from the Sabie River, both of which were infested with a total of nine parasites.

A high level of variability in anchors (Fig. 5.4) and body size (Fig. 5.5) is reported for *L. cyprinacea* specimens collected in this study. This could be visibly observed when comparing individuals from the two sites in Phongolo Floodplain population, which had the highest number of parasites among all the localities. Some of the anchors had a cuticular sheath covering (see Figs. 5.4f, j) that may influence the shape of the anchoral processes if not carefully unsheathed (see Fig. 5. 4g). There were at least four different anchor shapes morphologies given as follows:

I. A pair of unbranched ventral arms and bifurcated dorsal arms (Figs. 5.4a, b, d)
II. A pair of unbranched ventral arms and unbranched dorsal arms (Fig. 5.4i)
III. A pair of unbranched ventral arms and incompletely branched dorsal arms (Figs. 5.4c, e, f, g, h, j)
IV. Posterior dorsal processes which were more curved inwardly in some specimens than in others (see Figs. 5.4b, d).

5.4.2. Molecular analysis

A total of 20 new sequences were generated from this study. Detailed information on novel sequences, their hosts, and localities are given in Table 5.2. Newly generated sequences were genetically similar to all other *L. cyprinacea* sequences on GenBank as well as the *L. cruciata* sequences from Hua et al. (2019), which the authors in their study reported to be conspecific with *L. cyprinacea* (Hua et al., 2019).

For partial 18S analysis, a total of 27 sequences (see Table 5.2) used in the 18S alignment resulted in a length of 1,389 base pairs. All newly generated sequences were 100% identical. *Lernaea cyprinacea* sequences in the alignment were 99.59–100% (1–3 bp) similar to novel sequences from this study (see Table 5.5). Phylogenetic tree topologies were similar for BL and ML analyses. Novel sequences from the present study clustered in cades with the other *L. cyprinacea* sequences from GenBank (Fig. 5.7).

In the phylogenetic analyses for the partial 28S gene region, the alignment (with a total of 26 sequences) yielded a length of 824 base pairs. Novel sequences from this study showed 99.58–100% similarity (3 bp). All other *L. cyprinacea* sequences in the alignment (except KY346866) showed 99.29–100% similarity (1–5 bp) with newly generated sequences (see Table 5.6). The *L. cyprinacea* sequence from Australia (KY346866) was the least similar to the newly generated sequences, having 98.29–93.71% similarity (9–12 bp) (Table 5.6). Tree topologies for BI and ML analyses showed that newly generated sequences clade with other *L. cyprinacea* sequences from GenBank (Fig. 5.8).

Three partial COI sequences (1,154 bp from Barberspan, 1,154 bp from Takwasa, and 618 bp Nyamiti sites) were newly generated from this study; DNA amplification of the Shokwe samples was unsuccessful. As the primers by Hua et al. (2019) were unsuccessful in amplification of samples from the Nyamiti, a different primer set by Cheng et al. (2019) was used for amplification of the newly generated sequence from the Nyamiti site. The resultant sequence is relatively shorter and a slightly different fragment of the COI gene compared to newly generated sequences from the other two localities. Two sets of alignments were therefore made in order to observe the variability between all newly generated sequences and among the rest of the *L. cyprinacea* sequences in the alignment.

The first alignment with all three newly generated sequences yielded a length of 1,343 bp and confirmed that the portion of the COI gene amplified for the Nyamiti sequence was slightly different and thus not completely comparable to all the other partial COI sequences used in the alignment. The Nyamiti sequence differed from the Barberspan and Takwasa sequences by 62 bases, and from the rest of the *Lernaea* sequences by 75–90 bases (see Appendix Table A2). Overlapping alignment regions revealed that most of the variable nucleotide positions translated for the same amino acids—a silent mutation, and only one missense mutation (a nucleotide substitution that translates for a different amino acid) was

observed for the Nyamiti sequence when compared to all other *Lernaea* sequences in the overlapping region. A second missense mutation was only observed in the extended DNA fragments of the Nyamiti sequence that overlap with the complete mitochondrial DNA sequence (KM235194) used in the alignment. This partial COI sequence generated from the Nyamiti locality in this study was with a different primer set and didn't match completely with the other newly generated COI sequences. It will however be made available on GenBank, along with the other newly generated sequences for further comparative evolutionary studies but will not be used in the final phylogenetic analyses in this study.

The second alignment made use of only newly generated sequences from Barberspan and Takwasa, which were amplified with the same primers and consistent with the other COI sequences of *L. cyprinacea*; yielding an alignment length of 1,154 bp. Comparing all other sequences in this second alignment, a total of five missense mutations were observed. Newly generated sequences were 99.91% similar (1 bp) and differed with 7–22 bases (98.05–99.30% similar) from all other *Lernaea* sequences in the alignment (see Table 5.7). As observed in the COI evolutionary tree constructed (Fig. 5.9), sequences from Australia, China, and South Africa clustered in separated sister clades. The sequences for *L. cruciata* (*Gambusia holbrooki* Girard, 1859, the host) from China also clustered away from the other China specimens.

5.5. Discussion

In South Africa, parasitisation of fishes by the invasive *L. cyprinacea* had been reported as far back as the 1980s (van As & Basson, 1984). Until 2017, *L. cyprinacea* had been recorded from 11 fishes belonging to the Cichlidae and Cyprinidae families from Barberspan (Harts River), Crocodile (West) River, Douglas Wier (Vaal River), Lowveld Fisheries Station, Mogalakwena River, Nyamiti Pan (Phongolo River), Olifants River, Orange River, Selati River, and the Vaal River (see Smit et al., 2017 and the references therein). This invasive parasite was also reported from the endangered endemic rocky kurper, *S. bainsii* (Anabantidae), for the first time in South Africa by Chakona et al. (2019), from the Eastern Cape Province, South Africa. Fishes from the Cichlidae and Cyprinidae families seem to be the most preferred host group for *L. cyprinacea* (Robinson & Avenant-Oldewage, 1996).

Table 5.5: Genetic divergence of aligned 18S sequences used in the *Lernaea cyprinacea* alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study). Sequences from the present study are boldface in a grey shade. *Lamproglena orientalis* Markevich, 1936 was used as the outgroup. Abbreviations: AR – Argentina; AU – Australia; BP – Barberspan (Harts River); CN – China; JP – Japan; LSR – Lower Sabie River (Incomati System, Kruger National Park); NP – Nyamiti Pan (Phongolo Floodplain); TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	**P44K *Lernaea cyprinacea* TW ZA**		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**3**	**0**	**0**	**2**	**2**	**1**	**0**	**0**	**0**	**1**	**2**	**2**	**0**	**0**	**0**	**39**	**39**	**37**
2	**P45K *Lernaea cyprinacea* TW ZA**	**100**		**0**	**0**	**0**	**0**	**0**	**0**	**2**	**3**	**0**	**0**	**2**	**2**	**1**	**0**	**0**	**0**	**1**	**2**	**2**	**0**	**0**	**0**	**43**	**43**	**41**
3	**P61K *Lernaea cyprinacea* NP ZA**	**100**	**100**		**0**	**0**	**0**	**0**	**0**	**2**	**3**	**0**	**0**	**2**	**2**	**1**	**0**	**0**	**0**	**1**	**2**	**2**	**0**	**0**	**0**	**37**	**37**	**36**
4	**P64K *Lernaea cyprinacea* NP ZA**	**100**	**100**	**100**		**0**	**0**	**0**	**0**	**2**	**3**	**0**	**0**	**2**	**2**	**1**	**0**	**0**	**0**	**1**	**2**	**2**	**0**	**0**	**0**	**37**	**37**	**36**
5	**P65K *Lernaea cyprinacea* NP ZA**	**100**	**100**	**100**	**100**		**0**	**0**	**0**	**2**	**3**	**0**	**0**	**2**	**2**	**1**	**0**	**0**	**0**	**1**	**2**	**2**	**0**	**0**	**0**	**37**	**37**	**36**
6	**P69K *Lernaea cyprinacea* BP ZA**	**100**	**100**	**100**	**100**	**100**		**0**	**0**	**2**	**3**	**0**	**0**	**2**	**2**	**1**	**0**	**0**	**0**	**1**	**2**	**2**	**0**	**0**	**0**	**37**	**37**	**36**
7	**P70K *Lernaea cyprinacea* LSR ZA**	**100**	**100**	**100**	**100**	**100**	**100**		**0**	**2**	**3**	**0**	**0**	**2**	**2**	**1**	**0**	**0**	**0**	**1**	**2**	**2**	**0**	**0**	**0**	**37**	**37**	**36**
8	**P72K *Lernaea cyprinacea* LSR ZA**	**100**	**100**	**100**	**100**	**100**	**100**	**100**		**2**	**3**	**0**	**0**	**2**	**2**	**1**	**0**	**0**	**0**	**1**	**2**	**2**	**0**	**0**	**0**	**37**	**37**	**36**
9	MH198053 *Lernaea cyprinacea* AR	**99.75**	**99.75**	**99.73**	**99.73**	**99.73**	**99.73**	**99.73**	**99.73**		1	2	2	3	4	2	2	2	2	3	3	4	2	1	0	19	20	18
10	MH198054 *Lernaea cyprinacea* AR	**99.62**	**99.62**	**99.59**	**99.59**	**99.59**	**99.59**	**99.59**	**99.59**	99.87		3	3	4	5	3	3	3	3	4	4	5	3	2	0	20	21	19
11	MT367695 *Lernaea cyprinacea* AU	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	99.72	99.58		0	2	2	1	0	0	0	1	2	2	0	0	0	37	37	36
12	MT367704 *Lernaea cyprinacea* AU	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	99.72	99.58	100		2	2	1	0	0	0	1	2	2	0	0	0	37	37	36
13	DQ107554 *Lernaea cyprinacea* CN	**99.86**	**99.86**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	99.62	99.49	99.84	99.84		2	3	2	2	2	3	4	2	2	1	0	44	44	42
14	DQ107555 *Lernaea cyprinacea* CN	**99.86**	**99.86**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	99.49	99.37	99.84	99.84	99.86		3	2	2	2	2	4	2	2	1	0	44	44	42
15	DQ107557 *Lernaea cyprinacea* CN	**99.93**	**99.93**	**99.92**	**99.92**	**99.92**	**99.92**	**99.92**	**99.92**	99.75	99.62	99.92	99.92	99.79	99.79		1	1	1	2	3	3	1	0	0	44	44	42
16	MH982196 *Lernaea cyprinacea* CN	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	99.75	99.62	100	100	99.86	99.86	99.93		0	0	1	2	2	0	0	0	39	39	37
17	MH982197 *Lernaea cyprinacea* CN	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	99.75	99.62	100	100	99.86	99.86	99.93	100		0	1	2	2	0	0	0	39	39	37
18	MH982198 *Lernaea cyprinacea* CN	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	99.75	99.62	100	100	99.86	99.86	99.93	100	100		1	2	2	0	0	0	39	39	37
19	MH982195 *Lernaea cyprinacea* CN	**99.93**	**99.93**	**99.92**	**99.92**	**99.92**	**99.92**	**99.92**	**99.92**	99.62	99.49	99.92	99.92	99.78	99.86	99.86	99.93	99.93	99.93		3	3	1	0	0	40	40	38
20	MH982203 *Lernaea cruciata* CN	**99.86**	**99.86**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	99.62	99.49	99.84	99.84	99.71	99.71	99.78	99.86	99.86	99.86	99.78		4	2	0	0	41	41	39
21	DQ107556 *Lernaea cyprinacea* CN	**99.86**	**99.86**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	**99.84**	99.49	99.37	99.84	99.84	99.86	99.86	99.79	99.86	99.86	99.86	99.78	99.71		2	1	0	44	44	42
22	KP235363 *Lernaea cyprinacea* JP	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	99.75	99.62	100	100	99.86	99.86	99.93	100	100	100	99.93	99.86	99.86		0	0	43	43	41
23	KY435939 *Lernaea cyprinacea* ZA	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	99.84	99.67	100	100	99.84	99.84	100	100	100	100	100	100	99.84	100		0	13	14	12
24	MN243682 *Lernaea cyprinacea* ZA	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100		7	8	6
25	DQ107552 *Lamproglena orientalis* CN	**97.18**	**96.92**	**97.11**	**97.1**	**97.1**	**97.1**	**97.11**	**97.09**	97.59	97.46	97.05	97.05	96.85	96.85	96.85	97.18	97.18	97.18	97.11	97.04	96.85	96.9	97.9	97.98		6	4
26	DQ107551 *Lamproglena orientalis* CN	**97.18**	**96.92**	**97.11**	**97.1**	**97.1**	**97.1**	**97.11**	**97.09**	97.46	97.34	97.05	97.05	96.85	96.85	96.85	97.18	97.18	97.18	97.11	97.04	96.85	96.9	97.7	97.69	99.57		2
27	DQ107550 *Lamproglea orientalis* CN	**97.33**	**97.07**	**97.19**	**97.18**	**97.18**	**97.18**	**97.19**	**97.17**	97.72	97.59	97.13	97.13	97	97	97	97.33	97.33	97.33	97.25	97.18	97	97.1	98	98.27	99.71	99.86	

Table 5.6: Genetic divergence of aligned 28S sequences used in the *Lernaea cyprinacea* Linnaeus, 1758 alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study). Sequences from the present study are boldface in a grey shade. *Lamproglena orientalis* Markevich, 1936 was used as the outgroup. Abbreviations: AR – Argentina; AU – Australia; BP – Barberspan (Harts River); CN – China; JP – Japan; LSR – Lower Sabie River (Incomati System, Kruger National Park); NP – Nyamiti Pan (Phongolo Floodplain); RO – Romania; TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	**P44Ka *Lernaea cyprinacea* TW ZA**	**99.51**	**0**	**3**	**3**	**3**	**0**	**3**	**3**	**0**	**0**	**0**	**1**	**1**	**2**	**0**	**2**	**1**	**0**	**1**	**0**	**0**	**9**	**256**	**264**	**264**	**262**
2	**P45Ka *Lernaea cyprinacea* TW ZA**	**100**		**3**	**3**	**3**	**0**	**3**	**3**	**0**	**0**	**0**	**1**	**1**	**2**	**0**	**2**	**1**	**0**	**1**	**0**	**0**	**9**	**256**	**264**	**264**	**262**
3	**P61Ka *Lernaea cyprinacea* NP ZA**	**99.58**	**99.58**		**0**	**0**	**3**	**0**	**0**	**3**	**3**	**3**	**4**	**4**	**5**	**3**	**5**	**4**	**3**	**4**	**1**	**1**	**12**	**255**	**263**	**263**	**261**
4	**P64Ka *Lernaea cyprinacea* NP ZA**	**99.58**	**99.58**	**100**		**0**	**3**	**0**	**0**	**3**	**3**	**3**	**4**	**4**	**5**	**3**	**5**	**4**	**3**	**4**	**1**	**1**	**12**	**255**	**263**	**263**	**261**
5	**P65Ka *Lernaea cyprinacea* NP ZA**	**99.58**	**99.58**	**100**	**100**		**3**	**0**	**0**	**3**	**3**	**3**	**4**	**4**	**5**	**3**	**5**	**4**	**3**	**4**	**1**	**1**	**12**	**255**	**263**	**263**	**261**
6	**P69Ka *Lernaea cyprinacea* BP ZA**	**100**	**100**	**99.58**	**99.58**	**99.58**		**3**	**3**	**0**	**0**	**0**	**1**	**1**	**2**	**0**	**2**	**1**	**0**	**1**	**0**	**0**	**9**	**256**	**264**	**264**	**262**
7	**P70Ka *Lernaea cyprinacea* LSR ZA**	**99.58**	**99.58**	**100**	**100**	**100**	**99.58**		**0**	**3**	**3**	**3**	**4**	**4**	**5**	**3**	**5**	**4**	**3**	**4**	**1**	**1**	**12**	**255**	**263**	**263**	**261**
8	**P72Ka *Lernaea cyprinacea* LSR ZA**	**99.58**	**99.58**	**100**	**100**	**100**	**99.58**	**100**		**3**	**3**	**3**	**4**	**4**	**5**	**3**	**5**	**4**	**3**	**4**	**1**	**1**	**12**	**255**	**263**	**263**	**261**
9	KX908211 *Lernaea cyprinacea* AR	**100**	**100**	**99.58**	**99.58**	**99.58**	**100**	**99.58**	**99.58**		0	0	1	1	2	0	2	1	0	1	0	0	9	256	264	264	262
10	MT371347 *Lernaea cyprinacea* AU	**100**	**100**	**99.53**	**99.53**	**99.53**	**100**	**99.53**	**99.53**	100		0	1	1	2	0	2	1	0	0	0	0	2	246	254	254	252
11	MT371367 *Lernaea cyprinacea* AU	**100**	**100**	**99.53**	**99.53**	**99.53**	**100**	**99.53**	**99.53**	100	100		1	1	2	0	2	1	0	0	0	0	2	246	254	254	252
12	DQ107548 *Lernaea cyprinacea* CN	**99.86**	**99.86**	**99.44**	**99.44**	**99.44**	**99.86**	**99.44**	**99.44**	99.86	99.84	99.84		2	3	1	3	2	1	2	0	0	10	256	264	264	262
13	MH982210 *Lernaea cyprinacea* CN	**99.86**	**99.86**	**99.43**	**99.43**	**99.43**	**99.86**	**99.43**	**99.43**	99.86	99.84	99.84	99.72		3	1	3	2	1	2	0	0	10	257	265	265	263
14	DQ107547 *Lernaea cyprinacea* CN	**99.72**	**99.72**	**99.31**	**99.31**	**99.31**	**99.72**	**99.31**	**99.31**	99.72	99.68	99.68	99.58	99.58		2	4	1	2	3	1	1	11	256	264	264	262
15	MH982204 *Lernaea cyprinacea* CN	**100**	**100**	**99.58**	**99.58**	**99.58**	**100**	**99.58**	**99.58**	100	100	100	99.86	99.86	99.72		2	1	0	1	0	0	9	256	264	264	262
16	MH982208 *Lernaea cyprinacea* CN	**99.72**	**99.72**	**99.29**	**99.29**	**99.29**	**99.72**	**99.29**	**99.29**	99.72	99.68	99.68	99.58	99.58	99.43	99.72		3	2	3	1	1	11	258	266	266	264
17	DQ107546 *Lernaea cyprinacea* CN	**99.86**	**99.86**	**99.44**	**99.44**	**99.44**	**99.86**	**99.44**	**99.44**	99.86	99.84	99.84	99.72	99.72	99.86	99.86	99.58		1	2	0	0	10	255	263	263	261
18	MH982215 *Lernaea cyprinacea* CN	**100**	**100**	**99.58**	**99.58**	**99.58**	**100**	**99.58**	**99.58**	100	100	100	99.86	99.86	99.72	100	99.72	99.86		1	0	0	9	256	264	264	262
19	KP235364 *Lernaea cruciata* JP	**99.86**	**99.86**	**99.44**	**99.44**	**99.44**	**99.86**	**99.44**	**99.44**	99.86	100	100	99.72	99.72	99.58	99.86	99.58	99.72	99.86		0	0	9	257	265	265	263
20	DQ107556 *Lernaea cyprinacea* RO	**100**	**100**	**99.76**	**99.76**	**99.76**	**100**	**99.76**	**99.76**	100	100	100	100	100	99.76	100	99.76	100	100	100		0	0	51	51	51	50
21	KP235363 *Lernaea cyprinacea* RO	**100**	**100**	**99.76**	**99.76**	**99.76**	**100**	**99.76**	**99.76**	100	100	100	100	100	99.76	100	99.76	100	100	100	100		0	51	51	51	50
22	KY435939 *Lernaea cyprinacea* AU	**98.71**	**98.71**	**98.29**	**98.29**	**98.29**	**98.71**	**98.29**	**98.29**	98.7	99.68	99.68	98.57	98.56	98.43	98.7	98.41	98.57	98.7	98.71	100	100		262	270	270	268
23	MN243682 *Lernaea cyprinacea* CN	**68.28**	**68.28**	**68.44**	**68.44**	**68.44**	**68.16**	**68.44**	**68.44**	67.76	65.74	65.74	68.28	67.63	68.28	67.76	67.51	68.4	67.76	67.88	87.91	87.91	66.71		52	54	52
24	DQ107552 *Lamproglena orientalis* CN	**67.8**	**67.8**	**67.97**	**67.97**	**67.97**	**67.69**	**67.97**	**67.97**	67.29	65.25	65.25	67.8	67.16	67.8	67.29	67.04	67.93	67.29	67.4	87.91	87.91	66.25	93.67		4	2
25	DQ107551 *Lamproglema orientalis* CN	**67.8**	**67.8**	**67.97**	**67.97**	**67.97**	**67.69**	**67.97**	**67.97**	67.29	65.25	65.25	67.8	67.16	67.8	67.29	67.04	67.93	67.29	67.4	87.91	87.91	66.25	93.42	99.51		2
26	DQ107550 *Lamproglema orientalis* CN	**68.05**	**68.05**	**68.21**	**68.21**	**68.21**	**67.93**	**68.21**	**68.21**	67.53	65.53	65.53	68.05	67.41	68.05	67.53	67.29	68.17	67.53	67.65	88.15	88.15	66.5	93.67	99.76	99.76	

Table 5.7: Genetic divergence of aligned COI sequences used in the *Lernaea cyprinacea* Linnaeus, 1758 alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study). Sequences from the present study are boldface in a grey shade. *Sinergasilus polycolpus* (Markevich, 1940) was used as the outgroup. Abbreviations: AU – Australia; BP – Barberspan (Harts River); CN – China; TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

		1	**2**	3	4	5	6	7	8	9	10	11	12
1	**P44Kh *Lernaea cyprinacea* TW ZA**		**1**	**8**	**8**	**12**	**14**	**15**	**14**	**14**	**14**	**22**	**318**
2	**P69Kh *Lernaea cyprinacea* BP ZA**	**99.91**		**7**	**7**	**11**	**13**	**14**	**13**	**13**	**13**	**21**	**319**
3	MT371896 *Lernaea cyprinacea* AU	**99.2**	**99.3**		0	7	9	10	9	8	8	17	270
4	MT371895 *Lernaea cyprinacea* AU	**99.2**	**99.3**	100		7	9	10	9	8	8	17	270
5	KM235194 *Lernaea cyprinacea* CN	**98.96**	**99.05**	99.3	99.3		12	13	12	12	12	16	321
6	MH982216 *Lernaea cyprinacea* CN	**98.76**	**98.85**	99.1	99.1	98.94		1	6	14	14	18	315
7	MH982219 *Lernaea cyprinacea* CN	**98.67**	**98.76**	99	99	98.85	99.91		7	15	15	19	315
8	MH982220 *Lernaea cyprinacea* CN	**98.76**	**98.85**	99.1	99.1	98.94	99.47	99.38		14	14	18	315
9	MH982226 *Lernaea cruciata* CN	**98.76**	**98.85**	99.2	99.2	98.94	98.76	98.67	98.76		0	20	310
10	MH982227 *Lernaea cruciata* CN	**98.76**	**98.85**	99.2	99.2	98.94	98.76	98.67	98.76	100		20	310
11	MH982221 *Lernaea cyprinacea* CN	**98.05**	**98.14**	98.29	98.29	98.58	98.4	98.31	98.4	98.23	98.23		315
12	KR263117 *Sinergasilus polycolpus* CN	**72.44**	**72.36**	72.89	72.89	72.18	72.05	72.05	72.05	72.49	72.49	72.05	

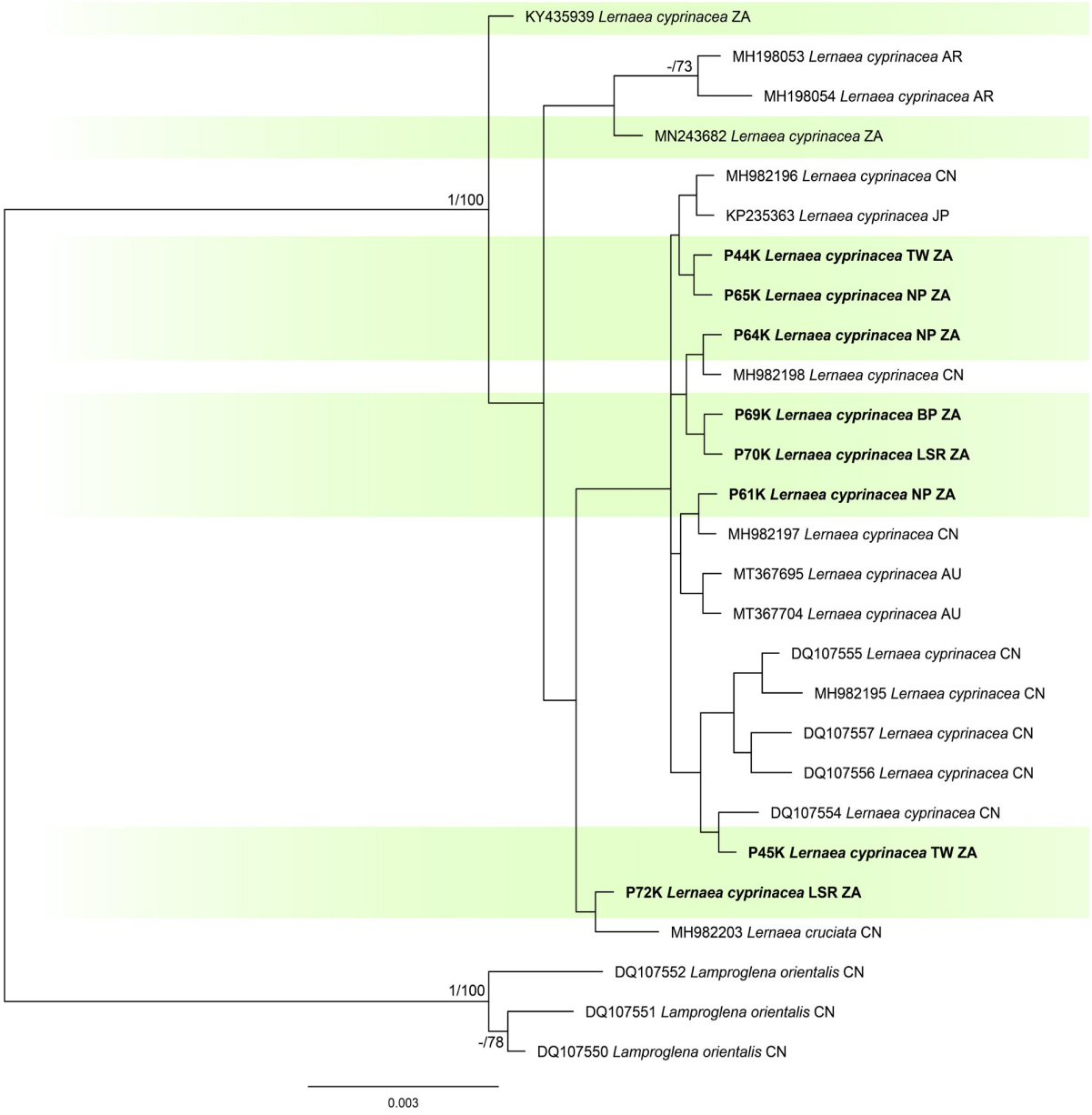


Figure 5.7: Phylogenetic relationship of *Lernaea cyprinacea* Linnaeus, 1758 based on partial 18S rRNA gene alignments. All African species are highlighted in a different coloured gradient; newly generated sequences are represented in bold. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lamproglena orientalis* was used as the outgroup. Abbreviations: AR – Argentina; AU – Australia; BP – Barberspan (Harts River); CN – China; JP – Japan; LSR – Lower part of Sabie River (Incomati System, Kruger National Park); NP – Nyamiti Pan (Phongolo Floodplain); TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

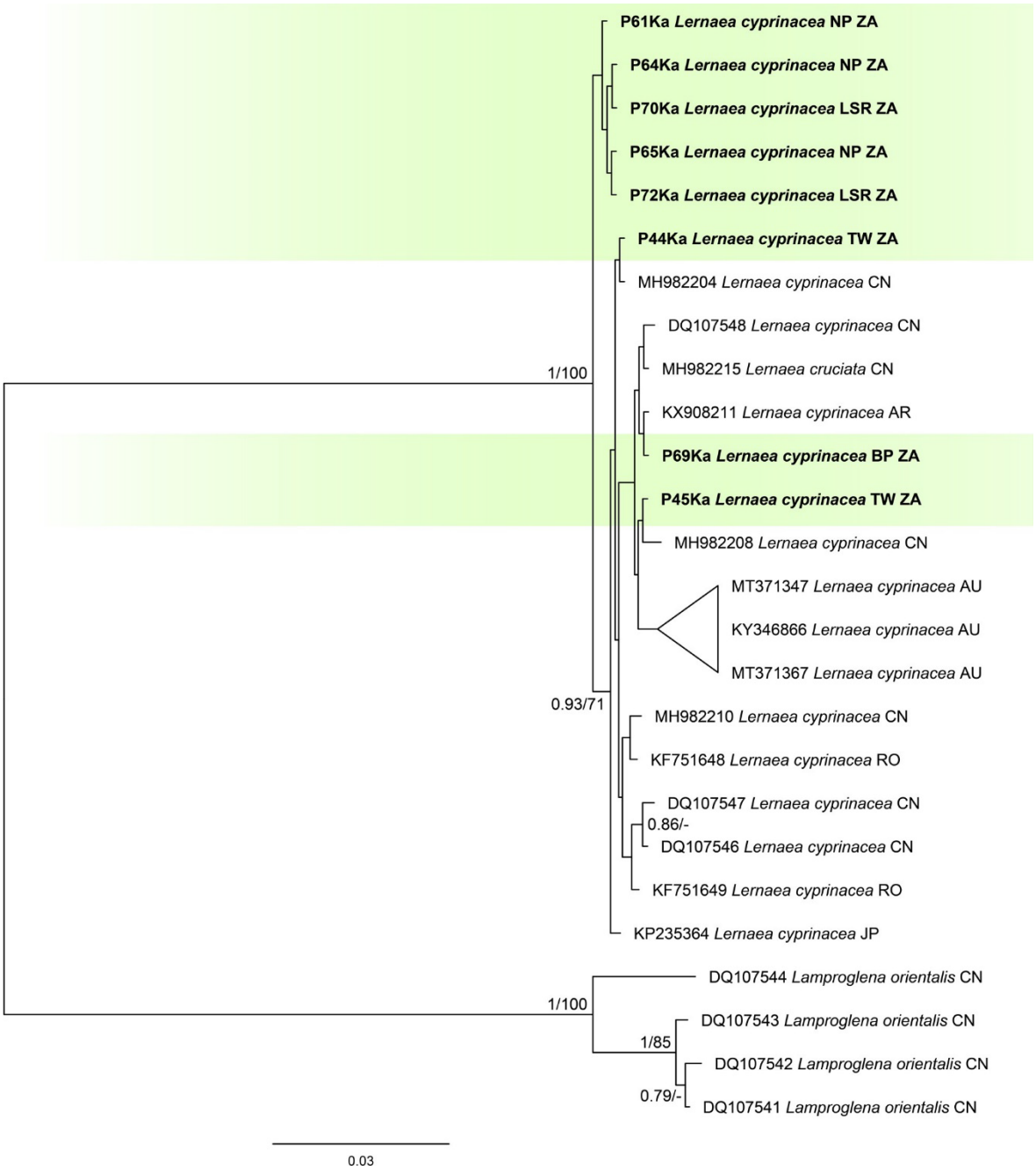


Figure 5.8: Phylogenetic relationship of *Lernaea cyprinacea* Linnaeus, 1758 based on partial 28S rRNA gene alignments All African species are highlighted in a different coloured gradient; newly generated sequences are represented in bold. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lamproglena orientalis* was used as the outgroup. Abbreviations: AR – Argentina; AU – Australia; BP – Barberspan (Harts River); CN – China; JP – Japan; LSR – Lower part of Sabie River (Incomati System, Kruger National Park); RO – Romania; NP – Nyamiti Pan (Phongolo Floodplain); TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

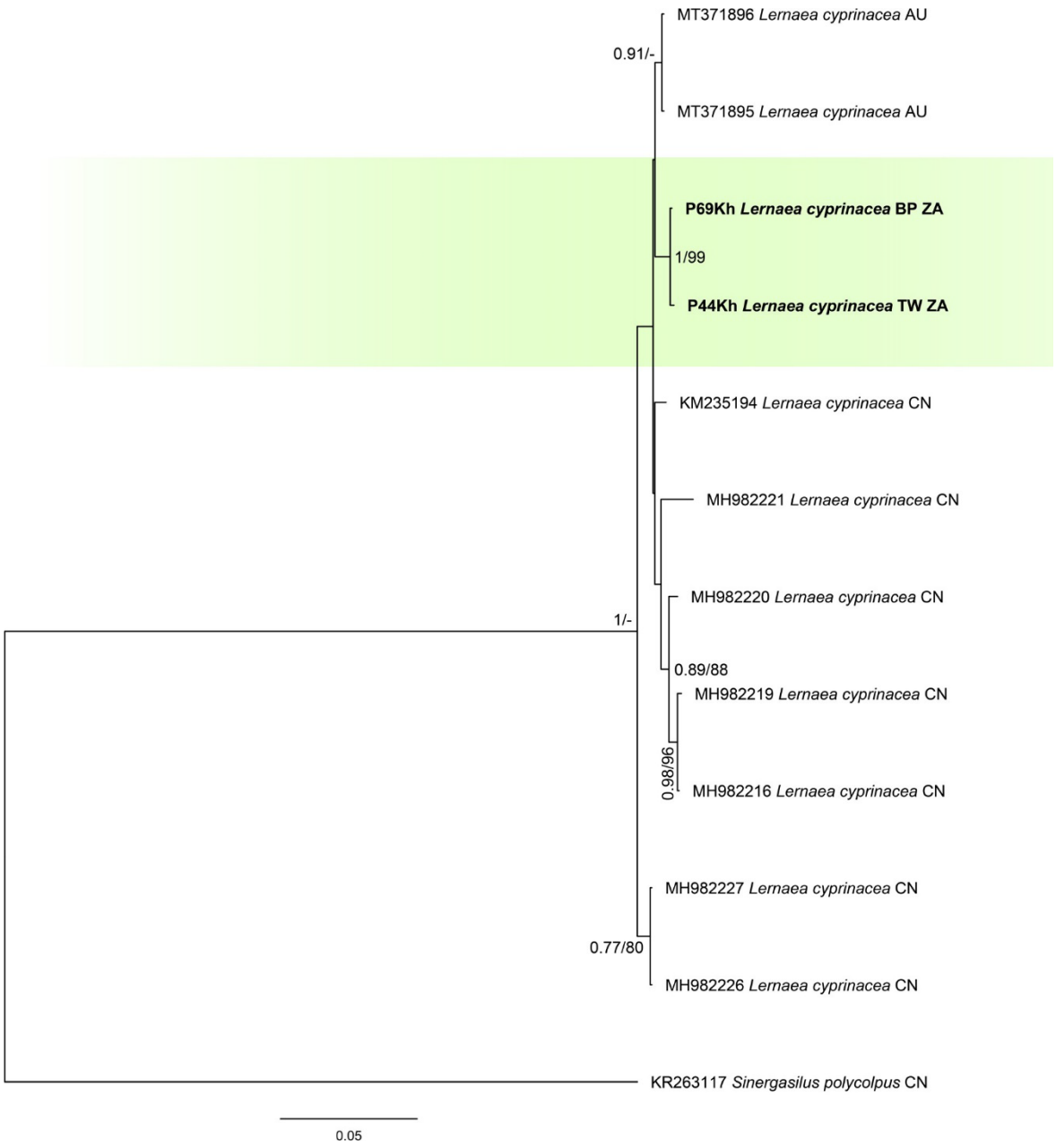


Figure 5.9: Phylogenetic relationship of *Lernaea cyprinacea* Linnaeus, 1758 based on partial COI mitochondrial gene alignments. All African species are highlighted in a different coloured gradient; newly generated sequences are represented in bold. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Sinergasilus polycolpus* was used as the outgroup. Abbreviations: AU – Australia; BP – Barberspan (Harts River); CN – China; TW – Takwasa, Venterskroon (Vaal River); ZA – South Africa.

5.5.1. Host reports from current study

From this study, *L. cyprinacea* was collected from its type host the invasive common carp, *Cyprinus carpio* (Cyprinidae); the Mozambique tilapia, *O. mossambicus*, (Cichlidae); the moggel, *L. umbratus*, (Cyprinidae); and the smallmouth yellowfish, *L. aeneus* (Cyprinidae). According to Smit et al. (2017), there had been no South African records of *L. cyprinacea* from any of the invasive cichlids that may have potentially been responsible for its introduction. This study provides the first report of *L. cyprinacea* from its co-introduced host *C. carpio,* in South Africa. The parasitisation of *O. mossambicus* by *L. cyprinacea*, has been well reported (van As & Basson, 1984; Barson et al., 2008; Welicky et al., 2017), and it had the highest infestation rates, similar to reports by Barson et al. (2008). There have also been reports of *L. umbratus* as a host of *L. cyprinacea* from the Olifants River by Robinson and Avenant-Oldewage (1996). To the best of our knowledge, there have been no published reports of *L. cyprinacea* from the smallmouth yellowfish *L. aeneus* (Cyprinidae), in South Africa, and is thus reported from this study for the first time. The current study corroborates the presence of *L. cyprinacea* in the Harts River System (Barberspan), the Phongolo River and Floodplain (Nyamiti Pan and Shokwe Pan), and in the Vaal River (Takwasa Youth camp) but reports the Shokwe Pan and the Takwasa sites as new distribution records. Before now, there had also been no reports of *L. cyprinacea* from the Incomati System. This study, therefore, provides a new distribution record for the invasive copepod in the Sabie River of the Incomati System.

5.5.2. El Niño-Southern Oscillation (ENSO) event on Lernaea cyprinacea

Between 2015 and 2016, South Africa suffered a severe drought as a result of an El Niño-Southern Oscillation (ENSO) event, which reduced the water levels and increased the salinity of freshwater environments (Welicky et al., 2017; De Necker et al., 2021, 2022). In their study on the drought-associated absence of *L. cyprinacea*, Welicky et al. (2017) examined Mozambique tilapia, *O. mossambicus* that were caught in the Nyamiti Pan before 2013 and during 2016 (the drought season). They reported that the prevalence of *L. cyprinacea* before the drought was 100%, but no infestation in the Mozambique tilapia population was recorded during the drought. Before the drought, Lake Nyamiti, permanently received water through controlled flooding from the Pongolapoort Dam (Acosta et al., 2020). According to De Necker et al. (2021, 2022), the Nyamiti Lake started receiving water from local rainfall and small floods from the Usuthu River, after the ENSO and drought ended in 2017. These authors reported that the Nyamiti Pan (referred to as Lake Nyamiti in De Necker et al., 2021, 2022) experienced a decreased salinity in 2017 because of the inflow of freshwater as well as a recolonisation of its invertebrate fauna (De Necker et al., 2021). At the time of sampling, water had been released once, from the Pongolapoort Dam into the Phongolo River and thus the Nyamiti. This, therefore, suggests that the Nyamiti received fish

again from the Usuthu River as well as the Phongolo River, which could have led to the reinfestation of *L. cyprinacea* in the Nyamiti site. The mean abundance and intensity (12.5) of *L. cyprinacea* from the current study were the highest at Nyamiti, further showing that *L. cyprinacea* is able to quickly populate any given water body.

Since its first official report in South Africa by van As and Basson (1984), this parasite, which exists in major river systems, continues to extend its distribution to different sites in South Africa as well as its host range to more native host species—some of which are threatened, like *O. mossambicus* (see Welicky et al., 2017).

5.5.3. Morphological variability in Lernaea cyprinacea

Measurements from specimens collected during the study revealed that these anchor worms vary greatly in body lengths and in the length of the anchor processes. Because of the variable range of anchor shapes and the curve in posterior dorsal processes of some specimens, the authors included the width of the posterior dorsal processes as well as the width of the ventral arms as valuable measurements to be included when comparing populations. Generally, specimens from this study were larger than specimens reported by Nagasawa et al. (2007), Nagasawa (2013), Nagasawa and Torii (2014), and Nagasawa and Katahira (2019), but within the range of specimens reported by Hua et al. (2019), although Hua et al. (2019) also recorded specimens that were larger than 12 mm. The *L. cyprinacea* population from this study was, however, smaller than records by Koyuncu and Dönmez (2006) (17 mm) and Hossain et al. (2018) (20–25 mm). This shows that *L. cyprinacea* can grow to varying sizes. Comparing other measured parameters with Hua et al. (2019), the South African population of *L. cyprinacea* was within the range of the Chinese population reported by Hua et al. (2019) (see Table 5.4).

From the current study, it was observed that members of the South African population varied in the length and shape of the anchor processes, even individuals collected from the same host. This is consistent with reports by Hua et al. (2019) and Zhu et al. (2021). Other authors have suggested that the differences in the anchors may be because of the point of tissue attachment (Fryer, 1968; Amin, 1981; Robinson & Avenant-Oldewage, 1996). Specimens from this study also showed varying ranges of anchor processes, similar to Hua et al. (2019). The posterior dorsal processes ranged from (1.24–2.63 mm) in width, and specimens with a more strongly curved posterior dorsal processes had the smallest width between its processes.

Some of the *L. cyprinacea* individuals collected from this study were covered with filamentous algae, diatoms and “necklaces” of stalked sessile ciliates (see Figs. 5.3a, d, 5.5–5.6). These epizoic associations are often reported with parasitic crustaceans (see Fryer, 1965; Amin, 1981; van As & Viljoen, 1984; Winemiller & Winsborough, 1990; van As, 2019).

5.5.4. Molecular analysis

Phylogenetic analyses for all datasets (partial 18S, 28S, and COI) revealed very little variability among *L. cyprinacea* specimens. For the 18S analyses, newly generated sequences had no variabilities within their bases and generally showed very little variation between all other *L. cyprinacea* sequences used in the alignment. The phylogenetic tree constructed for the partial 18S analysis had newly generated sequences clustering in several smaller clades with other *L. cyprinacea sequences.* Although these parasites are not host-specific, the 28S analyses showed that newly generated sequences from the Mozambique tilapia host *O. mossambicus* were 100% identical, even though they were from different sampling localities and from two separate systems (the Phongolo Floodplain and the Incomati System). This is further observed with these sequences clustering separately from the other newly generated sequences from Barberspan (*L. umbratus* host) and Takwasa (*L. aeneus* host). This separation observed in the Barberspan, Tawkasa, Phongolo, and Incomati populations probably highlights multiple levels of *L. cyprinacea* invasion into South Africa, indicating that not all individuals of *L. cyprinacea* arrived in South Africa at the same time.

Furthermore, the *L. cruciata* sequences from China, which were collected from hosts in the family Poeciliidae, clustered separately from the other sequences from China that were either from hosts in the family Cyprinidae or Xenocyprididae. It seems to be evident that the concept of parasite-host co-evolution is strongly supported using molecular analysis. As localities and hosts are possible factors in understanding the evolutionary relationship and intraspecific variability among *Lernaea* species, more molecular and morphological research is needed to further explore these relationships among *Lernaea* species. The current study only successfully amplified one sequence from the Nyamiti Pan (which had varying anchor morphotypes), which was only possible using a different primer set, as such, the morphovariants could not be molecularly compared. A more in-depth study is thus recommended to understand how these morphovariants of *L. cyprinacea* compare molecularly.

5.6. Conclusion

This study highlights the morphological variability in anchor shapes and sizes of *Lernaea cyprinacea* specimens and provides genetic data for *L. cyprinacea* from South Africa, using three partial genes (18S, 28S, and COI) as molecular markers. It further highlights the possibility of slight genetic variability based on host species, but more studies are required to test this theory. Three new localities from three river systems in South Africa as well as a new host are reported in this study. This is also the first report of *L. cyprinacea* from its co-introduced host, *C. carpio*, in South Africa.

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CHAPTER 6

New records of an invasive fish parasitic copepod in Africa: substantial range extension, new host, and molecular characterisation of *Neoergasilus japonicus* (Harada, 1930) (Ergasilidae: Cyclopoida)

6.1. Abstract

The fish parasitic copepod, *Neoergasilus japonicus* (Harada, 1930) has, to date, been reported from all continents except Australia and Antarctica. This parasite is typically found attached to the fins, and less frequently on the gills of their hosts (from several families). Its introduction and distribution have been attributed to the exotic fish trade, aquaculture, sports fishing, and angling. In Africa, there are published reports of *N. japonicus* from Algeria, and unpublished records from South Africa. Although this parasite species has been reported in several countries, there are very limited genetic data available to support its morphological characteristics amongst the different populations and the respective distribution records. A total of 23 fishes were sampled from three South African water bodies: five tigerfish *Hydrocynus vittatus* (Castelnau, 1861) from the Olifants River in Kruger National Park, and 18 *Micropterus salmoides* (Lacepède, 1802) from the Boskop and Potchefstroom impoundments in the Mooi River (part of the Orange-Vaal River System). A total of 14 adult female copepods were collected from the gills and fins of the hosts and preserved in 70% ethanol. *Micropterus salmoides* from Boskop Dam had the highest prevalence (50%), and the highest mean infestation intensity was recorded for *H. vittatus* specimens from the Olifants River. Specimens from this study were morphologically and genetically identified as *N. japonicus.* Molecular analyses yielded a total of eight newly generated sequences from partial 18S, 28S, and COI genes. This study is the first integrative study of *N. japonicus* from Africa and the southern hemisphere. Additionally, this is the first report of *N. japonicus* from a characin host, *H. vittatus*. The three sampling sites from southern Africa are new locality records and the first published records from sub-Saharan Africa for *N. japonicus*.

6.2. Introduction

Neoergasilus Yin, 1956, a genus of freshwater parasitic copepods from the family Ergasilidae, currently has nine accepted species (Walter & Boxshall, 2023). In the paper on freshwater ergasilids from China, which included the description of two species – *Neoergasilus longispinosus* Yin, 1956 and *N. inflatus* Yin, 1956 (now known as *N. squaliobarbi* (Dogiel & Akhmerov, 1952)), Yin (1956) erected the genus *Neoergasilus* based on the morphological differences observed in the first leg of these parasites. At the same time, *Ergasilus japonicus* Harada, 1930, described from Taiwan, was also moved to this new genus due to observed similarities with the other *Neoergasilus* specimens (Yin, 1956).

This species, now known as *Neoergasilus japonicus* (Harada, 1930), is an euryxenous parasite (Kvach et al., 2022) that exhibits high fecundity and infestation rates (Kim & Choi, 2003). Only ovigerous females are parasitic, whereas the copepodites, males, and immature females are free-living. Non-ovigerous females can detach and reattach to hosts (Pónyi & Molnar, 1969), potentially facilitating the spread and translocation between hosts and regions (Soylu & Soylu, 2012). Due to its low host specificity, *N. japonicus* can parasitise both exotic and native fish species (Nagasawa & Obe, 2015), with reports on centrarchids, cichlids, cyprinids, ictalurids, and percids across various regions of the world (Mugridge et al., 1982; Hayden & Rogers, 1998; Hudson & Bowen, 2002) (Table 6.1). Most recently, *N. japonicus* was reported parasitising Chinese sleepers *Perccottus glenii* Dybowski, 1877 (Odontobutidae) in Latvia (Kvach et al., 2020) and in the Lviv region of Ukraine (Kvach et al., 2022).

Neoergasilus japonicus, is the most prevalent and widespread species in its genus. Hanek (1968) reported *N. japonicus* from Europe just over a decade after Yin's (1956) description, and reports since then have shown that this parasite is spreading through several European countries (see Table 6.1). Just over 20 years after its first mention in Europe, *N. japonicus* was reported from fishes in Cuba, Central America, by Prieto et al. (1985) and it also quickly dispersed across different states in North and Central America (see Suárez-Morales & Mercado-Salas, 2013; Marshall et al., 2019; and the references therein). Three decades after Prieto et al.'s (1985) report, *N. japonicus* was reported from Algeria in Africa (Berrouk et al., 2018), which is the first published record from Africa. A year later, it was recorded from Peru, South America, by Mendes Marques and Murrieta Morey (2019), the only published report of this parasite currently available from the southern hemisphere. To the best of our knowledge, there has to date been no report of this ergasilid species in Australia and Antarctica, but it is reported as an invasive species from the other five continents (Table 6.1). Although the first published record of *N. japonicus* from Africa was in 2018 (Berrouk et al., 2018), an unpublished report (conference abstract) from Smit et al. (2018) suggests that this parasite was found in South Africa prior to 2017. In a dissertation by Mokonyane (2020), a single *N. japonicus*

specimen was reported to have parasitised the common carp, *Cyprinus carpio* Linnaeus, 1758 from a survey of the Kwena Dam (Mpumalanga, South Africa) in 2016.

The introduction of *N. japonicus* into different parts of the world is presumed to be through aquaculture and transportation of infested fishes via the aquarium trade (Hudson & Bowen, 2002) and sports fishing within continents (Alfonso & Belmonte, 2010). In Europe and North America, *N. japonicus* was reported to have been introduced through commercial fish introductions from Asia (Pónyi & Molnár, 1969; Hudson & Bowen, 2002). Suárez-Morales et al. (2010) suggested that aquaculture-related activities are the points of introduction of this parasite into southeast Mexico. Mendes Marques and Murrieta Morey (2019) further mentioned that the introduction of *N. japonicus* into Peru could most likely be linked to tilapia aquaculture practices, as well as possible host switching within these cultures.

Despite its global distribution and importance as an invasive parasite, molecular information on *N. japonicus* is extremely limited. At this time of writing, only 15 GenBank submissions for sequences of *N. japonicus* are available, with five each for fragments of the ribosomal RNA gene regions (18S and 28S), and mitochondrial DNA gene region (COI) respectively. Of the 10 partial ribosomal RNA sequences available, eight are from the Czech Republic (four each for 18S and 28S) (Ondračková et al., 2019; Kvach et al., 2021), and the remaining two are unpublished 18S and 28S sequences from South Korea. For the COI gene, there is only a single published sequence from South Korea (Baek et al., 2016) and four other submissions from the USA, published as supplementary sequence data (Vasquez et al., 2021). Additionally, three other *Neoergasilus* sp. GenBank submissions from the USA are available for the partial COI gene (Vasquez et al., 2021), and a single sequence from the USA is also available in the Barcode of Life Database (BOLD, 2023).

Across different regions and host species, some authors have reported slight morphological variations among *N. japonicus* specimens (Hayden & Rogers, 1998; Alfonso & Belmonte, 2010). This emphasises the importance of distribution reports being accompanied by morphological and molecular data, especially with the recent advances in integrative studies. The main aims of this section of the study are to utilise an integrative approach, linking distribution records of *N. japonicus* from southern Africa with morphological and molecular data, using partial 18S, 28S, and COI genes regions as molecular markers, and providing new host and locality records for this species.

Table 6.1: An updated global distribution checklist for published records of *Neoergasilus japonicus* (Harada, 1930) with the most recent host names and families. Data from the present study are presented in bold.

Continent	Country	Locality	Host	Host Family	Reference
Africa	Algeria	Beni Haroun Dam	*Carassius carassius* (Linnaeus, 1758); *Cyprinus carpio* Linnaeus, 1758; *Luciobarbus callensis* (Valenciennes, 1842)	Cyprinidae	Berrouk et al. (2018)
	Algeria	Barrage de Oued Charef, Foum El Khanga reservoir, Souk Ahras	*Luciobarbus callensis* (Valenciennes, 1842)	Cyprinidae	Boucenna et al. (2018)
	Algeria	Beni Haroun Dam	*Cyprinus carpio* Linnaeus, 1758	Cyprinidae	Berrouk et al. (2020)
	South Africa	**Boskop Dam**	***Micropterus salmoides* (Lacepède, 1802)**	**Centrarchidae**	**Present study**
	South Africa	**Potchefstroom Dam**	***Micropterus salmoides* (Lacepède, 1802)**	**Centrarchidae**	**Present study**
	South Africa	**Olifants River, Kruger National Park**	***Hydrocynus vittatus* Castelnau, 1861**	**Alestidae**	**Present study**
Asia	China				Yin (1956)
	China	Xianyang	*Carassius auratus* (Linnaeus, 1758)	Cyprinidae	HongWei et al. (2010)
	India	Nursery ponds in Krishna district, Andhra Pradesh	*Labeo rohita* (Hamilton, 1822)	Cyprinidae	Kumari et al. (2009)
	Japan	Chizuka pond in Fukuyama City, Hiroshima Prefecture	Free-living copepods		Urawa et al. (1980)
	Japan	Hokkaido Island	*Carassius auratus* (Linnaeus, 1758)	Cyprinidae	Nagasawa et al. (1989)
	Japan	Nagasaki Prefecture	*Lepomis macrochirus* Rafinesque, 1819	Centrarchidae	Yoshikoshi & Kô (1991)
	Japan	Ashida River system, Hiroshima Prefecture	*Lepomis macrochirus* Rafinesque, 1819; *Micropterus salmoides* (Lacepède, 1802)	Centrarchidae	Nagasawa & Inoue (2012)
	Japan	Komosawa Pond, Asaricho, Gotsu, Shimane	*Lepomis macrochirus* Rafinesque, 1819	Centrarchidae	Nagasawa & Nitta (2013)
	Japan	Kagamiyama, Higashihiroshima, Hiroshima	*Lepomis macrochirus* Rafinesque, 1820	Centrarchidae	Nagasawa & Obe (2013)
	Japan	Takayacho Kodani, Higashihiroshima, Hiroshima	*Nipponocypris sieboldii* (Temminck & Schlegel, 1846)	Xenocyprididae	Nagasawa et al. (2014)

Continent	Country	Locality	Host	Host Family	Reference
	Japan	Yoshiimachi Higashiya, Takasaki	*Lepomis macrochirus* Rafinesque, 1819; *Micropterus salmoides* (Lacepède, 1802)	Centrarchidae	Nagasawa & Sato (2015)
	Japan	Haebaru Reservoir, Okinawa-jima Island, Okinawa Prefecture	*Coptodon zillii* (Gervais, 1848)	Cichlidae	Nagasawa & Uyeno (2015)
	Japan	Harinoki Pond, Tomino, Shinano, Kamiminochi District	*Micropterus dolomieu* Lacepède, 1802	Centrarchidae	Nagasawa & Obe (2015)
	Japan	Lake Jō-numa, Tatebayashi, Gunma Prefecture	*Cyprinus carpio* Linnaeus, 1758; *Gnathopogon elongatus* (Temminck & Schlegel, 1846); *Lepomis macrochirus* Rafinesque, 1819	Centrarchida; Cyprinidae; Gobionidae	Nagasawa & Sato (2016)
	Taiwan	Jitsugetsutan Lake, Yuchi Township, Nantou County	*Hemiculter leucisculus* (Basilewsky, 1855) [recorded as *Cultriculus kneri*]; and *Pseudorasbora parva* (Temminck & Schlegel, 1846)	Cyprinidae	Harada (1930)
Europe	Britain	Carterslodge Pond and Furnace Pond on Slaugham Common, Haywards Heath	*Abramis brama* (Linnaeus, 1758); *Cyprinus carpio* Linnaeus, 1758; *Perca fluviatilis* Linnaeus, 1758; *Rutilus rutilus* (Linnaeus, 1758); *Scardinius erythrophthalmus* (Linnaeus, 1758); *Tinca tinca* (Linnaeus, 1758)	Cyprinidae; Leuciscidae; Percidae; Tincidae	Mugridge et al. (1982)
	Czech Republic	Danube, Main and Rhine rivers; Hvězda and Rohlik, Danube basin	*Lepomis gibbosus* (Linnaeus, 1758)	Centrarchidae	Ondračková et al. (2019, 2021)
	Czech Republic	River Dyje floodplain, South Moravia	*Rutilus rutilus* (Linnaeus, 1758)	Leuciscidae	Kvach et al. (2016)
	Czech Republic	Morava River basin (Babice 2 & U Jezu)	*Lepomis gibbosus* (Linnaeus, 1758); *Rutilus rutilus* (Linnaeus, 1758); *Scardinius erythrophthalmus* (Linnaeus, 1758)	Centrarchidae; Leuciscidae	Kvach et al. (2021)
	England	Shrubbery Lake	*Leucaspius delineatus* (Heckel, 1843)	Leuciscidae	Beyer et al. (2005)
	Finland	Lakes Leppävesi, Peurunka, Saravesi and Vatia	*Rutilus rutilus* (Linnaeus, 1758)	Leuciscidae	Tuuha et al. (1992)
	Finland	Lake Jyväsjärvi	*Rutilus rutilus* (Linnaeus, 1758)	Leuciscidae	Vainikka et al. (2009)

Continent	Country	Locality	Host	Host Family	Reference
	France	Lac de Créteil	*Perca fluviatilis* Linnaeus, 1758; *Rutilus rutilus* (Linnaeus, 1758); *Sander lucioperca* (Linnaeus, 1758); *Scardinius erythrophthalmus* (Linnaeus, 1758)	Leuciscidae; Percidae	Lescher-Moutoué (1979)
	France	Lake Grand Laoucien	Free-living copepods		Alekseev et al. (2021)
	Germany	Großer Döllnsee Lake (Großdöllner See)	*Rutilus rutilus* (Linnaeus, 1758)	Leuciscidae	Knopf & Hölker (2005)
	Hungary	Tisa River, tributaries of the Tisa, Danube River, Dinnyés fish ponds	*Alburnus alburnus* (Linnaeus, 1758); *Carassius carassius* (Linnaeus, 1758); *Lepomis gibbosus* (Linnaeus, 1758); *Leuciscus idus* (Linnaeus, 1758); *Rutilus rutilus* (Linnaeus, 1758)	Centrarchidae; Cyprinidae; Leuciscidae	Pónyi & Molnár (1969)
	Iran	Fish stores in Kerman	*Poecilia sphenops* Valenciennes, 1846	Poeciliidae	Mirzaei et al. (2016)
	Italy	Agri, Basento, Bradano, Crati, Neto, Ofanto, Passante, Sinni rivers	Free-living copepods		Alfonso & Belmonte (2010)
	Latvia	University Pond in Daugavpils	*Perccottus glenii* Dybowski, 1878	Odontobutidae	Kvach et al. (2020)
	Russia	Lake Glubokoye	*Gymnocephalus cernua* (Linnaeus, 1758); *Scardinius erythrophthalmus* (Linnaeus, 1758)	Leuciscidae; Percidae	Sokolov et al. (2017)
	Slovakia	Latorica near Kráľovský Chlmec, Trebišov District	*Sander lucioperca* (Linnaeus, 1758)	Percidae	Hanek (1968)
	Turkey	Lake Sapanca, northeast Marmara region	*Scardinius erythrophthalmus* (Linnaeus, 1758)	Leuciscidae	Soylu & Soylu (2012); Kuş & Soylu (2013); Çanayaz et al. (2018)
	Ukraine	Lake Inzhenerne	*Perccottus glenii* Dybowski, 1880	Odontobutidae	Kvach et al. (2022)
	Ukraine	Lower Dnieper irrigation canal	*Lepomis gibbosus* (Linnaeus, 1758)	Centrarchidae	Kvach et al. (2023)
	Wales	Private fishery	*Abramis brama* (Linnaeus, 1758); *Rutilus rutilus* (Linnaeus, 1758); *Scardinius erythrophthalmus* (Linnaeus, 1758); and hybrid bream	Centrarchidae; Leuciscidae	Elsheikha & Beech (2017)
North America	Cuba				Prieto et al. (1985); Prieto (1991)
	Mexico	Presa Los Arquitos, Aguascalientes	Free-living copepods		Suárez-Morales & Mercado-Salas (2013)

Continent	Country	Locality	Host	Host Family	Reference
	Mexico	Laguna de Cajititlán (Cajititlán Lagoon)	*Chapalichthys encaustus* (Jordan & Snyder, 1899); *Gambusia affinis* (Baird & Girard, 1853); *Goodea atripinnis* Jordan, 1880	Goodeidae; Poeciliidae	Velazquez-Ornelas et al. (2021); Velázquez-Ornelas & Suarez-Morales (2022)
	Mexico	Angostura Dam, Columbus Lakes and Río Lagartero (Chiapas state)	*Chiapaheros grammodes* (Taylor & Miller, 1980); *Vieja breidohri* (Werner & Stawikowski, 1987); *Vieja hartwegi* (Taylor & Miller, 1980)	Cichlidae	Suárez-Morales et al. (2010); Paredes-Trujillo et al. (2020)
	USA	Earthen ponds, Auburn University, Alabama	*Ictalurus furcatus* (Valenciennes, 1840)	Ictaluridae	Truong & Bullard (2021)
	USA	Auburn University Fisheries Research Unit (Alabama Agricultural Experiment Station), Alabama	*Ictalurus punctatus* (Rafinesque, 1818); *Lepomis macrochirus* Rafinesque, 1819; *Lepomis microlophus* (Günther, 1859); *Micropterus salmoides* (Lacepède, 1802)	Centrarchidae; Ictaluridae	Hayden & Rogers (1998)
	USA	Lake Huron, Michigan	*Ambloplites rupestris* (Rafinesque, 1817); *Carassius auratus* (Linnaeus, 1758); *Cyprinus carpio* Linnaeus, 1758; *Ictalurus punctatus* (Rafinesque, 1818); *Lepomis cyanellus* Rafinesque, 1819; *Lepomis gibbosus* (Linnaeus, 1758); *Lepomis macrochirus* Rafinesque, 1819; *Micropterus dolomieu* Lacepède, 1802; *Micropterus salmoides* (Lacepède, 1802); *Perca flavescens* (Mitchill, 1814); *Pimephales promelas* Rafinesque, 1820	Centrarchidae; Cyprinidae; Ictaluridae; Leuciscidae; Percidae	Hudson & Bowen (2002)
	USA	Oneida Lake, New York	*Catostomus commersonii* (Lacepède, 1803); *Lepomis cyanellus* Rafinesque, 1819; *Lepomis macrochirus* Rafinesque, 1819	Catostomidae, Centrarchidae	Marshall et al. (2019)
	USA	Missouri River	Free-living copepods		Dickerson et al. (2010)
South America	Peru	Fish pond located in the facilities of the Instituto de Investigaciones de la Amazonía Peruana (IIAP), Tarapoto	*Colossoma macropomum* (Cuvier, 1816)	Serrasalmidae	Mendes Marques & Murrieta Morey (2019)

6.3. Materials and methods

6.3.1. Sampling

A total of 23 fishes belonging to two species were caught between 2019 and 2022. Ten largemouth bass *Micropterus salmoides* (Lacepède, 1802), an invasive fish species in South Africa, were sampled from Boskop Dam (26°32'41.1"S; 27°07'10.3"E) and eight from Potchefstroom Dam (26°40'02.8"S; 27°05'46.9"E) in the Mooi River, a tributary of the Vaal River (Fig. 6.1). Five African tigerfish *Hydrocynus vittatus* Castelnau, 1861, a native species, were caught from the Olifants River (24°03'27.6"S; 31°35'59.5"E) in the Kruger National Park, South Africa. Fish sampling was done using seine nets and rod and reel techniques. Fishes were humanely killed and dissected (ethical clearance: NWU-00600-19-A5) and the gills, fins, and body surface were examined for parasites immediately with the aid of Zeiss Stemi 305 compact stereomicroscope. Observed parasitic crustaceans were removed using fine paint brushes, needles, and tweezers and preserved in 70% ethanol for further morphological and molecular analyses.

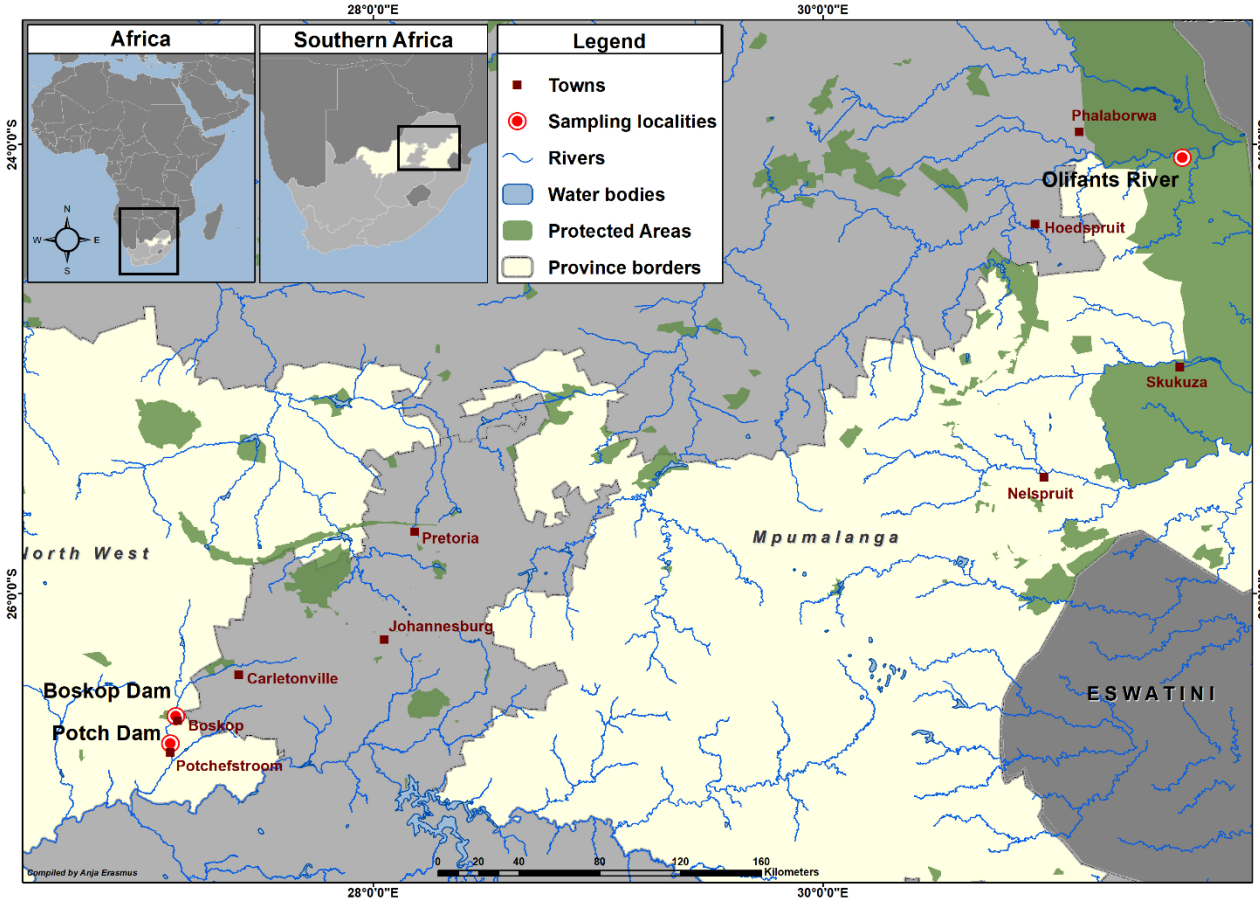


Figure 6.1: Map of sampling localities for *Neoergasilus japonicus* (Harada, 1930) in South Africa. Sample sites are indicated with red circles.

Necessary permits were received for this study from SANPARKS scientific services; the Department of Rural, Environmental and Agricultural Development (NW 8065/03/2019) and the Department of Economic Development, Tourism and Environmental Affairs (01/30201). Host nomenclature is from FishBase (Froese & Pauly, 2023) (www.fishbase.org), and parasites and host authorities are not included in the reference list.

6.3.2. Morphological analysis

For morphological analyses, selected *N. japonicus* specimens were cleared in lactic acid for 30 minutes and stained in lignin pink for 20–30 minutes. Appendages were carefully dissected off using very fine needles with the aid of a Zeiss Stemi 508 stereomicroscope. Photomicrographs were taken using a ZEISS Axiocam ERc 55 camera attached to the Zeiss stereomicroscope. Measurements of whole organisms were made using the Labscope Mat Ver. 2.8.4 software of the stereomicroscope and expressed in millimetres as mean ± standard deviation (with range in parentheses). For a more detailed morphological analysis, dissected appendages were studied in temporary glycerine slide mounts with the aid of a Nikon Eclipse *Ni* microscope (Nikon Instruments, Tokyo, Japan), in both bright-field and phase-contrast settings. The z-dimensional stacking (Z-stack) function was applied for differential interference contrast (DIC) photomicrographs of morphological structures taken with the aid of the Nikon DS-Fi1 camera mounted on the compound microscope. Photomicrographs of these appendages were taken using the NIS-Elements BR Ver. 4.60 software. Specimens for scanning electron microscopy (SEM) were dehydrated and dried in a graded ethanol and hexamethyldisilazane (HMDS) series, respectively. Dried specimens were mounted on carbon tape stubs, sputter-coated with gold/palladium (SPI-module, SPI Supplies, Pennsylvania, USA), and viewed with a JEOL Winsem JSM IT200. Specimen identification was confirmed based on the morphological descriptions provided by Hayden and Rogers (1998), Kim and Choi (2003), Alfonso and Belmonte (2010), and Marshall et al. (2019). Voucher material will be deposited in the National Museum Bloemfontein (NMB) and in the parasite collection of the Water Research Group, North-West University (NWU), Potchefstroom, South Africa.

6.3.3. Infestation rates

Quantitative descriptors of parasite infestation included: prevalence (P), mean intensity (MI), and mean abundance (MA), calculated according to Bush et al. (1997).

6.3.4. Molecular analysis

An egg string was removed for molecular analyses from a single specimen collected from Boskop Dam and Olifants River, respectively. For DNA extractions, the manufacturer's protocol for the PCRBIO Rapid Extract PCR Kit (PCRBiosystems, Analytical Solutions,

Randburg, South Africa) was adapted as follows: 20 µl of lysis buffer, 10 µl of buffer containing protease, and 150 µl molecular grade water for dilution. A whole adult specimen without eggs, from the Olifants River, was also used for DNA extraction following the Macherey-Nagel tissue kit (GmbH & Co. KG, Sandton, South Africa) manufacturer's protocol.

Genetic markers for this study included two partial ribosomal RNA genes; 18S and 28S, and one partial mitochondrial DNA gene, cytochrome *c* oxidase 1 (COI). Primers used for 18S (18SF and 18SR) and 28S (28SF and 28SR) partial gene amplification were those designed by Song et al. (2008). Two sets of primers were used for partial COI gene amplification: the universal mitochondrial DNA primers: LCO1490 and HCO2198 (Folmer et al., 1994); and cop-COX1+20 (Chang & Min, 2005) and HCO2612 (Machida et al., 2004) (see Table 6.2).

Polymerase Chain Reaction (PCR) mixtures for all DNA amplifications contained 3 µl DNA, 1.25 µl each for forward and reverse primers, 12.5 µl DreamTaq polymerase, and 7 µl of molecular grade water, making a total volume of 25 µl. Thermocycler conditions for 18S and 28S rRNA amplification followed Song et al. (2008), and COI followed Hayes et al. (2021). One percent agarose gel electrophoresis was carried out to visualise PCR products. Positive PCR products were sent to a commercial sequencing company, Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa, for purification and sequencing in both directions. Obtained sequences were viewed, edited, and assembled using Geneious Prime v. 2022.2.2. Consensus sequences generated were queried for similarities with other GenBank sequences using the Basic Local Alignment Search Tool (BLAST). All *Neoergasilus* sequences (published and unpublished) were downloaded from GenBank, however, only sequences associated with peer-reviewed publications were selected as representatives of the other genera in the family, Ergasilidae. *Lernaea cyprinacea* Linnaeus, 1758 (Lernaeidae) was used as the outgroup (Table 6.3). The Barcode of Life Database (BOLD, 2023) was utilised for COI submissions that may not be available on GenBank (Table 6.3).

Using Geneious Prime ver. 2022.2.2, all sequences were aligned with default parameters from MAFFT v7.490 (Katoh et al., 2002; Katoh & Standley, 2013) and trimmed; genetic divergence tables for alignments showing base pair differences and percentage identity of nucleotides were calculated. Alignments were exported as *Nexus* and *Phylip* files for Bayesian Inference (BI) and Maximum likelihood (ML) analyses, respectively.

Computation of likelihood scores and estimation of best fitting nucleotide substitution models for phylogenetic analyses used Akaike Information Criteria (AIC) implemented in jModel test 2.1.3 (Posada, 2008; Darriba et al., 2012). The model suggested for 18S, 28S, and COI was a general time-reversible model incorporating invariant sites and gamma distributed among-site rate variations (GTR + I + G).

Table 6.2: List of primers used for DNA amplification of South African *Neoergasilus japonicus* (Harada, 1930), with references. Codes: P36R and P46K are specimens from the Olifants River (OR), Kruger National Park; P43R is the specimen from Boskop Dam (BD), Mooi River.

Gene regions	Specimen codes	Localities	Primers	Sequences	Sources
18S	P36R P43R P46K	OR BD OR	18SF	5′-AAG GTG TGM CCT ATC AAC T-3′	Song et al. (2008)
			18SR	5′-TTA CTT CCT CTA AAC GCT C-3′	
28S	P36R* P43R* P46K*	OR BD OR	28SF	5′-ACA ACT GTG ATG CCC TTA G-3′	
			28SR	5′-TGG TCC GTG TTT CAA GAC G-3′	
COI	P43R**	BD	LCO1490	5′-GGTCAACAAATCATAAAGATATT GG-3′	Folmer et al. (1994)
			HCO2198	5′-TAAACTTCAGGGTGACCAAAAAA TCA-3′	
	P46K***	OR	cop-COX1+20	5′-GAC TAA TCA TAA AGA TAT TGG TAC-3′	Chang & Min (2005)
			HCO2612	5′-AGG CCT AGG TGT ATW GGG AAA-3′	Machida et al. (2004)

* to indicate a 28S reaction using 28SF & 28SR primers;
** to indicate a COI reaction using LCO1490 & HCO2198 primers;
*** to indicate a reaction using cop-COX1+20 & HCO2612 primers.

Table 6.3: List of sequences included in the phylogenetic analyses. The taxa in bold are newly generated sequences, all other sequences are from the GenBank and Barcode of Life Database (BOLD) databases. *Lernaea cyprinacea* Linnaeus, 1758 (in grey shade) was used as the outgroup.

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Acusicola margulisae	*Amphilophus citrinellus*, *Oreochromis* sp., *Parachromis managuensis*, *Poecilia exicana*	Nicaragua	MN852694	MN852851	MN854870	Santacruz et al. (2020)
Ergasilus anchoratus	*Pseudobagrus fulvidraco*	China	DQ107564	DQ107528	-	Song et al. (2008)
Ergasilus branini	*Misgurnus anguillicaudatus*	China	DQ107572	DQ107532	-	Song et al. (2008)
Ergasilus hypomesi	*Acanthogobius hasta*	China	DQ107573	DQ107539	-	Song et al. (2008)
Ergasilus peregrinus	*Siniperca chuatsi*	China	DQ107577	DQ107531	-	Song et al. (2008)
Ergasilus scalaris	*Tachysurus dumerili*	China	DQ107565	DQ107538	-	Song et al. (2008)
Ergasilus sieboldi	*Perca fluviatilis*	Czech Republic	MW810238	MW810242	-	Kvach et al. (2021)
Ergasilus sieboldi	*Sparus aurata*	Egypt	-	OM812074	-	Abdel-Radi et al. (2022)
Ergasilus sp.	Free-living	South Korea	-	-	KR049035	Baek et al. (2016)
Ergasilus sp.	*Mugil liza*	Argentina	-	-	KU557411	Castro-Romero et al. (2016)
Ergasilus timidus	*Acanthorhodeus taenianalis*	China	DQ107569	DQ107535	-	Song et al. (2008)
Ergasilus wilsoni	Free-living	South Korea	-	-	KR049036	Baek et al. (2016)
Ergasilus yaluzangbus	*Gymnocypris stewartii*	China	DQ107578	DQ107540	-	Song et al. (2008)
****Ergasilus yandemontei*	*Odontesthes hatcheri*	Argentina	MT969345	-	-	Waicheim et al. (2021)
Neoergasilus japonicus	***Hydrocynus vittatus***	**Olifants River, South Africa**	**P36R**	**P36Ra**	-	**Present study**
Neoergasilus japonicus	***Hydrocynus vittatus***	**Olifants River, South Africa**	**P46K**	**P46Ka**	**P46Kg**	**Present study**

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Neoergasilus japonicus	***Micropterus salmoides***	**Boskop Dam, Vaal River, South Africa**	**P43R**	**P43Ra**	**P43Rb**	**Present study**
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MH167969	MH167967	-	Ondračková et al. (2019)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MH167970	MH167968	-	Ondračková et al. (2019)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MW810236	MW810240	-	Kvach et al. (2021)
Neoergasilus japonicus	*Lepomis gibbosus, Scardinius erythrophthalmus*	Czech Republic	MW810237	MW810241	-	Kvach et al. (2021)
***Neoergasilus japonicus*	Collected by plankton net	USA	-	-	MZ964933 – MZ964936	Vasquez et al. (2021)
Neoergasilus japonicus	Free-living	South Korea	-	-	KR049037	Baek et al. (2016)
**Neoergasilus japonicus*	*Notropis heterolepis*	USA	-	-	ZOOPS113-18	BOLD (2023)
Paraergasilus brevidigitus	*Cyprinus carpio*	China	DQ107576	DQ107530	-	Song et al. (2008)
Paraergasilus longidigitus	*Abramis brama, Perca fluviatilis, Scardinius erythrophthalmu*	Czech Republic	MW810239	MW810243	-	Kvach et al. (2021)
Paraergasilus medius	*Ctenopharyngodon idellus*	China	DQ107574	DQ107529	-	Song et al. (2008)
Pseudergasilus parasiluri	*Tachysurus fulvidraco*	China	DQ107567	DQ107536	-	Song et al. (2008)
Sinergasilus major	*Ctenopharyngodon idella*	China	DQ107560	DQ107524	-	Song et al. (2008)
Sinergasilus major	*Silurus glanis*	Hungary	MZ047814	MZ047815	-	Dos Santos et al. (2021)
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	China	DQ107563	DQ107525	-	Song et al. (2008)
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	China	-	-	KR263117	Feng et al. (2016)
Sinergasilus undulatus	*Cyprinus carpio*	China	DQ107561	DQ107526	-	Song et al. (2008)
Sinergasilus undulatus	*Cyprinus carpio*	China	-	-	MW080644	Hua et al. (2021)

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Lernaea cyprinacea	*Carassius auratus, Chanodichthys ilishaeformis, Cyprinus carpio,*	China	MH982195	MH982204	MH982220	Hua et al. (2019)

* taxon from the Barcode of Life Database (BOLD);
** only able to access abstract and sequences that were published as supplementary data;
*** *Ergasilus yandemontei* (Published on GenBank as *Ergasilus* sp.).

Analyses for BI were performed on the computational resource, CIPRES Science Gateway v 3.3 (Miller et al., 2010). Bayesian Inference trees were generated with the aid of MrBayes v. 3.2.7a (Ronquist et al., 2012), running two independent Markov Chain Monte Carlo (MCMC) runs of four chains for 10^7 generations and sampling tree topologies every 10^3 generations. Burn-in parameters were set to the first 25000 generations. Maximum Likelihood (ML) trees were generated using PhyML v. 3.0 (Guindon et al., 2010), run on the ATGC bioinformatics platform (available from http://www.atgc-montpellier.fr/phyml/); with fixed substitution parameters keyed from jModel AIC console estimation, and bootstrap values of 1000 repetitions. Phylogenetic trees were visualised in FigTree v 1.4.4 (Rambaut, 2012) and combined tree topologies were represented using Adobe Illustrator CC 2022.

6.4. Results

6.4.1. Taxonomy

Order Cyclopoida Burmeister, 1834
Family Ergasilidae von Nordmann, 1832
Genus *Neoergasilus* Yin, 1956
(Figs. 6.2–6.4)

Type species. *Neoergasilus japonicus* (Harada, 1930).

Generic remarks.

Parasitic copepods collected during this study were identified as belonging to the genus *Neoergasilus* Yin, 1956. This identification was based on the following generic characteristics: the morphology of the first swimming leg is prolonged ventrally, covering the third thoracic segment, and reaching up to the fourth and fifth thoracic segment in some individuals (see Fig. 6.2a); the presence of the characteristic large spatulate spine extending from the second exopodite of the first leg, lying parallel to and extending past the third exopodite (Figs. 6.2e,

6.4a); furthermore, a triangular tooth is present between the exo- and endopod of the first leg projecting posteriorly from the basis of the first leg (Figs. 6.2e, 6.4a).

In addition to the above-mentioned generic characteristics, the genus *Neoergasilus* further differs from the other three Ergasilidae genera and species reported in Africa. Compared to the genus *Ergasilus* von Nordmann, 1832, *Neoergasilus* has short, greatly curved antennae with a prominent spine on the distal edge of the first antennal segment (Fig. 6.2c), which *Ergasilus* lacks. Additionally, the antennal claws of *Neoergasilus* do not end in three prongs like those of *Paraergasilus* Markewitsch, 1937 (Figs. 6.2b, 6.3b, d). Finally, the genus *Dermoergasilus* Ho & Do, 1982, has a cuticular sheath covering its antennae that is absent in *Neoergasilus* (Figs. 6.2b, 6.3b, d).

***Neoergasilus japonicus* (Harada, 1930)**

Type host(s). Hemiculter leucisculus (Basilewsky, 1855) [syn. *Culter leucisculus* Basilewsky, 1855; the original name given in Harada, 1930 was *Cultriculus kneri*] and *Pseudorasbora parva* (Temminck & Schlegel, 1846).

Type locality. Jitsugetsutan Lake (sun moon lake), Yuchi Township, Nantou County, Taiwan.

Host families (published globally). **Alestidae (present study),** Catostomidae, **Centrarchidae (present study)**, Cichlidae, Cyprinidae, Gobionidae, Goodeidae, Ictaluridae, Leuciscidae, Odontobutidae, Percidae, Poeciliidae, Serrasalmidae, Tincidae, Xenocyprididae (see Table 6.1).

Other hosts (unpublished from South Africa). Chetia flaviventris Trewavas, 1961, *Clarias gariepinus* (Burchell, 1822), *Coptodon rendalli* (Boulenger, 1897), *Cyprinus carpio* Linnaeus, 1758, *Hypophthalmichthys molitrix* (Valenciennes, 1844), *Labeo rosae* Steindachner, 1894, *Labeobarbus marequensis* (Smith, 1841), *Marcusenius macrolepidotus* (Peters, 1852) (no longer in South Africa), *Micralestes acutidens* (Peters, 1852), *Micropterus salmoides* (Lacepède, 1802), *Oreochromis mossambicus* (Peters, 1852), *Schilbe intermedius* Rüppell, 1832, *Synodontis zambezensis* Peters, 1852.

Unpublished BOLD host. Notropis heterolepis Eigenmann & Eigenmann, 1893.

Localities excluding South Africa. Africa [Algeria]; Europe [Czech Republic, England, Finland, France, Germany, Hungary, Italy, Latvia, Romania, Russia, Turkey (transcontinental), Ukraine, Wales]; Asia [China, Japan, Taiwan]; North America [Cuba, Mexico, USA]; South America [Peru] (see Table 6.1).

Localities in South Africa. **Boskop Dam and Potchefstroom Dam [Present study]** in the Mooi River, and the **Olifants River**, Kruger National Park **[Present study].** Unpublished reports from South Africa include: Crocodile River (Kwena Dam); Olifants

and Limpopo River System (Flag Boshielo Dam, Lapalala River, Limpopo River, Loskop Dam, Molepo Dam, Nandoni Dam, Nwanedi-Luphephe Dam, Phalaborwa Barrage, Stanford Lake, Tibani Dam), and the Vaal River (Vaal Dam).

Material examined.

South Africa. Eight adult females (two dissected, one egg string sequenced from a dissected specimen), from Boskop Dam (26°32'41.1"S; 27°07'10.3"E) and Potchefstroom Dam (26°40'02.8"S; 27°05'46.9"E), 2022, from fins of *Micropterus salmoides* (one from the gills); six adult females (one dissected, three used for SEM, one sequenced, and one egg string sequenced from the dissected specimen), from Olifants River (24°03'27.6"S; 31°35'59.5"E), 2021, from the gills of *Hydrocynus vittatus.*

Infestation rates. Total prevalence 34.8% (8/23), mean intensity 1.75 (14/8), mean abundance 0.61 (14/23) (see Table 6.4 for a detailed breakdown).

Description of adult female (Figs. 6.2–6.4).

Measurements (n=12) are given as: total length (from anterior of cephalosome to posterior of furcal rami, excluding furcal rami seta) 0.794 ± 0.056 (0.673–0.857) mm, cephalothorax length 0.459 ± 0.038 (0.402–0.513) mm, cephalothorax width 0.263 ± 0.019 (0.229–0.296) mm, urosome length 0.205 ± 0.014 (0.168–0.225) mm.

Body shape cyclopiform (Figs. 6.2a, 6.3a). Prosome comprising cephalosome fused with first pedigerous somite and three free pedigerous somites gradually reducing in size, bearing legs 2 to 4, each leg pair linked by an intercoxal plate. Urosome consisting of greatly reduced fifth pedigerous somite, non-pedigerous genital double-somite, and three free abdominal somites (Fig. 6.3e); distal margins of genital and abdominal somites ornamented with spinules. Furcal rami as long as second and third abdominal segments, armed with four setae of unequal lengths, median seta the longest.

Cephalosome elongate, eyespot present (Fig. 6.3b), ornamentation absent. Antennules (Figs. 6.2b, 6.3b) six-segmented with each segment bearing setation. Antennae (Figs. 6.2b, c, 6.3b, d) four-segmented, first segment the widest, with conspicuous spine and a sensory pore at distal margin (Fig. 6.2c); second segment twice as long as it is wide, bears single small spiniform seta with broad base and sensory pore at distal third of segment (Figs. 6.2c, 6.3d); third segment greatly curved; fourth segment the shortest, ending in a claw. Mandible possesses two denticulated blades (Fig. 6.3c); maxillule not segmented, possesses two setae (Fig. 6.2d); maxilla with broad proximal segment and elongate distal segment terminating in numerous teeth at its convex margin (Figs. 6.2d, 6.3c).

Both rami of legs 1–3 three-segmented (Figs. 6.4a–c). Leg 1 (Figs. 6.2e, 6.4a) reaching the fourth and fifth thoracic segment, possesses a triangular tooth on posterior

margin of basis between endopod and exopod; second exopodite with broadly rounded large spatulate spine, longer than third exopodite. Leg 2 (Fig. 6.4b) with single spine each on first and third exopodite; leg 3 (Fig. 6.4c) without spine on third exopodite; leg 4 (Fig. 6.2f) two-segmented, basiopodite bears a seta; leg 5 single segmented with three setae of unequal lengths (Fig. 6.3e). Armature formula of legs 1–4 given in Table 6.5.

Table 6.4: Table of infestation data of *Neoergasilus japonicus* (Harada, 1930) sampled from two hosts and three localities in South Africa. Abbreviations: KNP – Kruger National Park, MA – mean abundance, MI – mean intensity, MR – Mooi River, N(E) – number of hosts examined, N(I) – number of hosts Infested, OR – Olifants River, PC – number of parasites collected, P – prevalence, SOI – site of infestation, ZA – South Africa.

Sampling sites	Host	Family	SOI	N(E)	N(I)	PC	%P	MA	MI
Potchefstroom Dam, MR, ZA	*Micropterus salmoides*	Centrarchidae	Fins	8	1	1	12.5	0.13	1
Boskop Dam, MR, ZA	*Micropterus salmoides*	Centrarchidae	Fins; gills	10	5	7	50	0.7	1.4
Olifants River, OR, KNP, ZA	*Hydrocynus vittatus*	Alestidae	Gills	5	2	6	40	1.2	3

Table 6.5: Spine-setae formula for swimming legs of *Neoergasilus japonicus* (Harada, 1930) from South Africa. Number of spines in Roman numerals, number of setae in Arabic numerals. Spine with asterisks* represents the generic spatulate spine on the second exopodite of leg 1.

	Coxa	Basis	Exopod	Endopod
Leg 1	0-0	I-0	I-0; I*-1; II-5	0-1; 0-1; II-4
Leg 2	0-0	I-0	I-0; 0-1; I-6	0-1; 0-2; I-4
Leg 3	0-0	I-0	I-0; 0-1; I-6	0-1; 0-2; I-4
Leg 4	0-0	I-0	0-4	0-2

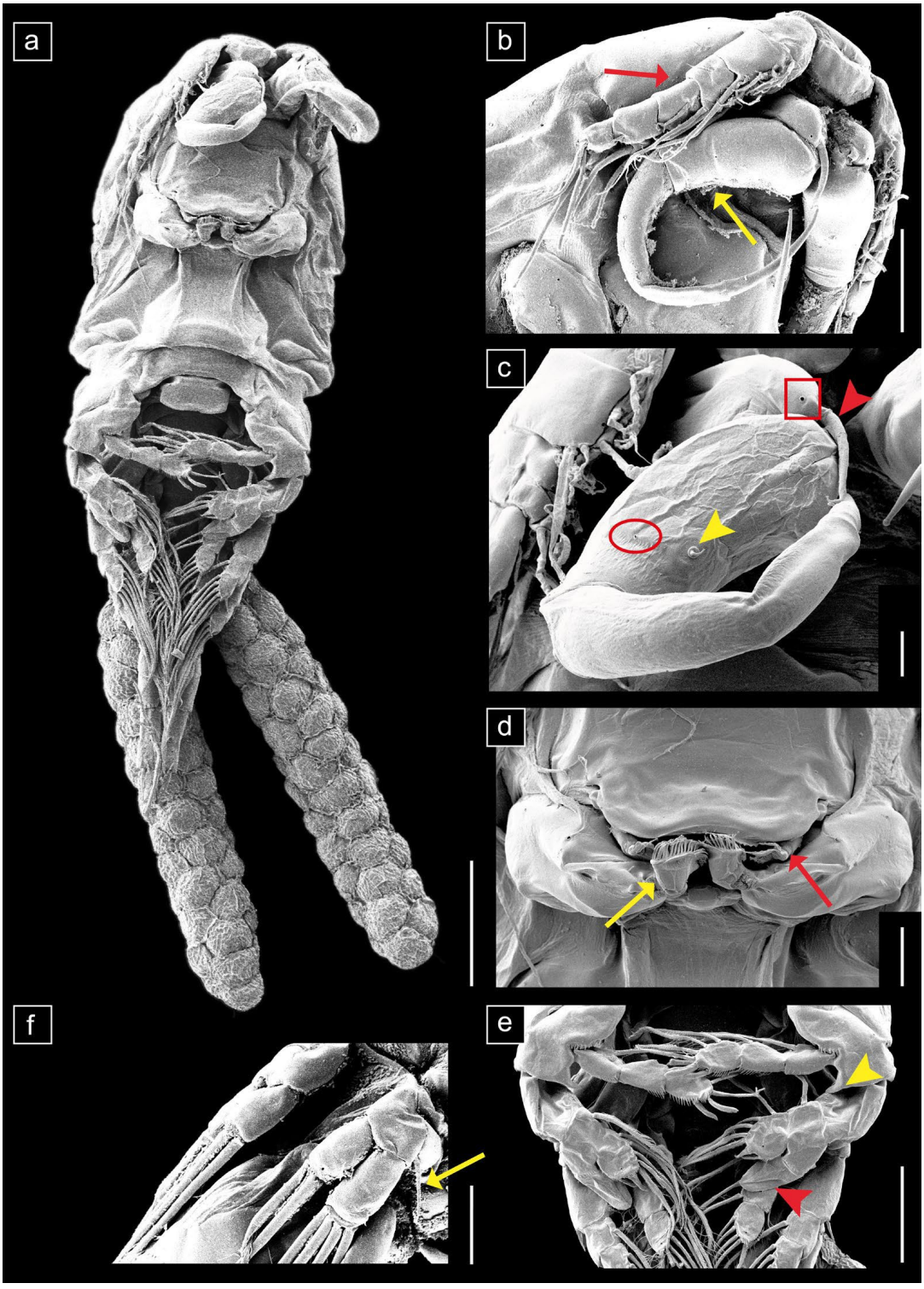


Figure 6.2: Scanning electron microscope images of *Neoergasilus japonicus* (Harada, 1930) from *Hydrocynus vittatus* from the Olifants River: (a) full ventral image of ovigerous female; (b) antennule (red arrow) and antenna (yellow arrow); (c) antennae showing conspicuous spine (red arrowhead) and sensory pore (red square) on first segment, single small seta (yellow arrowhead) and sensory pore (red circle) on second segment; (d) maxillule (red arrow), maxilla (yellow arrow); (e) leg 1 showing triangular tooth at distal end of basis (yellow arrowhead), and large spatulate spine of second exopodite (red arrowhead); (f) leg 4 showing seta on basal segment (yellow arrowhead). Scale bars: (a) 100 µm; (b, e) 50 µm; (c) 10 µm; (d, f) 20 µm.

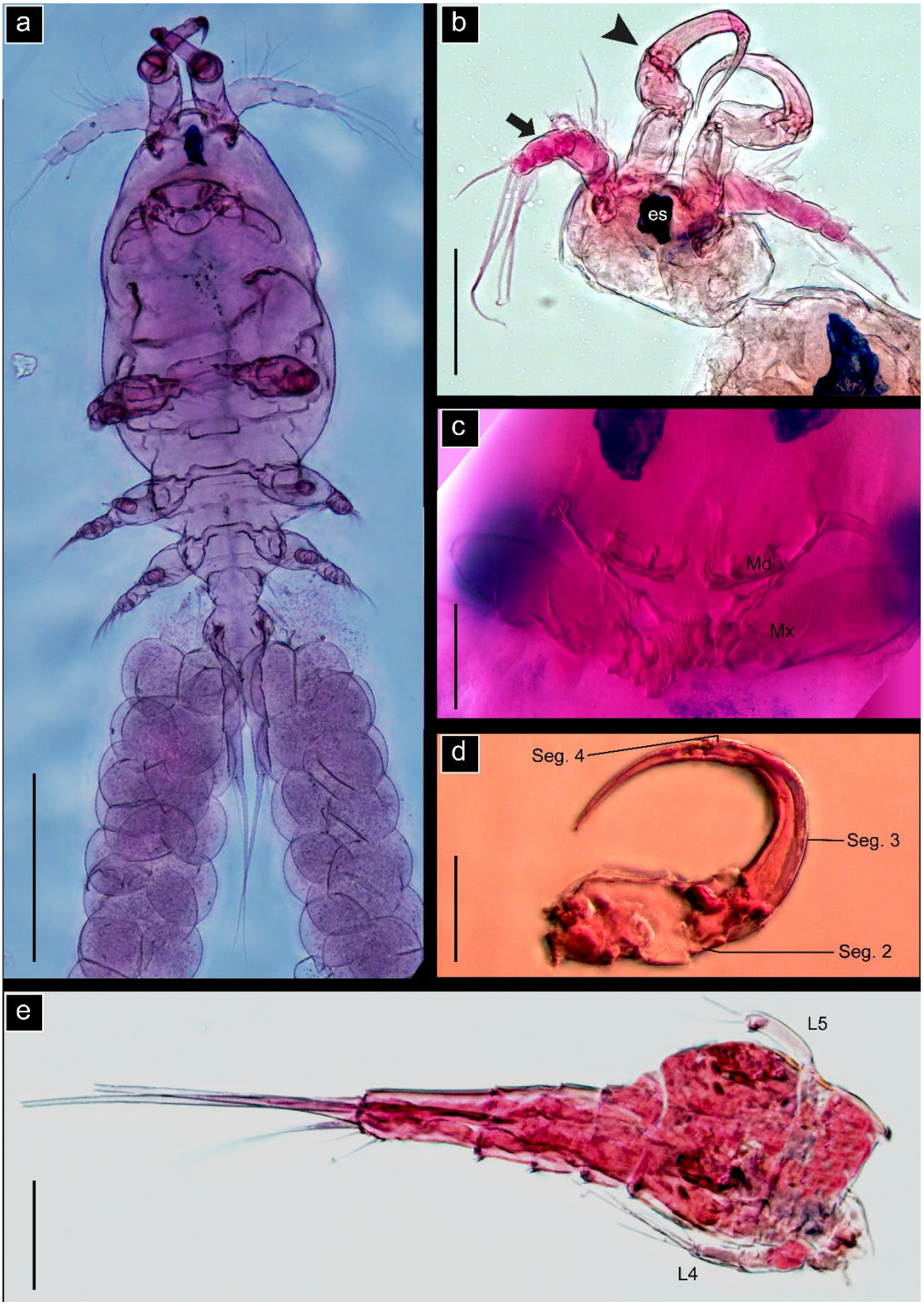


Figure 6.3: Light photomicrographs of *Neoergasilus japonicus* (Harada, 1930) specimens collected from *Micropterus salmoides* from the Mooi River and stained with lignin pink, applying the z-dimensional stacking function of the differential interference contrast microscope: (a) full image of female with egg strings attached, ventral; (b) head region showing eyespot (es), antennae (black arrowhead), and antennule (black arrow); (c) mouth part dissection highlighting mandible (md), and maxilla (mx); d) antenna showing segments (seg. 2–4); (e) urosome and furcal rami with part of 4th thoracic segment showing leg 5 (L5), and leg 4 (L4). Scale bars: (a) 200 µm; (b) 100 µm; (c–e) 50 µm.

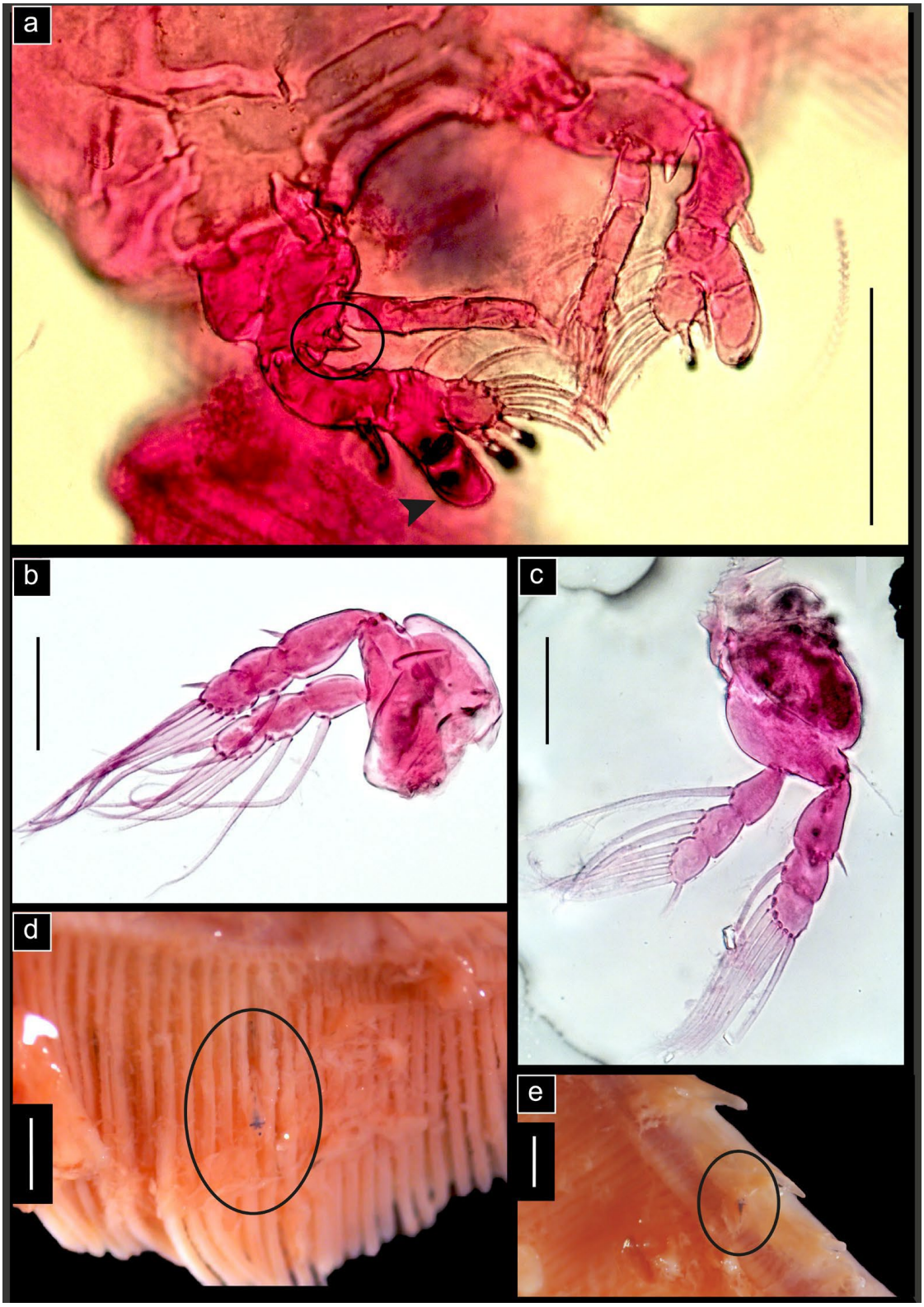


Figure 6.4: Light microscopy photomicrographs of legs 1–3 (a–c) from dissections of *Neoergasilus japonicus* (Harada, 1930) specimens stained with Lignin pink, and photomicrographs of *N. japonicus* on gills of *Hydrocynus vittatus* (d & e) from the Olifants River in the Kruger National Park, South Africa: (a) legs 1 with triangular tooth on distal margin of basis, between endopod and exopod highlighted (black circle), and large spatulate spine of second exopodite (black arrowhead); (b) leg 2; (c) leg 3; (d) *N. japonicus* on the gill filament (black circle); (e) *N. japonicus* on the gill arch (black circle). Scale bars: (a) 100 µm; (b–c) 50 µm; (d–e) 1 mm.

6.4.2. Molecular analysis

Alignments with newly generated sequences for partial 18S, 28S, and COI resulted in lengths of 1387 bp for 18S, 750 bp for 28S, and 1215 bp for COI. The newly generated sequences formed clades with other published and unpublished *N. japonicus* sequences available on GenBank and BOLD, further confirming its identity (Figs. 6.5–6.7).

Newly generated 18S sequences from Olifants River (1,301 bp and 1,351 bp) and Mooi River (1,361 bp) specimens, were 100% identical. Nucleotide substitutions were observed in the alignment of newly generated sequences and the Czech Republic and South Korea sequences (Table 6.6). A difference of 1–4 bp was observed when the newly generated sequences from this study were compared with other *N. japonicus* sequences in the alignment (Table 6.7). Phylogenetic trees for Bayesian Inference and Maximum Likelihood had similar tree topologies. All *N. japonicus* sequences formed a separate clade from the other Ergasilidae genera (Fig. 6.5). Newly generated partial 18S sequences clustered in a sub-clade from other *N. japonicus* sequences (Czech Republic and South Korea) used in the alignment.

From the alignment of partial 28S *N. japonicus*, newly generated sequences (692 bp and 693 bp for Olifants River specimens, and 686 bp for Boskop Dam specimens) were 100% identical. Novel sequences were also 100% identical with the Czech Republic Rohlik sequence (MH167968) and differed from other *N. japonicus* sequences in the alignment by 1–2 bases (Table 6.8). From the alignment, four positions of nucleotide substitution among *Neoergasilus* 28S sequences were observed from this study (Table 6.6). Tree topologies for 28S were similar for BI and ML analyses. All *N. japonicus* sequences claded separately from the other Ergasilidae sequences (Fig. 6.6). Newly generated sequences clustered in a sub-clade from the rest of the *N. japonicus* sequences used in the alignment.

Newly generated COI sequences from the Olifants River and Boskop Dam were 1178 bp and 698 bp long, respectively. The partial mitochondrial DNA (COI) alignment from this study showed the following variability: a 2 bp difference among newly generated sequences; a 1–4 bp difference between newly generated sequences and other *Neoergasilus* sequences from the United States of America (USA); and a 40 bp difference between newly generated sequences and the *N. japonicus* sequence from South Korea (see Table 6.9). Using an invertebrate mitochondrion translation, two types of mutations (missense and silent) were observed in the COI alignment from this study. A total of four missense mutations from five nucleotide substitutions observed in this COI alignment: three nucleotide substitutions from the South Korea sequence and two from a USA sequence (Table 6.10). Several silent mutations were observed in this study (Appendix Table A3), two of which were positions of nucleotide substitutions in the newly generated sequences (Table 6.10).

Table 6.6: Genetic divergences among aligned 18S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study and The Czech Republic). Sequences from the present study are presented in bold and grey shade, *Lernaea cyprinacea* (MH982195) was used as the outgroup. Abbreviations: AR – Argentina, BB – Babice, BD – Boskop Dam, CN – China, CZ – Czech Republic, HU – Hungary, HV – Hvezda, JZ – Jezu, KNP – Kruger National Park, KR – South Korea, NI – Nicaragua, OR – Olifants River, RH – Rohlik, VR – Vaal River, ZA – South Africa. *Given as *Ergasilus* sp. on GenBank.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	**P36R OR KNP *Neoergasilus japonicus* ZA**		**0**	**0**	**3**	**1**	**1**	**3**	**1**	**34**	**28**	**11**	**10**	**31**	**38**	**19**	**11**	**30**	**30**	**33**	**38**	**33**	**20**	**26**	**26**	**26**	**26**	**133**
2	**P43R BD VR *Neoergasilus japonicus* ZA**	**100**		**0**	**4**	**1**	**2**	**3**	**2**	**38**	**31**	**13**	**13**	**34**	**40**	**22**	**13**	**34**	**32**	**37**	**42**	**38**	**22**	**29**	**29**	**30**	**30**	**139**
3	**P46K2 OR KNP *Neoergasilus japonicus* ZA**	**100**	**100**		**3**	**1**	**1**	**3**	**1**	**35**	**29**	**11**	**11**	**32**	**39**	**20**	**11**	**31**	**31**	**35**	**40**	**35**	**21**	**27**	**27**	**28**	**28**	**136**
4	MW810237 *Neoergasilus japonicus* JZ CZ	**99.77**	**99.71**	**99.78**		2	2	0	2	36	32	14	14	35	41	23	14	35	31	36	41	37	21	28	28	29	29	139
5	MH167970 *Neoergasilus japonicus* RH CZ	**99.92**	**99.93**	**99.93**	99.85		0	2	0	35	29	11	11	32	39	20	11	31	31	35	40	35	21	27	27	28	28	136
6	MW810236 *Neoergasilus japonicus* BB CZ	**99.92**	**99.85**	**99.93**	99.85	100		2	0	37	30	12	12	33	39	21	12	33	31	36	41	37	21	28	28	29	29	139
7	MH167969 *Neoergasilus japonicus* HV CZ	**99.77**	**99.78**	**99.78**	100	99.85	99.85		2	34	31	13	13	34	41	22	13	33	31	35	40	35	21	27	27	28	28	136
8	KR048752 *Neoergasilus japonicus* KR	**99.92**	**99.85**	**99.93**	99.85	100	100	99.85		37	30	12	12	33	39	21	12	33	31	36	41	37	21	28	28	29	29	139
9	MN852694 *Acusicola margulisae* NI	**97.39**	**97.21**	**97.41**	97.35	97.42	97.28	97.49	97.28		38	42	35	44	49	33	42	44	33	42	46	43	45	35	35	35	35	143
10	DQ107564 *Ergasilus anchoratus* CN	**97.85**	**97.72**	**97.85**	97.64	97.86	97.79	97.71	97.8	97.21		31	24	39	28	24	31	37	33	33	34	32	33	9	9	12	13	140
11	DQ107572 *Ergasilus branini* CN	**99.15**	**99.04**	**99.19**	98.97	99.19	99.12	99.04	99.12	96.91	97.72		14	37	41	21	4	36	37	34	37	35	19	31	31	32	34	143
12	DQ107573 *Ergasilus branini* CN	**99.23**	**99.04**	**99.19**	98.97	99.19	99.12	99.04	99.12	97.43	98.24	98.97		31	35	19	14	34	31	32	35	33	19	23	23	24	26	141
13	DQ107577 *Ergasilus peregrinus* CN	**97.62**	**97.5**	**97.63**	97.42	97.64	97.57	97.49	97.58	96.77	97.13	97.28	97.72		50	33	36	39	43	36	39	37	41	39	39	38	38	148
14	DQ107565 *Ergasilus scalaris* CN	**97.08**	**97.06**	**97.11**	96.98	97.12	97.13	96.97	97.13	96.4	97.94	96.99	97.43	96.33		32	41	45	42	40	43	41	43	27	27	28	29	146
15	MW810238 *Ergasilus sieboldi* CZ	**98.54**	**98.38**	**98.52**	98.31	98.52	98.45	98.38	98.46	97.58	98.24	98.46	98.6	97.58	97.65		21	23	27	27	32	28	27	23	23	24	27	135
16	DQ107569 *Ergasilus timidus* CN	**99.15**	**99.04**	**99.19**	98.97	99.19	99.12	99.04	99.12	96.91	97.72	99.71	98.97	97.35	96.99	98.46		36	37	34	37	35	19	31	31	32	34	144
17	DQ107578 *Ergasilus yaluzangbus* CN	**97.7**	**97.5**	**97.71**	97.42	97.71	97.57	97.56	97.58	96.77	97.28	97.36	97.5	97.14	96.7	98.31	97.36		34	35	39	35	38	33	33	35	38	140
18	*MT969345 *Ergasilus yandemontei* AR	**97.71**	**97.65**	**97.71**	97.72	97.71	97.72	97.71	97.73	97.58	97.58	97.27	97.73	96.82	96.9	98.03	97.27	97.5		33	37	34	36	30	30	33	36	141
19	DQ107574 *Paraergasilus medius* CN	**97.46**	**97.28**	**97.41**	97.35	97.42	97.35	97.42	97.35	96.91	97.58	97.5	97.65	97.35	97.06	98.02	97.5	97.43	97.58		7	3	38	33	33	35	38	142
20	DQ107576 *Paraergasilus brevidigitus* CN	**97.08**	**96.91**	**97.04**	96.98	97.05	96.98	97.05	96.99	96.62	97.5	97.28	97.43	97.13	96.84	97.65	97.28	97.14	97.27	99.49		8	41	35	35	37	40	145
21	MW810239 *Paraergasilus longidigitus* CZ	**97.46**	**97.21**	**97.41**	97.28	97.42	97.28	97.42	97.28	96.84	97.65	97.43	97.58	97.28	96.99	97.94	97.43	97.43	97.5	99.78	99.41		39	34	34	36	39	142
22	DQ107567 *Pseudergasilus parasiluri* CN	**98.46**	**98.38**	**98.45**	98.45	98.45	98.45	98.45	98.46	96.69	97.58	98.6	98.6	96.99	96.84	98.02	98.6	97.21	97.35	97.21	96.99	97.13		33	33	34	36	151
23	MZ047814 *Sinergasilus major* HU	**98**	**97.87**	**98**	97.94	98.01	97.94	98.01	97.94	97.43	99.34	97.72	98.31	97.13	98.02	98.31	97.72	97.58	97.8	97.58	97.43	97.5	97.58		0	3	7	137
24	DQ107560 *Sinergasilus major* CN	**98**	**97.87**	**98**	97.94	98.01	97.94	98.01	97.94	97.43	99.34	97.72	98.31	97.14	98.02	98.31	97.72	97.58	97.8	97.58	97.43	97.5	97.58	100		3	7	137
25	DQ107563 *Sinergasilus polycolpus* CN	**98**	**97.8**	**97.93**	97.87	97.93	97.87	97.93	97.87	97.43	99.12	97.65	98.24	97.21	97.94	98.24	97.65	97.43	97.58	97.43	97.28	97.36	97.5	99.78	99.78		6	136
26	DQ107561 *Sinergasilus undulatus* CN	**98**	**97.8**	**97.93**	97.87	97.93	97.87	97.93	97.87	97.43	99.05	97.5	98.09	97.21	97.87	98.02	97.5	97.21	97.35	97.21	97.06	97.14	97.36	99.49	99.49	99.56		142
27	MH982195 *Lernaea cyprinacea* CN	**89.98**	**89.96**	**90.12**	89.95	90.14	89.95	90.14	89.96	89.68	89.89	89.68	89.82	89.31	89.46	90.25	89.6	89.89	89.63	89.75	89.53	89.75	89.1	90.1	90.11	90.18	89.75	

Table 6.7: Genetic divergences among aligned 28S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from the present study and The Czech Republic). Sequences from the present study are presented in bold and in grey shade, *Lernaea cyprinacea* (MH982204) was used as the outgroup. Abbreviations: BB – Babice, BD – Boskop Dam, CN – China, CZ – Czech Republic, HU – Hungary, HV – Hvezda, JZ – Jezu, KNP – Kruger National Park, KR – South Korea, NI – Nicaragua, OR – Olifants River, RH – Rohlik, VR – Vaal River, ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	**P36Ra OR KNP *Neoergasilus japonicus* ZA**		**0**	**0**	**0**	**1**	**1**	**1**	**3**	**63**	**82**	**48**	**44**	**60**	**40**	**51**	**44**	**80**	**57**	**59**	**57**	**41**	**80**	**94**	**83**	**81**	**216**
2	**P43Ra BD VR *Neoergasilus japonicus* ZA**	**100**		**0**	**0**	**1**	**1**	**1**	**3**	**63**	**82**	**48**	**44**	**60**	**40**	**51**	**44**	**80**	**57**	**59**	**57**	**41**	**80**	**94**	**83**	**81**	**216**
3	**P46Ka OR KNP *Neoergasilus japonicus* ZA**	**100**	**100**		**0**	**1**	**1**	**1**	**3**	**63**	**82**	**48**	**44**	**60**	**40**	**51**	**44**	**80**	**57**	**59**	**57**	**41**	**80**	**94**	**83**	**81**	**216**
4	MH167968 *Neoergasilus japonicus* RH CZ	**100**	**100**	**100**		1	1	1	0	63	81	46	42	59	37	51	42	76	55	59	55	39	80	92	81	79	213
5	MH167967 *Neoergasilus japonicus* HV CZ	**99.84**	**99.84**	**99.84**	99.84		0	2	1	62	80	45	41	58	36	50	41	75	54	58	54	38	79	91	80	78	212
6	MW810241 *Neoergasilus japonicus* JZ CZ	**99.85**	**99.85**	**99.85**	99.84	100		2	1	62	80	45	41	58	38	50	41	77	54	58	54	38	79	91	80	78	215
7	MW810240 *Neoergasilus japonicus* BB CZ	**99.92**	**99.92**	**99.92**	99.92	99.76	99.77		1	64	81	46	43	59	40	52	43	78	55	60	55	40	80	92	81	79	216
8	KR048823 *Neoergasilus japonicus* KR	**99.57**	**99.56**	**99.57**	100	99.84	99.85	99.92		63	83	47	43	61	41	51	43	79	56	59	56	40	80	93	82	80	216
9	MN852851 *Acusicola margulisae* NI	**90.13**	**90.13**	**90.13**	89.76	89.92	90.24	90	90.13		71	50	43	49	51	53	53	79	45	49	49	49	67	82	71	71	204
10	DQ107528 *Ergasilus anchoratus* CN	**88.13**	**88.03**	**88.13**	87.02	87.18	87.9	87.75	87.99	88.89		74	63	64	76	72	78	100	72	72	71	78	9	25	12	11	229
11	DQ107532 *Ergasilus branini* CN	**93.05**	**92.99**	**93.05**	92.63	92.79	93.19	93.04	93.2	92.18	89.31		32	43	23	42	19	77	47	51	55	18	71	87	74	75	214
12	DQ107539 *Ergasilus hypomesi* CN	**93.63**	**93.58**	**93.63**	93.27	93.43	93.8	93.57	93.78	93.27	90.9	95.38		41	35	39	35	68	47	45	49	32	55	75	62	62	214
13	DQ107531 *Ergasilus peregrinus* CN	**91.3**	**91.23**	**91.3**	90.53	90.69	91.21	91.14	91.16	92.32	90.74	93.78	94.07		46	45	46	66	43	46	42	45	59	80	66	69	209
14	DQ107538 *Ergasilus scalaris* CN	**94.2**	**94.15**	**94.2**	94.06	94.22	94.24	94.02	94.06	92.01	89	96.67	94.93	93.33		45	30	73	55	56	59	9	74	91	78	79	206
15	MW810242 *Ergasilus sieboldi* CZ	**92.07**	**92.07**	**92.07**	91.83	91.99	92.2	91.97	92.07	91.4	88.82	93.48	93.94	93	93		43	65	48	48	49	45	70	89	75	78	216
16	DQ107535 *Ergasilus timidus* CN	**93.63**	**93.58**	**93.63**	93.27	93.43	93.8	93.57	93.78	91.71	88.73	97.25	94.94	93.34	95.66	93.32		74	52	52	58	25	75	91	78	77	214
17	DQ107540 *Ergasilus yaluzangbus* CN	**88.51**	**88.41**	**88.51**	87.92	88.08	88.44	88.29	88.65	87.73	85.65	88.95	90.24	90.52	89.51	89.95	89.38		71	74	73	74	94	111	98	97	212
18	DQ107530 *Paraergasilus brevidigitus* CN	**91.74**	**91.67**	**91.74**	91.17	91.33	91.82	91.67	91.88	92.95	89.58	93.2	93.2	93.77	92.03	92.53	92.47	89.8		9	8	56	67	84	71	71	205
19	MW810243 *Paraergasilus longidigitus* CZ	**91.01**	**91.01**	**91.01**	90.53	90.69	91.16	90.93	91.01	92.27	89.04	92.24	93.15	92.99	91.46	92.48	92.09	88.82	98.62		15	58	66	83	70	70	205
20	DQ107529 *Paraergasilus medius* CN	**91.74**	**91.67**	**91.74**	91.17	91.33	91.82	91.67	91.88	92.32	89.73	92.04	92.91	93.91	91.45	92.38	91.61	89.51	98.83	97.7		60	64	83	70	72	207
21	DQ107536 *Pseudergasilus parasiluri* CN	**94.06**	**94.01**	**94.06**	93.74	93.9	94.24	94.02	94.2	92.32	88.71	97.4	95.37	93.48	98.7	93	96.38	89.37	91.88	91.16	91.3		75	91	78	79	207
22	MZ047815 *Sinergasilus major* HU	**87.75**	**87.75**	**87.75**	87.18	87.34	87.9	87.75	87.75	89.38	98.62	89.14	91.59	90.96	88.67	89.05	88.53	85.74	89.74	89.89	90.2	88.51		21	8	11	229
23	DQ107524 *Sinergasilus major* CN	**86.4**	**86.28**	**86.4**	85.26	85.42	86.23	86.08	86.54	87.17	96.37	87.43	89.16	88.42	86.83	86.18	86.85	84.07	87.84	87.37	87.99	86.83	96.77		19	22	240
24	DQ107525 *Sinergasilus polycolpus* CN	**87.99**	**87.88**	**87.99**	87.02	87.18	87.9	87.75	88.13	88.89	98.26	89.31	91.04	90.45	88.71	88.35	88.73	85.94	89.73	89.35	89.87	88.71	98.77	97.24		9	228
25	DQ107526 *Sinergasilus undulatus* CN	**88.28**	**88.18**	**88.28**	87.34	87.5	88.2	88.05	88.42	88.89	98.4	89.16	91.04	90.01	88.57	87.89	88.87	86.08	89.73	89.35	89.58	88.57	98.31	96.8	98.69		230
26	MH982204 *Lernaea cyprinacea* CN	**69.41**	**69.41**	**69.41**	68.54	68.69	69.55	69.48	69.41	70.18	67.56	69.69	69.69	70.4	70.82	68.7	69.69	70.1	70.96	70.96	70.68	70.68	67.56	66.01	67.71	67.42	

Table 6.8: Genetic divergences among aligned COI sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/BOLD/Sequence ID, Taxon and Country (sites are included for sequences from the present study and The Czech Republic). Sequences from the present study are bold in a grey shade, *Lernaea cyprinacea* (MH982220) was used as the outgroup. Abbreviations: AR – Argentina, BD – Boskop Dam, CN – China, KNP – Kruger National Park, KR – South Korea, NI – Nicaragua, OR – Olifants River, US – United States, VR – Vaal River, ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	**P43Rb BD VR *Neoergasilus japonicus* ZA**		**2**	**40**	**1**	**1**	**0**	**4**	**1**	**119**	**105**	**107**	**126**	**136**	**128**	**138**
2	**P46Kg OR KNP *Neoergasilus japonicus* ZA**	**99.7**		**40**	**1**	**1**	**0**	**4**	**1**	**114**	**105**	**107**	**119**	**219**	**212**	**139**
3	KR049037 *Neoergasilus japonicus* KR	**93.13**	**93.13**		39	39	40	42	39	103	107	108	112	115	112	131
4	MZ964933 *Neoergasilus* sp. US	**99.85**	**99.84**	93.30		0	1	4	0	117	106	108	125	129	123	135
5	MZ964936 *Neoergasilus japonicus* US	**99.85**	**99.84**	93.30	100		1	4	0	117	106	108	125	129	123	135
6	MZ964935 *Neoergasilus japonicus* US	**100**	**100**	93.13	99.85	99.85		4	1	118	105	107	126	130	124	136
7	MZ964934 *Neoergasilus* sp. US	**99.43**	**99.41**	92.78	99.43	99.43	99.43		4	121	108	110	128	133	125	139
8	*ZOOPS113-18 *Neoergasilus japonicus* US	**99.84**	**99.84**	93.30	100	100	99.84	99.41		111	106	108	116	123	117	135
9	MN854870 *Acusicola margulisae* NI	**82.78**	**82.57**	82.30	82.22	82.22	82.07	81.65	82.46		114	115	154	138	133	149
10	KR049036 *Ergasilus wilsoni* KR	**81.96**	**81.96**	81.62	81.79	81.79	81.96	81.44	81.79	80.41		14	124	118	99	138
11	KR049035 *Ergasilus* sp. KR	**81.62**	**81.62**	81.44	81.44	81.44	81.62	81.10	81.44	80.24	97.59		128	117	102	138
12	KU557411_*Ergasilus* sp. AR	**81.25**	**81.26**	80.76	80.92	80.92	80.76	80.50	81.59	77.08	78.69	78.01		137	133	142
13	MW080644 *Sinergasilus undulatus* CN	**80.52**	**81.41**	80.24	80.40	80.4	80.24	79.83	80.57	80.03	79.73	79.90	79.61		192	146
14	KR263117 *Sinergasilus polycolpus* CN	**81.66**	**82**	80.76	81.31	81.31	81.16	81.04	81.52	80.75	82.99	82.47	80.21	84.2		141
15	MH982220 *Lernaea cyprinacea* CN	**75.53**	**75.57**	73.90	75.05	75.05	74.86	74.35	75.05	73.25	72.51	72.51	73.61	74.34	75.22	

*Sequence from Barcode of Life Database (BOLD)

Table 6.9: Positions of nucleotide substitution in *Neoergasilus japonicus* (Harada, 1930) from this study used in alignments, for partial ribosomal RNA genes (18S and 28S). Information in bold are positions of variability for newly generated sequences.

Gene region	Sequence ID / Accession number	Locality	Position in alignment	Nucleotide substitutions
18S	MW810237; MH167970	Jezu and Rohlik, Czech Republic	388	T
	Other *Neoergasilus* sequences	Czech Republic, South Korea, South Africa		A
	P36R; P43R; P46K2	**Boskop Dam and Olifants River, South Africa**	**451**	**A**
	Other *Neoergasilus* sequences	Czech Republic, South Korea, South Africa		T
	MH167969; MH167970	Hvezda and Rohlik, Czech Republic	731	C
	Other *Neoergasilus* sequences	Czech Republic, South Korea, South Africa		T
	P46K2	Olifants River	1382	A
	Other *Neoergasilus* sequences	Czech Republic, South Korea, South Africa		G
28S	KR048823	South Korea	8	C
	Other *Neoergasilus* sequences	Czech Republic, South Korea, South Africa		A
	MW810240	Babice, Czech Republic	440	K
	Other *Neoergasilus* sequences	Czech Republic, South Korea, South Africa		T
	MH167967; MW810241	Hvezda and Jezu, Czech Republic	552	G
	Other *Neoergasilus* sequences	Czech Republic, South Korea, South Africa		A
	P36Ra; P43Ra; P46K2a	**Boskop Dam and Olifants River, South Africa**	**740**	**C**
	Other *Neoergasilus* sequences	Czech Republic, South Korea, South Africa		A

Table 6.10: Sites of nucleotide variation in the alignment of partial COI *Neoergasilus* sequences highlighting all silent mutations from newly generated sequences and missense mutations in *Neoergasilus* partial COI alignment from the present study. Invertebrate mitochondrion translation was used, and alignment was without stop codons. Divergent nucleotides in bold, amino acids translations are given. Abbreviations: BD – Boskop Dam (from the Mooi River); KR – South Korea; OR – Olifants River (in the Kruger National Park); US – The United States of America.

Sequence ID / GenBank Accession	Locality	Position in alignment	Nucleotide substitutions	Codons	Codon positions	3-letter Amino acid	Amino acid
Silent mutations from newly generated sequences in alignment from the present study							
P43Rb	BD	690	C	CA**C**	688–690	His	Histidine
P46Kg	OR		T	CA**T**			
P43Rb	BD	693	T	CC**T**	691–693	Pro	Proline
P46Kg	OR		A	CC**A**			
Missense mutations in *Neoergasilus* partial COI alignment from the present study							
KR049037	KR	275 & 276	TC	T**TC**	274–276	Phe	Phenylalanine
Other *Neoergasilus* sequences			AT	T**AT**		Tyr	Tyrosine
KR049037	KR	433	G	**G**TT	433–435	Val	Valine
Other *Neoergasilus* sequences			A	**A**TT		Ile	Isoleucine
MZ9464934	US	604	T	**T**AT	604–606	Tyr	Tyrosine
Other *Neoergasilus* sequences			G	**G**AT		Asp	Aspartic Acid
MZ9464934	US	649	N	**N**AC	649–651	Xaa	Any amino acid
Other *Neoergasilus* sequences			G	**G**AC		Asp	Aspartic Acid

Phylogenetic trees constructed for COI, using the BI and ML analyses, also showed similar tree topologies. All *Neoergasilus* sequences formed a separate clade from the other Ergasilidae genera (Fig. 6.7). Newly generated sequences clustered in a sub-clade with an *N. japonicus* sequence (MZ964935) from the USA.

6.5. Discussion

Different authors have reported some morphological variations in *N. japonicus* (Lescher-Moutoué,1979; Mugridge et al., 1982; Hayden & Rogers, 1988; Kim & Choi, 2003; Alfonso & Belmonte, 2010; Marshall et al., 2019). Mugridge et al. (1982) suggested that the minor differences observed between the British specimens from their study and the descriptions from other authors could indicate some level of sub-specific variation. In this study, specimens collected from the Boskop and Potchefstroom dams in the Mooi River were morphologically identical to those from the Olifants River in the Kruger National Park. However, some variations were observed among specimens from this study and those described in the literature.

For instance, the absence of a spine on the third exopodite of leg 3 (Fig. 6.4c) is consistent with reports by Mugridge et al. (1982), Hayden and Rogers (1988), Alfonso and Belmonte (2010), and Marshall et al. (2019), but not with Kim and Choi (2003). Additionally, the basis of the fourth leg in specimens observed from this study bears a short simple seta (Fig. 6.2f), differing from the authors above; but is consistent with Kim and Choi (2003), Alfonso and Belmonte (2010), as well as reports by Lescher-Moutoué (1979), although, Lescher-Moutoué reported this leg as the fifth leg instead of the fourth, and the seta on the basis as a short plumose seta and not the short simple seta reported in other literature. This seta may be easily missed during dissections and light microscopy observation but is visible with scanning electron microscopy. Yin (1956) and Kim and Choi (2003) all reported a spine on the exopod of the fourth leg which is absent in the reports from all the authors listed above too. Lescher-Moutoué (1979) observed this spine on exopod of leg 4 for only one out of the thirty adult female *N. japonicus* examined. This exopodal spine on leg 4 was also not observed in the present study. Alfonso and Belmonte (2010) reported plumose furcal rami setae and a bifid median furcal rami seta in the female *N. japonicus* specimens from Italy. This feature is also not observed in specimens from the current study and is not mentioned by any of the above-listed authors. Alfonso and Belmonte (2010) suggested that this might be a feature specific to the Italian population.

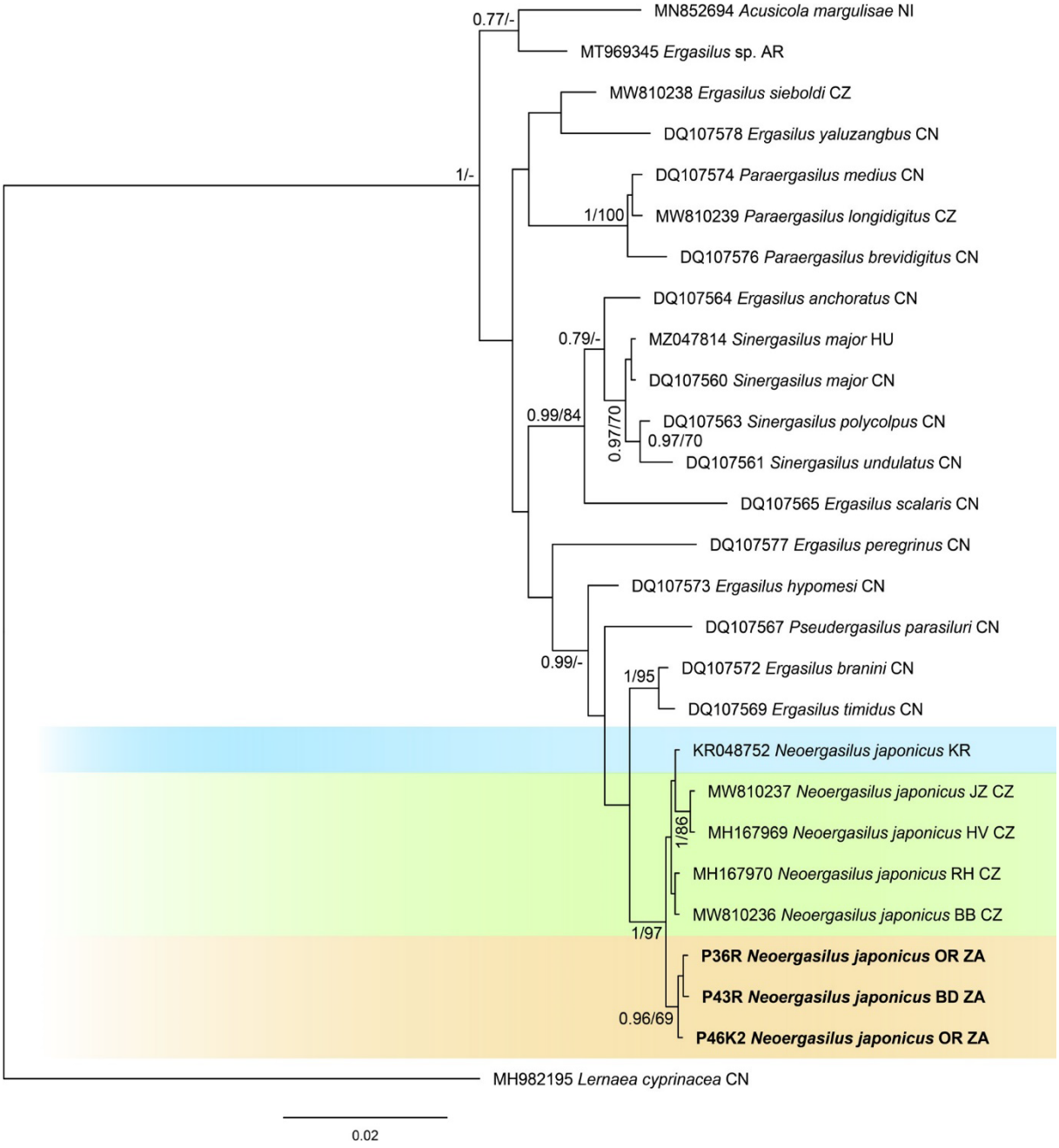


Figure 6.5: Phylogenetic tree of Ergasilidae copepods based on partial 18S rRNA gene alignments. Newly generated *Neoergasilus japonicus* (Harada, 1930) sequences are bold (highlighted in orange), and other published sequences are highlighted in light green (Czech Republic) and blue (South Korea). Nodal supports are place next to the corresponding branches for Bayesian Inference (>0.7) and Maximum Likelihood (>68%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: AR – Argentina, BB – Babice, BD – Boskop Dam, CN – China, CZ – Czech Republic, HU – Hungary, HV – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, OR – Olifants River, RH – Rohlik, ZA – South Africa.

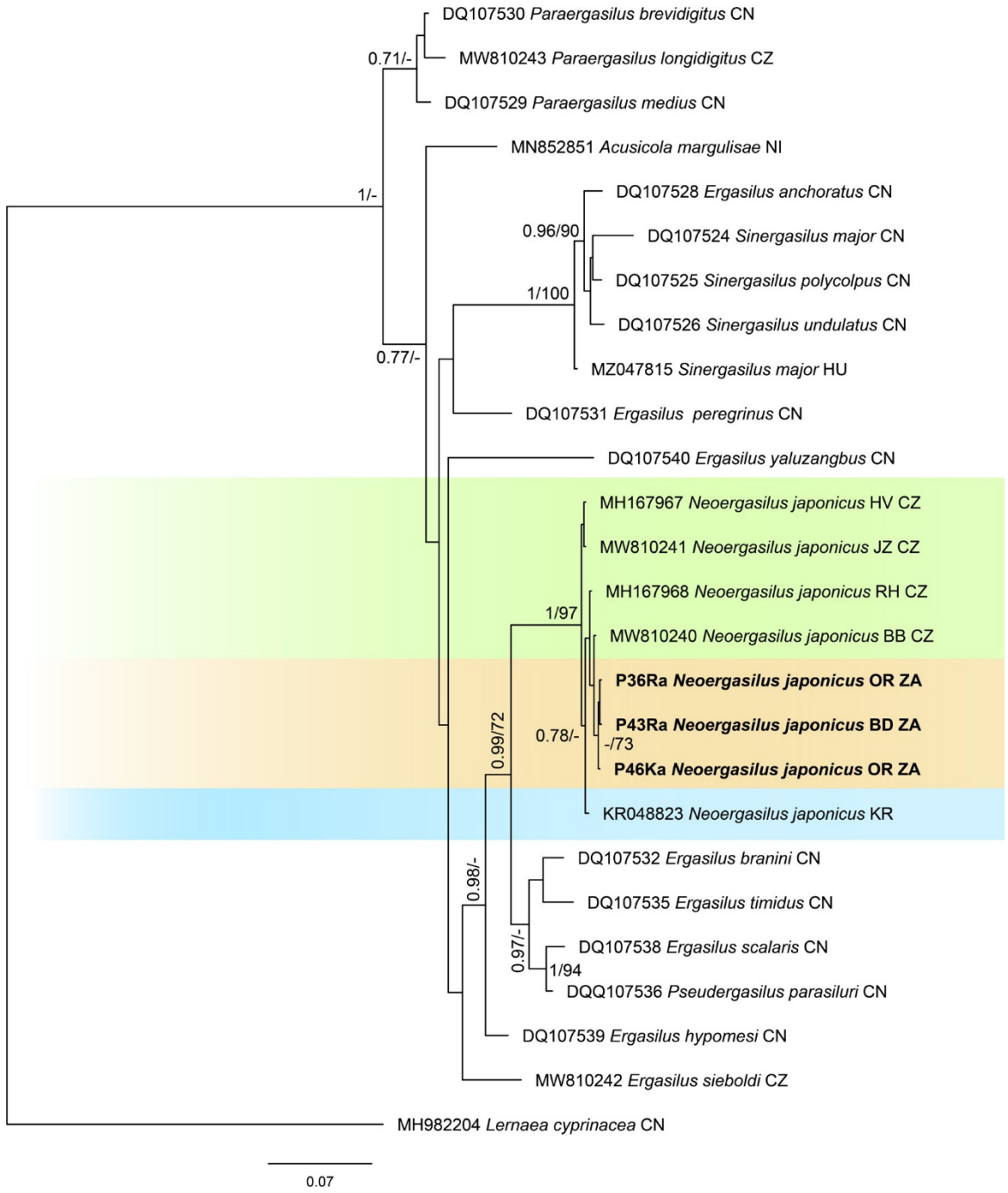


Figure 6.6: Phylogenetic tree of Ergasilidae copepods based on partial 28S rRNA gene alignments. Newly generated *Neoergasilus japonicus* (Harada, 1930) sequences are bold (highlighted in orange), and other published sequences are highlighted in light green (Czech Republic) and blue (South Korea). Nodal supports are placed next to the corresponding branches for Bayesian Inference (>0.7) and Maximum Likelihood (>68%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: BB – Babice, BD – Boskop Dam, CN – China, CZ – Czech Republic, EG – Egypt, HU – Hungary, HV – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, OR – Olifants River, RH – Rohlik, ZA – South Africa.

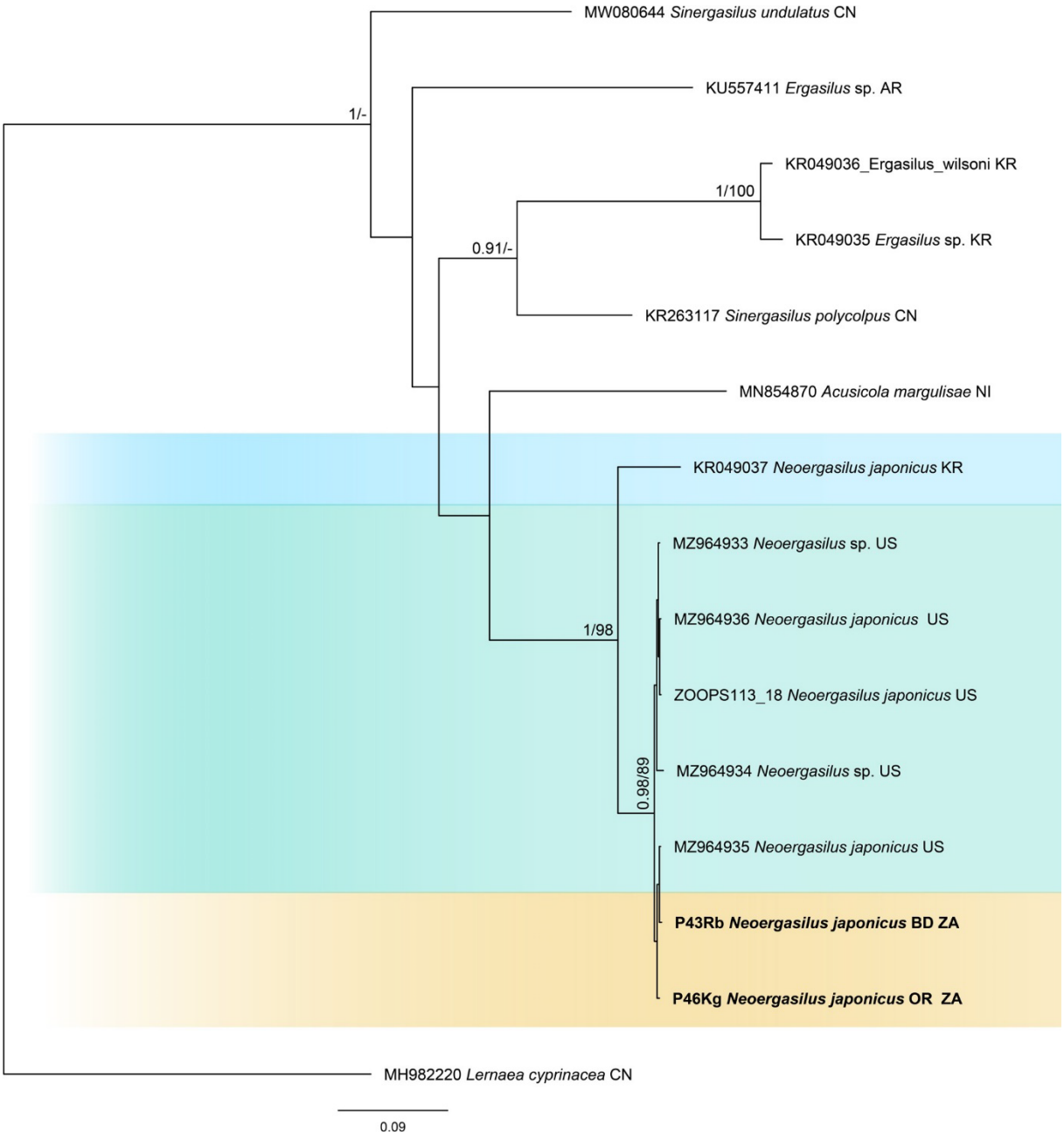


Figure 6.7: Phylogenetic tree of Ergasilidae copepods based on partial COI mtDNA gene alignments. Newly generated *Neoergasilus japonicus* (Harada, 1930) sequences are bold (highlighted in orange), and other published sequences are highlighted in blue (South Korea) and dark green (USA). Nodal supports are placed next to the corresponding branches for Bayesian Inference (>0.7) and Maximum Likelihood (>68%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: AR – Argentina, BD – Boskop Dam, CN – China, KR – South Korea, NI – Nicaragua, OR – Olifants River, US – United States of America, ZA – South Africa.

The Boskop and Potchefstroom impoundments in the Mooi River are new locality records for this parasite species in the Vaal River System, South Africa. In Boskop, half of the *M. salmoides* fish population from this study were infested with at least one parasite, resulting in a higher infestation prevalence than Olifants River, which had a prevalence of 40%. Even though Boskop had the highest number (n = 7) of parasites collected compared to the other sites, Olifants River had the highest mean intensity and abundance of parasitisation on *H. vittatus*. The total number of parasites reported in this study was relatively low when compared to what has been reported by other authors. For example, a single cyprinid, *Scardinius erythrophthalmus* (Linnaeus, 1758) (rudd), in France was reported to have up to 60 *N. japonicus* individuals (Lescher-Moutoué, 1979). Soylu and Soylu (2012) reported 52 specimens of *N. japonicus* on a single *S. erythrophthalmus* host, and Kuş and Soylu (2013) reported high infestation of *N. japonicus* on *S. erythrophthalmus* with a total of 743 *N. japonicus* individuals on 51 rudd hosts. Additionally, Ondračková et al. (2019) reported a 100% prevalence of *N. japonicus* from a centrarchid host, *Lepomis gibbosus* (Linnaeus, 1758), examined in 2017 from Hvezda and Rohlik sites, Czech Republic. *Neoergasilus japonicus* is typically found on the fins and occasionally on the gills, body surface, and nostrils of fish hosts, as reported by Ondračková et al. (2019). The parasites collected from *M. salmoides* in the present study were found on the fins, with the exception of one individual found on the gill arch; conversely, all parasites from *H. vittatus* were collected from the gill filaments and gill arches (Figs. 6.4d, e).

Neoergasilus japonicus has been reported from several continents on host fishes whose populations are already established in Africa either via accidental introductions, forage, aquaculture, or sports fishing. According to Weyl et al. (2020), the major pathways for the introduction of invasive species (parasites and hosts) in South Africa are intentional release, biological control, parasitic contaminants, and stowaways, with fisheries and pet trade being the major vectors. This invasive parasite might have been introduced to South Africa via any of the invasive fish hosts currently reported in South Africa. The largemouth bass, *M. salmoides*, for instance, is native to North America but has been introduced into all other continents except Antarctica. It was one of five centrarchid fishes that were introduced into South Africa for sports fishing and angling (Du Plessis, 1948; De Moor & Bruton, 1988; Ellender & Weyl, 2014; Smit et al., 2017; Truter et al., 2017; Khosa et al., 2019; Weyl et al., 2020). Of the remaining four centrarchids, the smallmouth bass *Micropterus dolomieu* (Lacepede, 1802) and the bluegill *Lepomis macrochirus* Rafinesque, 1819—both introduced in the 1930s, have been reported as hosts for *N. japonicus* in Asia, and North America (Table 6.1). These centrarchids may have also been points of introduction of *N. japonicus* into South Africa.

Du Plessis (1948) reported heavy losses in largemouth bass populations at the Jonkershoek fish hatchery in South Africa; this loss was attributed to the host's co-introduced parasite, *Acolpenteron ureteroecetes* Fischthal & Allison 1940 (Dactylogyridae), a parasite

found in the ureters of largemouth bass. This was the only record of parasitisation that existed for *M. salmoides* until 2017, when Truter et al. (2017) provided the first published peer-reviewed report of monogenean parasites from the largemouth bass in South Africa. To the best of our knowledge, other than the unpublished conference abstract (Smit et al., 2018) there have been no published records of the invasive copepod, *N. japonicus* from *M. salmoides* in South Africa. It is possible that the spread of *N. japonicus* to centrarchid hosts might also have been due to host switching from fishes of other families reported from Africa (see checklist in Table 6.1) in the event of a spillback to the invasive *M. salmoides.*

The family Cyprinidae is another group of invasive hosts in South Africa (Weyl et al., 2020). Currently, only the goldfish *Carassius auratus* (Linnaeus, 1758) and the common carp *Cyprinus carpio* have been reported as hosts for *N. japonicus* in Africa (Table 6.1). *Carassius auratus* is said to have been introduced into South Africa in the 1720s, from Asia on Dutch vessels (De Moor & Bruton, 1988), and its importation continues via pet trade for aquaria as an ornamental fish species (Weyl et al., 2020). *Cyprinus carpio* was introduced into South Africa from England in 1959 (see Smit et al., 2017). It currently occurs in several dams and mainstream rivers in all of the major river basins in South Africa and has been reported as a host to several invasive parasites in South Africa (Smit et al., 2017; Weyl et al., 2020). Only an unpublished dissertation records a single *N. japonicus* individual on the skin of *C. carpio* from the Kwena Dam, Mpumalanga Province, South Africa (Mokonyane, 2020),

There are other fishes that are invasive in South Africa and have been reported as hosts for *N. japonicus* in other continents. They include the European Perch *Perca fluviatilis* Linnaeus, 1758, (Percidae), introduced to South Africa in 1915 from England for angling in impoundments; and the Mosquitofish *Gambusia affinis* (Baird and Girard, 1853) (Poecilidae), introduced into South Africa in 1936 as a control for mosquitoes and also as prey for introduced gamefishes (De Moor & Bruton, 1988). Both of these fish species have been reported by other authors as hosts for *N. japonicus* (Table 6.1). Another host, the tench *Tinca tinca* (Linnaeus, 1758) (Tincidae), is a European fish species that was introduced into South Africa (Western Cape) in 1910 for angling (Weyl et al., 2020). Although its distribution seems to be confined to the Western Cape Province, it is the only other invasive cyprinid fish species, apart from the goldfish and common carp, to be reported as a host for *N. japonicus* in Britain in 1982 (Table 6.1). The introduction of tench in South Africa could serve as another potential point of introduction for *N. japonicus* to this country.

From their publications on invasive freshwater fish parasites and fauna from South Africa, Smit et al. (2017) and Weyl et al. (2020), respectively, reported the anchor worm *Lernaea cyprinacea* Linnaeus, 1758 and the fish louse, *Argulus japonicus* Thiele, 1900, originally described from Japan, as the only invasive parasitic Crustacea species officially reported to parasitise freshwater fishes in South Africa. The addition of *N. japonicus*, therefore, increases the number of invasive parasitic crustaceans in South Africa to three.

Like several other parasite species, *N. japonicus*, might have been in South Africa for a long period of time but may have not been reported. This copepod may have been missed because it is small in size (0.2–4.0 mm), and it is sometimes found parasitising smaller fish species that are caught with plankton nets (see Velázquez-Ornelas et al., 2021; Velázquez-Ornelas & Suarez-Morales, 2022). This copepod is able to use both exotic and native hosts, so it is possible that the impact of its host range and distribution is substantially more than what has been reported (published and unpublished).

The alestid characiform host, *Hydrocynus vittatus*, reported in this study as a new host record for *N. japonicus*, is endemic to Africa and might therefore represent a spillover from the invasive hosts. Other than the conference abstract (Smit et al., 2018) of *N. japonicus* on *Micralestes acutidens* (Peters, 1852), no other report of parasitisation on hosts from this family exists for *N. japonicus*. With its ability to parasitise across a wide range of host families and its fast spread, it is only a matter of time before this invasive parasite, increases its distribution in Africa.

As expected, the partial 18S and 28S *N. japonicus* sequences showed little variability in the alignments. While these genes are relatively conserved in understanding the evolutionary relationships of the species, the COI gene was used to confirm species identity and show variability within and among *Neoergasilus* populations. Previous studies suggested that ribosomal RNA genes are effective for species identification (Song et al., 2008) and for evaluating evolutionary relationships (Kim & Kim, 2000) within the family Ergasilidae. However, the effectiveness of the 18S gene, for instance, in determining evolutionary relationships has been suggested as an auxiliary tool to be used in combination with other molecular and morphological data (Wu et al., 2015). The study on the 18S and 28S phylogenetics of *N. japonicus* by Ondračková et al. (2019) reported single base differences between the sequences from different localities, but no variations among sequences from the same localities. The present study showed no genetic variations among sequences from the Olifants River and Mooi River for the ribosomal RNA genes. Tang et al. (2012) suggested that the 18S gene may be appropriate for understanding evolutionary relationships occurring at higher taxonomic scales but not for inferring species-level patterns.

In the present study, a more integrative multi-loci species delimitation approach was used, including fragments of two ribosomal RNA genes and especially the COI mitochondrial DNA gene. This COI gene has been suggested as an important tool to be used together with morphological studies for the resolution of genetic variability (Ondračková et al., 2019), and was reported to be a much better indicator of the meiofauna diversity by Tang et al. (2012).

From the present study, most nucleotide differences observed in the COI alignment with other *N. japonicus* sequences were silent mutations (nucleotide mutations that do not change the amino acids), with only a few resulting in missense mutations (nucleotide mutations that lead to different amino acids). The genetic variation of 40 bp between the South Korean

N. japonicus sequence and the newly generated sequences (Table 6.8) was largely attributed to silent mutations, which specifically accounted for more than 90% of the differences between the two sequences (37 bp) (Appendix Table A3). Further studies on nucleotide substitution and amino acid mutation could enhance our understanding of the genetic divergences and variabilities among sequences.

6.6. Conclusion

Parasitic copepods collected from this study were genetically and morphologically identified as *Neoergasilus japonicus*. Newly generated sequences were intraspecifically different from the other *Neoergasilus* sequences used in the alignments. This study provides the first morphological and molecular characterisation of *N. japonicus* from Africa and presents new host and locality records. The present study also provides novel molecular data of three gene regions for *N. japonicus* from Africa for future evolutionary and phylogenetic analyses.

6.7. References

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CHAPTER 7

A new species of *Neoergasilus* Yin, 1956 (Ergasilidae: Cyclopoida) parasitising the sharptooth catfish *Clarias gariepinus* (Burchell, 1822) (Clariidae) from South Africa

7.1. Abstract

Twenty-three specimens of an ergasilid copepod were collected from the gills of the sharptooth catfish *Clarias gariepinus* (Burchell, 1822) from the Great Fish River, Eastern Cape, South Africa. The morphology of its first leg and antennae conformed to that of species from the genus *Neoergasilus* Yin, 1956. The combination of the following characters, however, separates the South African specimens from the nine other congeners in the genus: an inflated cephalothorax; the presence of oval ornamentation anterior to the cephalosome; a knob-like process on the internal distal margin of the first exopodite of leg 1; leg 4 bearing a 2-segmented exopod and 3-segmented endopod; uniramous single-segmented fifth leg with a seta extending from the base of the thoracic segment, and three unequal setae on its free segment; and a median furcal rami seta with an array of spines. Supporting genetic data were generated using two partial ribosomal RNA genes, 18S and 28S, and one partial mitochondrial DNA gene, COI. Morphologically and genetically, specimens from this study were confirmed as members of the family Ergasilidae, but as a different species from *N. japonicus*. This species is thus new to science and described as *Neoergasilus* sp. nov. (Ergasilidae: Cyclopoida). This is the first *Neoergasilus* species described from the sharptooth catfish and from the southern hemisphere. This study adds to the limited information on representatives of the genus *Neoergasilus*. A key to all the species of the genus is also provided.

7.2. Introduction

Members of the parasitic crustacean family Ergasilidae von Nordmann, 1832 occur globally as parasites of freshwater, brackish, and marine fishes. There are currently 30 accepted genera in the family (Walter & Boxshall, 2023a). In Africa, all previously reported Ergasilidae species belong to four genera, namely *Dermoergasilus* Ho & Do, 1982 (marine and brackish water species), *Ergasilus* von Nordmann, 1832 (marine and freshwater species), *Neoergasilus* Yin, 1956 (invasive freshwater and brackish water species); and *Paraergasilus* Markevich, 1937 (freshwater species only) (Oldewage & van As, 1988a, b; Berrouk et al., 2018, 2020; Boucenna et al., 2018). During parasitological surveys in South Africa, specimens were collected that conformed to the characteristics of members of the genus *Neoergasilus*.

Like other copepods from the Ergasilidae, neoergasilids are found attached to host gills, as well as the nasal cavities, dorsal and anal fins of their hosts (Hayden & Rogers, 1998; Hudson & Bowen, 2002; Alekseev et al., 2021).

There are currently nine accepted *Neoergasilus* species (Walter & Boxshall, 2023b) described from China (*N. longispinosus* Yin, 1956); India (*N. ferozepurensis* Kumari, Khera & Gupta, 1988; *N. indicus* Vankara & Chikkam, 2010; *N. kherai* Battish & Brar, 1989; and *N. notopteri* Kumari, Khera & Gupta, 1988); Korea (*N. angustus* Kim & Choi, 2003 and *N. bullatus* Kim & Choi, 2003); Russia (*N. squaliobarbi* (Dogiel & Akhmerov, 1952)—syn. *N. inflatus* (see Smirnova, 1971)); and Taiwan (*N. japonicus* (Harada, 1930)). *Neoergasilus japonicus* is the only species that has been reported from several continents including Africa, Asia, Europe, North America, and South America (see Chapter 6).

Although the specimens from this study conform to the genus *Neoergasilus,* they differed morphologically from all other nine species in this genus. Furthermore, there is limited genetic data available for this genus with which to compare. At the time of writing, the only available sequences on GenBank are of *N. japonicus*: for 18S and 28S ribosomal RNA genes (Czech Republic: Ondračková et al., 2019; Kvach et al., 2021) and the mitochondrial DNA gene, COI (South Korea: Baek et al., 2016; and USA: Vasquez et al., 2021 supplementary data). There is also a GenBank submission from South Korea for 18S and 28S gene regions, but it is not associated with any peer-reviewed article (unpublished submission). This study, therefore, aims to morphologically and molecularly characterise a parasitic copepod species from the genus *Neoergasilus* not previously known to science.

7.3. Materials and methods

7.3.1. Sampling

Adult females (n = 23) were collected from the gills of 15 sharptooth catfish, *Clarias gariepinus,* caught with baited long lines in November 2018 from the Great Fish River, Eastern Cape, South Africa (33°19'49.3"S 26°59'54.2"E) (Fig. 7.1). Permits were obtained from the Department of Economic Development, Environmental Affairs and Tourism, Eastern Cape (permit no. CRO 20/18CR, CRO 22/18CR) and ethical clearance from the North-West University (Ethics No. NWU-00159-18-A5). Fish were physically examined for parasites attached to the body surface, fins, and gills, and were dissected and screened for parasites with the aid of a Zeiss Stemi 305 dissection microscope following standard methods for crustacean parasites in Dávidová and Smit (2018). Parasites were removed using fine paint brushes, needles, and tweezers, then cleaned in petri dishes containing freshwater, and fixed in 70% ethanol for further analyses. Host nomenclature is from FishBase (Froese & Pauly, 2023) (www.fishbase.org), and host and parasite authorities (except for the synonymy) are not included in the references.

7.3.2. Morphological analysis

For morphology, photomicrographs of uncleared specimens were taken with the aid of a Zeiss Stemi 508 stereomicroscope, cleared in lactic acid for 60 minutes and stained in lignin pink for 10–20 minutes. With the aid of the stereomicroscope, five adult female specimens were dissected on glass slides using fine needles, mounted temporarily with glycerine, and viewed with bright-field and phase-contrast microscopy functions of Nikon Eclipse *Ni* differential interference contrast (DIC) microscope (Nikon Instruments, Tokyo, Japan), enabling the z-dimensional stacking function. Photomicrographs of dissected appendages were taken using NIS-Elements BR Ver. 4.60 software of the DIC microscope and drawn with the aid of a drawing tube microscope attachment. Measurements of 10 randomly selected specimens were made using the Labscope Mat Ver. 2.8.4 microscope software connected to the Zeiss Stemi 508 stereomicroscope. All measurements are expressed in millimetres as mean ± standard deviation (with range in parentheses). Six ovigerous female specimens were selected for scanning electron microscopy (SEM). Specimens were dehydrated through a graded alcohol series; dried using hexamethyldisilazane (HMDS) or critically point dried; coated with gold palladium and studied with the aid of a JEOL Winsem JSM IT 200 at 5KVa respectively. Illustrations were electronically inked using a Wacom Intuos® Pro drawing tablet and Adobe Illustrator™ software package. Morphological terminologies for the description followed Abdelhalim et al. (1993) and Boxshall (2016).

7.3.3. Infestation rates

Calculations for infestation rates were according to Bush et al. (1997), including prevalence, mean intensity, and mean abundance.

7.3.4. Molecular analysis

For molecular analyses, two protocols were used for genomic DNA extractions. The Macherey- Nagel tissue kit (GmbH & Co. KG, Sandton, South Africa) manufacturer's protocol with an adaptation of the pre-lysis incubation period (3 hours ± 30 minutes in shaking incubator and a hold time of 2 hours), was used to extract genomic DNA from a selected ovigerous female specimen. The PCRBIO Rapid Extract PCR Kit (PCRBiosystems, Analytical Solutions, Randburg, South Africa) was also used for the extraction of genomic DNA using one egg string each from three separate specimens that had been selected for dissection, adapting the manufacturer's protocol as follows: 20 µl of lysis buffer, 10 µl of buffer containing protease, and 150 µl molecular grade water for dilution. DNA amplifications for partial ribosomal RNA gene regions used primers designed by Song et al. (2008): 18SF and 18SR (18S) and 28SF and 28SR (28S); and the partial mitochondrial DNA gene region (COI) was amplified with primers by Bucklin et al. (2010): cop- Col-1498-F and cop-Col-2198-R (Table 7.1). Each Polymerase Chain Reaction (PCR) was carried out in 25 µl volumes using: 12.5 µl

of DreamTaq PCR Master Mix (Thermo Fisher Scientific, South Africa); 1.25–2.5 µl of 10 µM of each primer (forward and reverse); and 2.5–10 µl of DNA product. The proportion of molecular grade water varied amongst the samples to make the final volume of 25 µl. DNA amplification was performed in a Benchmark TC9639 Thermal Cycler (Whitehead Scientific (Pty.) Ltd.) following conditions by Song et al. (2008) for 18S, and adapting the conditions by Hayes et al. (2021) for 28S and COI as follows: initial denaturation at 95°C for 5 min; followed by 40 cycles of 95°C denaturation for 30 sec, annealing of primers at 47°C for 30 sec, an initial extension at 72°C for 1 min; and the final extension at 72°C for 7 min. Negative controls were used to detect possible contamination of the reagents.

Amplicons of PCR were verified by 1% agarose gel electrophoresis along with a 1kb DNA ladder (BioLabs Inc.). Positive PCR products were sent to a commercial sequencing company, Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa, for purification and sequencing in both directions. Forward and reverse sequences received were checked for ambiguity, assembled, and edited using the bioinformatics software platform, Geneious Prime v. 2022.2.2 (Biomatters, Auckland, New Zealand; Kearse et al., 2012). Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to check similarity between newly generated sequences with submissions of *Neoergasilus* available on GenBank as well as other copepods from the family Ergasilidae. Due to the scarcity of sequences from this genus, published and unpublished *Neoergasilus* sequences were used in the alignment for phylogenetic analyses. However, only sequences associated with peer-reviewed publications from other genera in the family Ergasilidae were used and *Lernaea cyprinacea* Linnaeus, 1758 (Lernaeidae) was used as the outgroup in this study (see Table 7.2). Alignments were constructed using the default parameters of a multiple sequence alignment program, MAFFT (Katoh et al., 2002; Katoh & Standley, 2013) in Geneious Prime, trimmed, and all gaps were removed. For the best nucleotide substitution, a model GTR+I+G suggested based on the Akaike information criterion (AIC) from jModelTest 2.1.4 test (Posada, 2008; Darriba et al., 2012) was used.

Bayesian Inference (BI) trees were constructed in CIPRES Science Gateway v3.3 (available at https://www.phylo.org/) with default parameters of MrBayes v. 3.2.7a (Ronquist et al., 2012), running two independent Markov Chain Monte Carlo (MCMC) runs of four chains for ten million generations and sampling tree topologies every one thousand generations. Burn-in parameters were set to the first 25000 generations. Maximum likelihood (ML) trees were constructed using PhyML v3.0 (http://www.atgc-montpellier.fr/phyml/) (Miller et al., 2010). Nodal support for ML analyses was estimated at 100 bootstrap repetitions. The phylogenetic trees were visualised in FigTree v1.4.4 software (Rambaut, 2012). Pairwise genetic distance matrices, expressed as the percentage of bases which are identical and the number of bases which are not identical, were estimated in Geneious Prime.

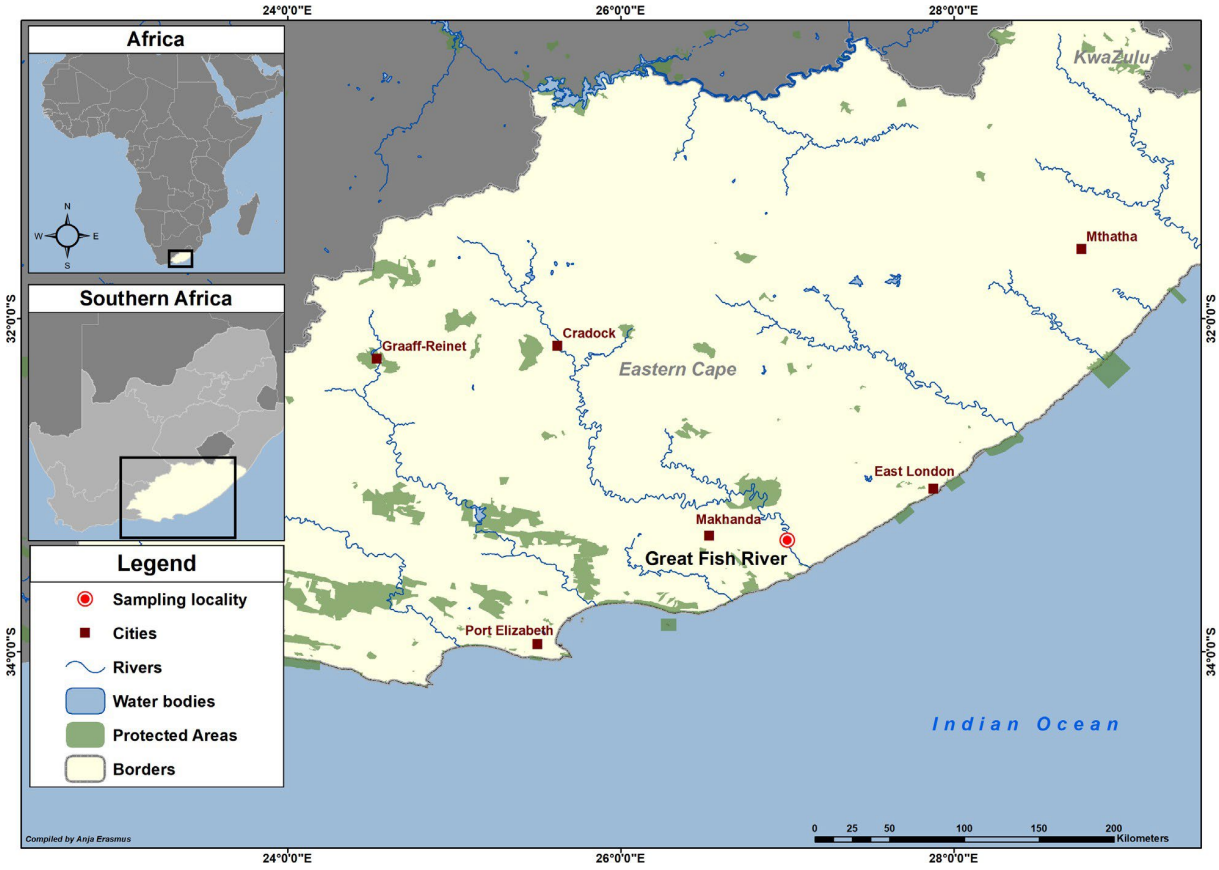


Figure 7.1: Map showing the sampling locality for the sharptooth catfish *Clarias gariepinus* (Burchell, 1822) in the Eastern Cape, South Africa, from which the *Neoergasilus* sp. nov. were collected.

Table 7.1: List of primers with references, annealing temperatures (T), and the number of cycles used for DNA amplification of South African *Neoergasilus* sp. nov. from the Great Fish River, South Africa. Abbreviations: K – specimens extracted using the Machery-Nachel tissue kit extraction method; R – specimens extracted using the PCRBio rapid kit extraction method

Gene regions	Specimen codes	Primers	Sequences	Annealing T (˚C)	Number of cycles	Sources
18S	P40R P47K P59R	18SF	5′-AAG GTG TGM CCT ATC AAC T-3′	54	30	Song et al. (2008)
		18SR	5′-TTA CTT CCT CTA AAC GCT C-3′			
28S	P50Ra2 P59Ra2	28SF	5′-ACA ACT GTG ATG CCC TTA G-3′	47	40	
		28SR	5′-TGG TCC GTG TTT CAA GAC G-3′			
COI	P50RJ P59RJ	cop-Col-1498-F	5′-GGGTGACCAAAAAATCARAA-3'	47	40	Bucklin et al. (2010)
		cop-Col-2198-R	5′-AAYCATAAAGAYATYGGDAC-3'			

Table 7.2: List of sequences included in the phylogenetic analyses. The taxa in bold are newly generated sequences, all other sequences are from the GenBank and Barcode of Life Database (BOLD) databases. *Lernaea cyprinacea* Linnaeus, 1758 (in grey shade) was used as the outgroup.

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Acusicola margulisae	*Amphilophus citrinellus*, *Parachromis managuensis*, *Oreochromis* sp.; *Poecilia mexicana*	Nicaragua	MN852694	MN852851	MN854870	Santacruz et al. (2020)
Ergasilus anchoratus	*Pseudobagrus fulvidraco*	China	DQ107564	DQ107528		Song et al. (2008)
Ergasilus briani	*Misgurnus anguillicaudatus*	China	DQ107572	DQ107532		Song et al. (2008)
Ergasilus hypomesi	*Acanthogobius hasta*	China	DQ107573	DQ107539		Song et al. (2008)
****Ergasilus parasiluri*	*Tachysurus fulvidraco*	China	DQ107567	DQ107536		Song et al. (2008)
Ergasilus peregrinus	*Siniperca chuatsi*	China	DQ107577	DQ107531		Song et al. (2008)
Ergasilus scalaris	*Tachysurus dumerili*	China	DQ107565	DQ107538		Song et al. (2008)
Ergasilus sieboldi	*Perca fluviatilis*	Czech Republic	MW810238	MW810242		Kvach et al. (2021)
Ergasilus sieboldi	*Sparus aurata*	Egypt		OM812074		Abdel-Radi et al. (2022)
Ergasilus sp.	Free-living	South Korea			KR049035	Baek et al. (2016)
Ergasilus sp.	*Mugil liza*	Argentina			KU557411	Castro-Romero et al. (2016)
Ergasilus tumidus	*Acanthorhodeus taenianalis*	China	DQ107569	DQ107535		Song et al. (2008)
Ergasilus wilsoni	Free-living	South Korea			KR049036	Baek et al. (2016)
Ergasilus yaluzangbus	*Gymnocypris stewartii*	China	DQ107578	DQ107540		Song et al. (2008)
****Ergasilus yandemontei*	*Odontesthes hatcheri*	Argentina	MT969345			Waicheim et al. (2021)
***Neoergasilus* sp. nov.**	***Clarias gariepinus***	**The Great Fish River, South Africa**	**P40R, P47K, P59R**	**P50Ra2, P59Ra2**	**P50RJ, P59RJ**	**Present study**

Taxon	Host	Locality	GenBank Accession Numbers			Reference
			18S	28S	COI	
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MH167969	MH167967		Ondračková et al. (2019)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MH167970	MH167968		Ondračková et al. (2019)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MW810236	MW810240		Kvach et al. (2021)
Neoergasilus japonicus	*Lepomis gibbosus, Scardinos erythrophthalmus*	Czech Republic	MW810237	MW810241		Kvach et al. (2021)
***Neoergasilus japonicus*	Collected by plankton net	USA			MZ964933–MZ964936	Vasquez et al. (2021)
Neoergasilus japonicus	Free-living	South Korea			KR049037	Baek et al. (2016)
**Neoergasilus japonicus*	*Notropis heterolepis*	USA			ZOOPS113-18	BOLD (2023)
Paraergasilus brevidigitus	*Cyprinus carpio*	China	DQ107576	DQ107530		Song et al. (2008)
Paraergasilus longidigitus	*Abramis brama, Perca fluviatilis, Scardinius erythrophthalmu*	Czech Republic	MW810239	MW810243		Kvach et al. (2021)
Paraergasilus medius	*Ctenopharyngodon idellus*	China	DQ107574	DQ107529		Song et al. (2008)
Sinergasilus major	*Ctenopharyngodon idella*	China	DQ107560	DQ107524		Song et al. (2008)
Sinergasilus major	*Silurus glanis*	Hungary	MZ047814	MZ047815		Dos Santos et al. (2021)
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	China	DQ107563	DQ107525		Song et al. (2008)
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	China			KR263117	Feng et al. (2016)
Sinergasilus undulatus	Cyprinus carpio	China	DQ107561	DQ107526		Song et al. (2008)
Sinergasilus undulatus	Cyprinus carpio	China			MW080644	Hua et al. (2021)
Lernaea cyprinacea	*Carassius auratus, Cyprinus carpio, Chanodichthys ilishaeformis*	China	MH982195	MH982204	MH982220	Hua et al. (2019)

* taxon from the Barcode of Life Database (BOLD);
** only able to access abstract and sequences that were published as supplementary data;
*** *Ergasilus yandemontei* (Published on GenBank as *Ergasilus* sp.); *Ergasilus parasiluri* (published on GenBank as its synonym *Pseudergasilus parasiluri*).

7.4. Results

7.4.1. Taxonomy

Order Cyclopoida Burmeister, 1834

Family Ergasilidae von Nordmann, 1832

Genus *Neoergasilus* Yin, 1956

Restricted synonymy.

Ergasilus Harada, 1930: 71–76 [not *Ergasilus* von Nordmann, 1832].

Neoergasilus Yin, 1956: 245–246; 267.—Yamaguti, 1963: 37.—Kabata, 1979: 81, 83.—Lescher-Moutoué, 1979: 111.—Mugridge et al. 1982: 533.—Kumari et al. 1988: 163.—Battish & Brar, 1989: 54–57.—Pu-ren & De-sheng, 1992: 1–3.—Kim & Choi, 2003: 71–83.—Vankara & Chikkam, 2010: 425–434.

Type-species. Ergasilus japonicus Harada, 1930 [now *Neoergasilus japonicus* (Harada, 1930)].

Generic remarks.

Individuals from the genus *Neoergasilus* are mainly characterised by the morphology of the first leg and antennae. The following generic characteristics differentiate specimens from this study from all other African Ergasilidae genera (*Ergasilus, Dermoergasilus,* and *Paraergasilus*). The first leg is elongate, reaching the fourth and fifth thoracic segments in some specimens; the basiopodite of the first legs bear a triangular process at the posterior margin between the endopod and exopod; and an enlarged spatulate spine is present on the outer margin of the second exopodal segment of the first leg (Yin, 1956; Kim & Choi, 2003). In addition, the antennae of *Neoergasilus* are short, slender, and strongly curved, with a conspicuous spine (also referred to as a seta in Kim & Choi (2003)) present on the basal or second segment. In addition to the above-listed characteristics, the antennae of *Paraergasilus* end in three claws compared to the single terminal claw of *Neoergasilus*; and the genus *Dermoergasilus* has a membrane sheath on the antennae that is absent in *Neoergasilus.* On the cephalothorax of some *Ergasilus* species, one to two oval ornamentations and an inverted T structure can be seen, however, no *Neoergasilus* species have been reported with more than one oval ornamentation and none have reported an inverted T structure.

***Neoergasilus* sp. nov**. (figs. 7.2–7.6).

Material examined.

Holotype. ovigerous female collected from the gills of *Clarias gariepinus* in the Great Fish River (33°19'49.3"S 26°59'54.2"E), Eastern Cape, South Africa, collected by M. Truter (2018). Holotype will be deposited in the collections of the National Museum, Bloemfontein, South Africa.

Paratypes. five ovigerous females, same host and collection data as holotype. Will be deposited in the collections of the National Museum, Bloemfontein, South Africa.

Other material. 12 ovigerous females, same host and collection data as holotype (six used for SEM; five dissected; one female and three egg strings used for DNA extraction). The remaining specimens will be in the possession of the Water Research Group at the North-West University (NWU).

Representative DNA sequences. Sequences, with the numbers of base pairs (bp), will be submitted to GenBank as follows:

18S: 1278, 1360, 1306 bp long sequences of three specimens.

28S: 672, 695 bp long sequences of two specimens.

COI: 655, 548 bp long sequences of two specimens.

Infestation rates. Total prevalence 46.7% (7/15), mean intensity 3.29 (23/7), mean abundance 1.53 (23/15).

Description of adult female (Figs. 7.2–7.6).

Measurements (n = 10) are given as: total length (from anterior of cephalothorax to posterior of furcal rami, excluding furcal rami seta) 0.70 ± 0.08 (0.54–0.78) mm, cephalothorax length 0.41 ± 0.06 (0.28–0.48) mm, cephalothorax width 0.30 ± 0.04 (0.21–0.36) mm, urosome length 0.15 ± 0.02 (0.12–0.18) mm.

Body cyclopiform with swollen cephalothorax (Figs. 7.2, 7.3a, 7.5a, c, 7.6a). Cephalothorax greatly expanded, 0.75 times as wide as long. Distinct lateral constriction defining cephalosome and second pedigerous somite. Anterodorsal oval ornamentation present. Thorax (Figs. 7.2, 7.3a) five segmented. First pedigerous somite completely fused with cephalosome; second pedigerous somite distinctly separated from cephalothorax; third and fourth somites gradually reducing in size; fifth somite largely reduced and grouped as part of urosome. Urosome (Figs. 7.4b, 7.6d) consists of fifth thoracic segment, genital double-somite, three free abdominal somites. Genital double somite, abdominal somites and furcal rami ornamented with a vertical row of minute spinules, observed ventrally. Each abdominal segment overlapping, slightly larger than the next; third abdominal somite with

dorsoventral incision, forming furcal ramus on each side; double U-shaped structure observed between rami at posteromedial margin of third abdominal somite. Furcal ramus rectangular with four setae of varying lengths: median seta the largest and longest, extending from the furcal ramus and ornamented with an array of spines (Figs. 7.4b, 7.6e). Egg sacs (Fig. 7.4c) 0.80 times the length of the body, multiseriate, occurring in pairs, with each sac containing approximately three columns of 8–10 eggs.

Antennule (Fig. 7.3c) 6-segmented, with simple setae of varying lengths. Setal armature formula: 3, 11, 5, 5, 2, 5 + 2 aesthetascs. Antenna (Figs. 7.3b, 7.5b, 7.6b) 4-segmented; first segment 1.5 times as long as its widest region, possesses small spine on posterodistal margin (Figs. 7.2b, 7.5b); second segment 0.90 times as long as first; only 0.58 times as wide as long, possesses sharply pointed stout spine on proximal inner margin and short seta on inner margin of segment (Figs. 7.3b, 7.5b, 7.6b); third segment longest, 1.2 times as long as first segment, slender, tapering, strongly curved; fourth segment greatly reduced: a short terminal claw about 0.6 times as long as first segment, armed with a short seta at its inner margin (Fig. 7.3b).

Mandible (Fig. 7.3d) with three denticulated blades; anterior blade shortest. Maxillule single-segmented (Fig. 7.3e), sightly elongate subtriangular lobe with two unequal setae and small spine. Maxilla 2-segmented (Figs. 7.3f, 7.6b), distal segment armed with numerous teeth on anterior margin.

Five pairs of swimming legs (Figs. 7.4a, d–g); each ramus for first three pairs of legs, 3-segmented; leg 4 exopod and endopod 2- and 3-segmented, respectively. Basiopodite of legs 1–4 with smooth, spiniform setae on outer margins, all other setae on exopodites and endopodites plumose; outer margins of endopodites and exopodites of legs 1–4 serrated; all spines of legs 1–4 serrated (except forked spines of third exopodite of leg 1). Leg 1 largest and longest, reaching up to fourth thoracic segment; basiopodite with distal horizontal row of 6–10 spines; second and third endopodites ornamented with 3–8 spines at distal margins (Fig. 7.6c); knob-like process present on inner distal margin of first endopodite (Figs. 7.4a, 7.6c); spatulate spine on outer margin of second exopodal segment sharply pointed, projecting inwards, longer than third exopodal segment and spines. Legs 2 and 3 (Figs. 7.4d, e) with same spine-setae armature; single pore towards outer margin, on third segments of endopod and exopod of legs 2 and 3. Table 7.3 gives armature formula of legs 1–4. Fifth leg (Fig. 7.4g) represented by a long seta at dorsolateral margin of fifth thoracic segment, and a free segment; free segment bears three setae of unequal lengths, one sub-terminal seta (the shortest) and two longer terminal setae.

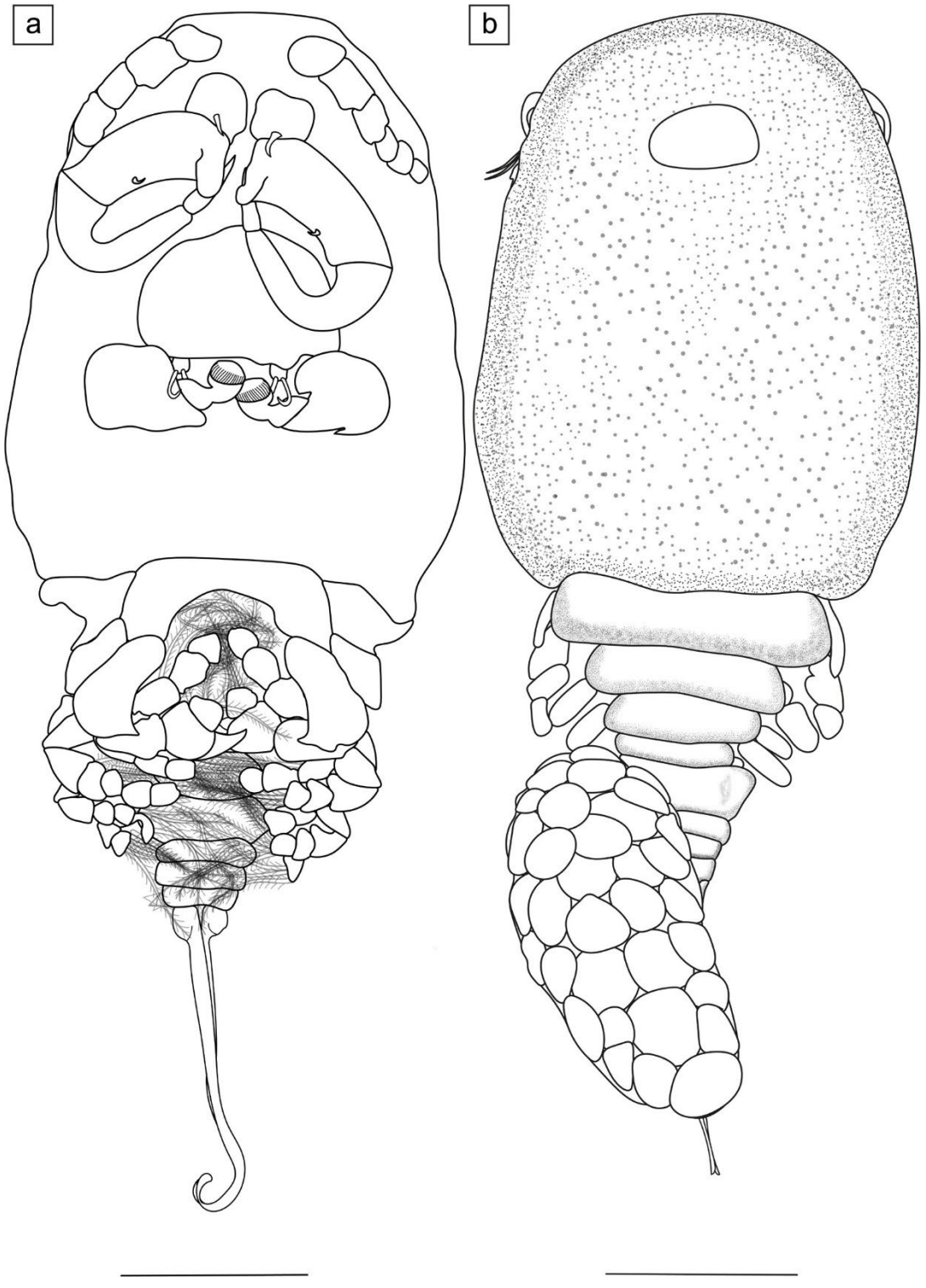


Figure 7.2: Illustrations of adult female *Neoergasilus* sp. nov. (a) ventral view, without eggs; (b) dorsal view; with one egg string exposing part of the urosome. Scale bars: (a & b) 100 µm.

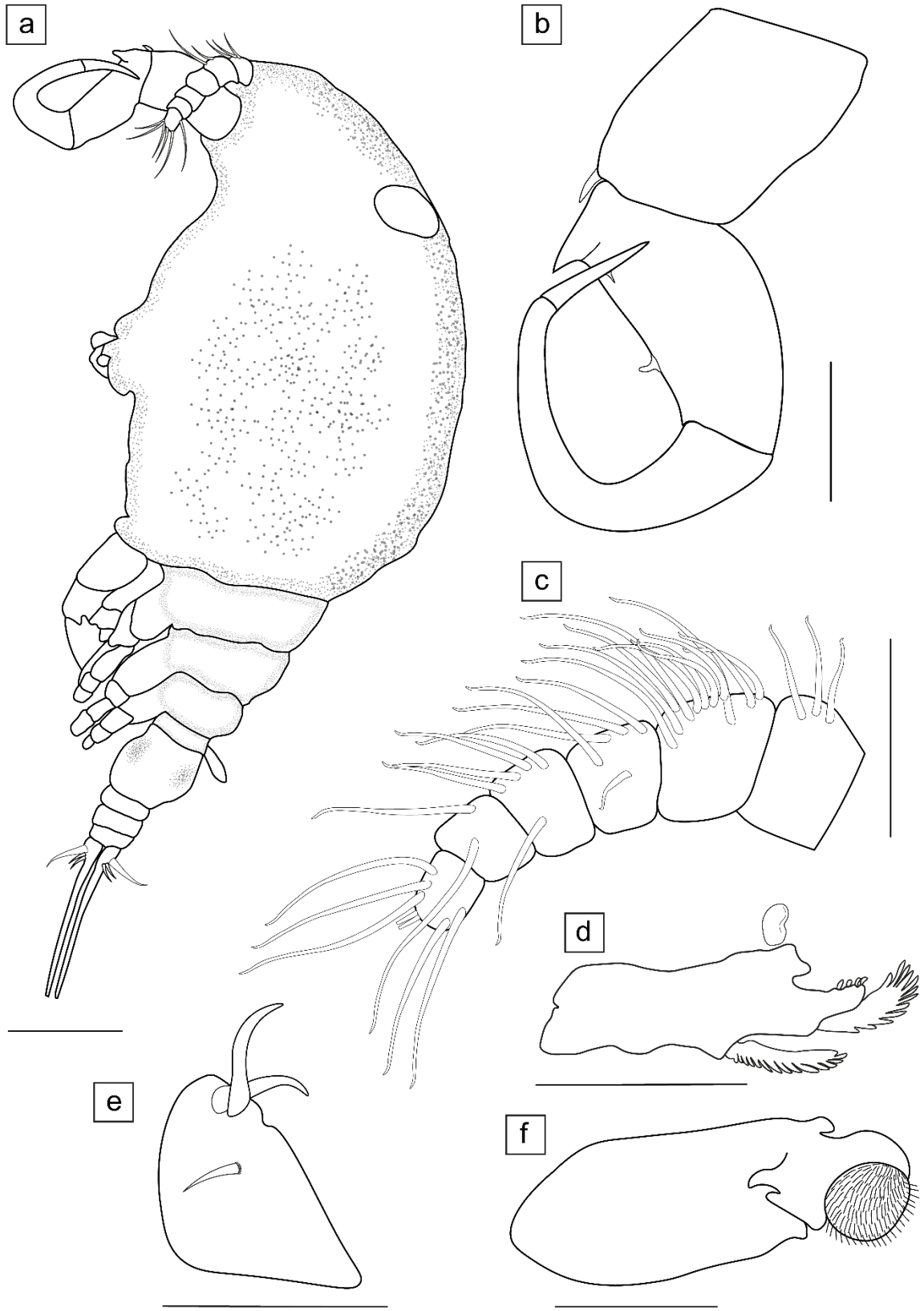


Figure 7.3: Illustrations of adult female *Neoergasilus* sp. nov. with drawings of its cephalic appendages. (a) lateral view, without eggs; (b) antenna; (c) antennule; (d) mandible; (e) maxillule; (f) maxilla. Scale bars: (a) 100 µm; (b, c, e, f) 50 µm; (d) 25 µm.

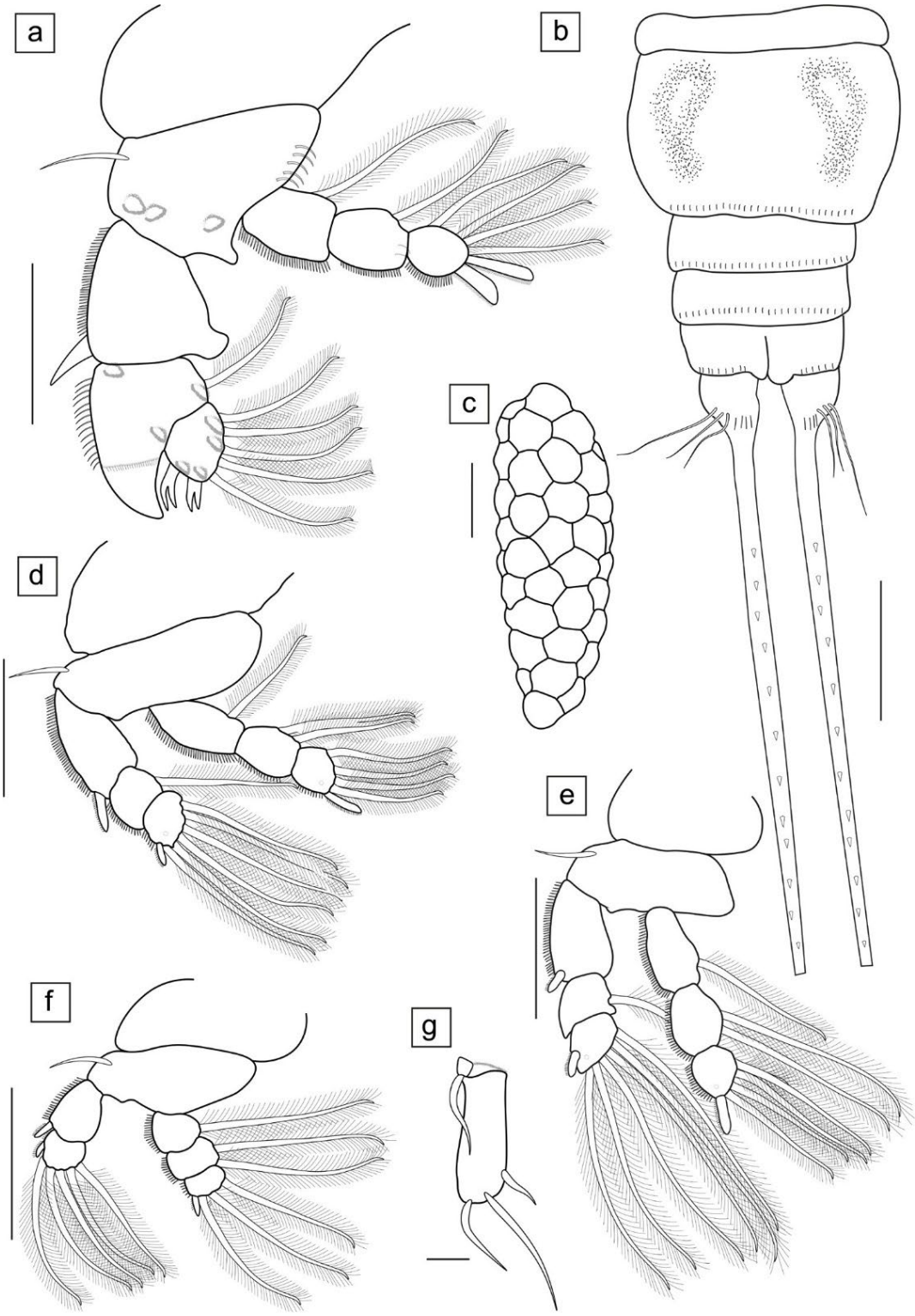


Figure 7.4: Illustrations of the legs, urosome, furcal rami and egg string of the adult female *Neoergasilus* sp. nov. (a) leg 1 showing the triangular process on the posterior margin of the basiopodite, the knob-like process on the inner distal margin of the first exopodite, and the spatulate spine on the outer distal margin of the second exopodite extending longer than the third exopodite spines; (b) urosome showing ventral row of spinules on genital double-somite, abdominal somites, and furcal rami with simple setae of varying sizes, and median seta of furcal rami with an array of spines; (c) egg string; (d) leg 2; (e) leg 3; (f) leg 4; (g) leg 5. Scale bars: (a– b, d–f) 50 µm; (c) 100 µm; (g) 10 µm.

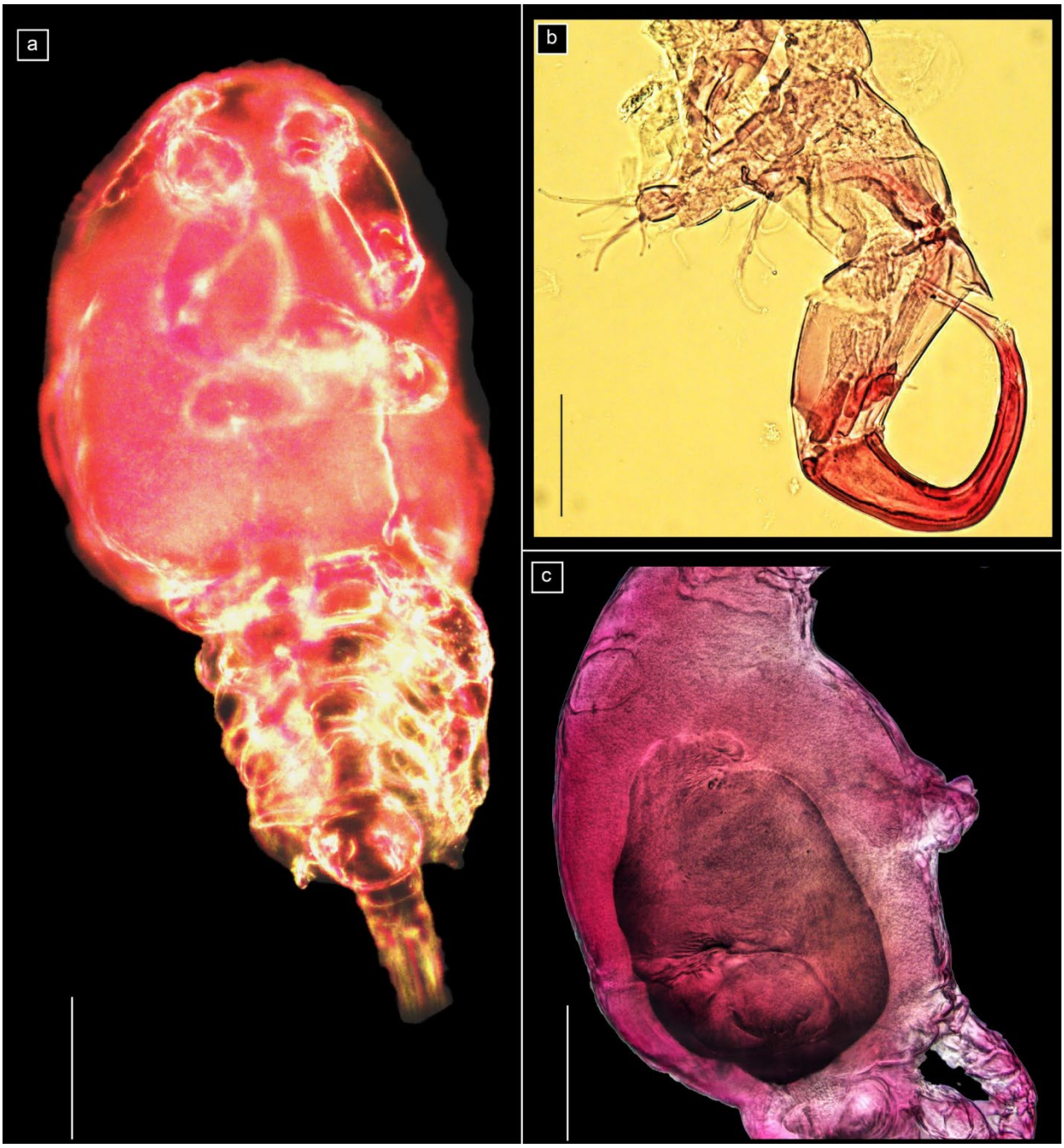


Figure 7.5: Light microscopy images of adult female *Neoergasilus* sp. nov. (a) ventral view using dark-field phase contrast image function; (b) differential interference contrast (DIC) microscope image of cleared and stained dissected antenna and antennule, applying the z-dimensional function; (c) DIC z-stack image of cephalothorax of cleared and stained *Neoergasilus* sp. nov. showing the oval ornamentation on the anterior of its cephalosome. Scale bars: (a, c) 100 µm; (b) 50 µm.

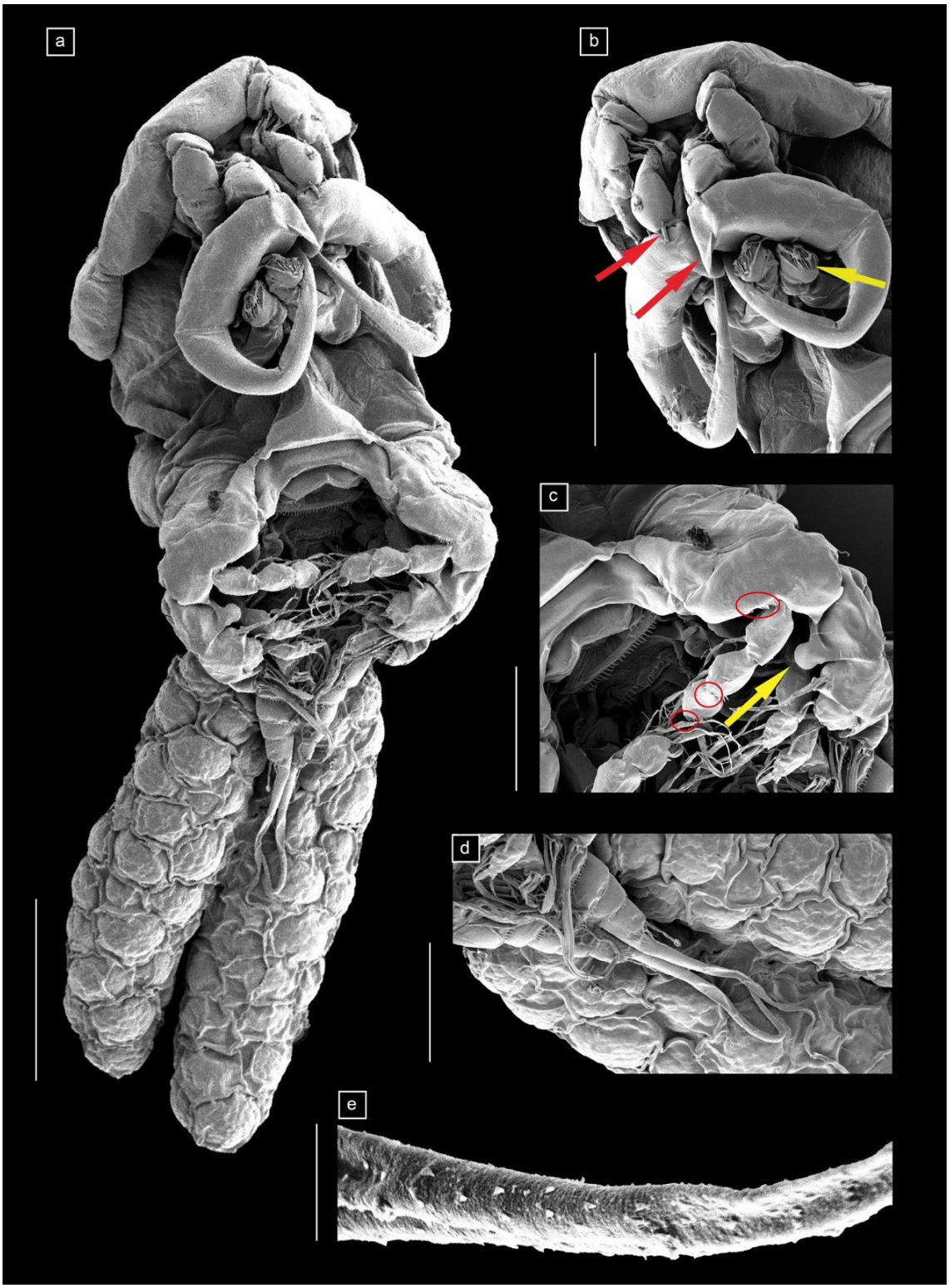


Figure 7.6: Scanning electron microscope (SEM) images of *Neoergasilus* sp. nov. adult female. (a) ventral view; with two egg strings; (b) ventral view of cephalosome showing spines in the antenna (red arrows) and maxilla (yellow arrow); (c) thoracic region showing denticles in segments of leg 1 (red circles) and knob-like process in inner margin of first exopodite of leg 1 (yellow arrow); (d) urosome; (e) median setae of furcal rami ornamented with an array of spines. Scale bars: (a) 100 µm; (b–d) 50 µm; (e) 5 µm.

Table 7.3: Spine-setae formula for swimming legs of *Neoergasilus* sp. nov. from South Africa.

Number of spines in Roman numerals, number of setae in Arabic numerals. Spine with asterisks* represents the generic spatulate spine.

	Coxa	Basis	Exopod	Endopod
Leg 1	0–0	I–0	I–0; I*–1; II–5	0–1; 0–1; II–4
Leg 2	0–0	I–0	I–0; 0–1; I–6	0–1; 0–2; I–4
Leg 3	0–0	I–0	I–0; 0–1; I–6	0–1; 0–2; I–4
Leg 4	0–0	I–0	I–0; I–4	0–1; 0–2; I–3

Remarks.

The new parasite species differs from the other nine known species by a combination of the following characteristics: an inflated cephalothorax 0.75 times as wide as it is long; the presence of an oval ornamentation anterior to the cephalosome; a knob-like process on the internal distal margin of the first exopodal segment of leg 1 and two forked spines on the third exopodal segment of leg 1; leg 4 bearing a 2-segmented exopod and 3-segmented endopod; an unsegmented fifth leg with a long seta with a broad base at the dorsolateral margin of the fifth thoracic segment, and free segment with three unequal setae; a long median seta of the furcal rami ornamented with an array of short spines; as well as the setation of the antennule; and the armature formula of the legs.

When compared to the other nine *Neoergasilus* species, the inflated cephalosome of *Neoergasilus* sp. nov. is similar to *N. squaliobarbi* and *N. bullatus*; the remaining seven species have a more elongate cephalosome. It differs, however, by the presence of an oval ornamentation at the anterior region of the cephalosome which is absent in *N. squaliobarbi* and *N. bullatus. Neoergasilus* sp. nov. has a stout knob-like process on the internal distal margin of the first exopodite of leg 1, which is absent in *N. squaliobarbi. Neoergasilus bullatus,* on the other hand, possesses a long blade-like inner distal process much longer than the segment itself and different from the knob-like process observed in *Neoergasilus* sp. nov. The antennae of *Neoergasilus* sp. nov. have a stout cone-shaped spine at the base of its second segment, which is absent in both *N. bullatus* and *N. squaliobarbi*. Additionally, the exopod and endopod of the fourth leg of *Neoergasilus* sp. nov. are 2- and 3-segmented respectively, sharing similarities with *N. bullatus*, but differing from *N. squaliobarbi,* which has a single-segmented exopod and 2- segmented endopod. The fifth leg of *Neoergasilus* sp. nov. is uniramous and single-segmented, with a seta on the margin of the thoracic segment, similar to *N. bullatus.* The free segment of *Neoergasilus* sp. nov. bears three unequal setae; *N. bullatus*, however, has only a long plumose terminal seta and a minute lateral seta. In the case of *N. squaliobarbi*, authors report an absent fifth leg.

Geographically, only *N. japonicus* has been officially reported in the southern hemisphere (Mendes Marques & Murrieta Morey, 2019) and in northern Africa (Berrouk et al., 2018; 2020). There is also an unpublished record of *N. japonicus* from *C. gariepinus* in South Africa (Smit et al., 2018). The species described in this study differs from *N. japonicus* with several morphological characters. The cephalosome is inflated compared to the more elongate cephalosome of *N. japonicus*. This species also has an oval ornamentation on the cephalothorax otherwise absent in the descriptions of *N. japonicus*. In addition to the elongate spine on the proximal margin of the first antennal segment, *Neoergasilus* sp. nov. is ornamented with a stout cone-shaped spine at the base of its second antennal segment which is absent on the antennae of *N. japonicus.* The fourth leg of *Neoergasilus* sp. nov. has a 2-segmented exopod and a 3-segmented endopod, whereas *N. japonicus* possesses a single-segmented endo- and exopod. Additionally, the spine-setae formulae of the legs are different for *Neoergasilus* sp. nov. and *N. japonicus* (leg armature formula for *N. japonicus* is given in Chapter 6).

Key to the species of Neoergasilus.

To date, the only available key to the species in the genus *Neoergasilus* was by Kumari et al. (1988), comprising only five species available at the time, two of which were newly described by the authors. At that point, Smirnova had already synonymised *N. inflatus* with *N. squaliobarbi* (see Smirnova, 1971). Since then, four more species have been described and a fifth *Neoergasilus* species, i.e., *Neoergasilus* sp. nov., is described in this study, increasing the total number to ten species. We, therefore, propose the key below to delineate species of the genus *Neoergasilus*, which was constructed using drawings and texts of original descriptions and supporting descriptions of these species.

This key is based on the morphological characters of the adult female:

1. Cephalothorax inflated (Fig. 7.7a) (at least 0.75 times as wide as long)……..2

   Cephalothorax elongate (Fig. 7.7b) (less than 0.75 times as wide as long)……...….4

2. Antennal claw short and broad with tooth-like projection on inner margin (Fig. 7.7d); first exopodite of leg 1 with smooth inner margin……***N. squaliobarbi***

   Antennal claw without tooth-like projection on inner margin (Fig. 7.7c, e); first exopodite of leg 1 with bulging process on inner margin……...…….3

3. Oval ornamentation on cephalothorax (Fig. 7.7a); knob-like process on inner margin of leg 1 first exopodite (Fig. 7.7f); elongate spine on proximal margin of first antennal segment with distally placed stout cone-shaped spine on second antennal segment……...……..***Neoergasilus* sp. nov.**

   Cephalothorax without oval ornamentation; blade-like process on inner margin of leg 1 first exopodite (Fig. 7.7g); first antennal segment with one large, scalpel-like distal spine (Fig. 7.7c)……***N. bullatus***

4. Leg 1 with bulging process on inner or outer margin of first exopodite……5

   Leg 1 without bulging process on inner or outer margin of first exopodite……..7

5. Knob-like process present (Fig. 7.7f) on inner margin of first exopodite of leg 1…..6

   Elongate blade-like process present (see Fig. 7.7g) on outer distal margin of first exopodite of leg 1; Leg 5 uniramous, single-segmented with two long terminal setae, one short lateral seta……***N. kherai***

6. Leg 5 uniramous, two-segmented with single seta on proximal segment and three unequal setae on distal segment.……..……...***N. indicus***

   Leg 5 uniramous, single-segmented with three setae; spines on outer margin of first exopodite less than half the length of second segment in legs 2–4…….***N. notopteri***

7. Leg 4 with single-segmented exo- and endopod; first antennal segment with elongate distal spine (Fig. 7.7e); spines on outer margin of first exopodite less than half the length of second segment in legs 2–4……..***N. japonicus***

   Leg 4 with at least 2-segmented endo- and exopod……8

8. Spines on outer margin of first exopodite longer than second segment in legs 2–4……..……9

   Spines on outer margin of first exopodite longer than third segment in legs 2–4; leg 5 with three equal setae……...……..…….***N. longispinosus***

9. Leg 5 uniramous with two setae; second antennal segment with one large, scalpel-like spine (Fig. 7.7c) towards proximal margin……..……..***N. ferozepurensis***

   Leg 5 with one dorsolateral seta on fifth pedigerous somite and three unequal setae on free segment; first antennal segment with one large, scalpel-like distal spine (Fig. 7.7c)……..***N. angustus***

The table of the characteristics compared throughout all ten *Neoergasilus* species, which was used in generating this key can be found in the appendix (Appendix Table A4).

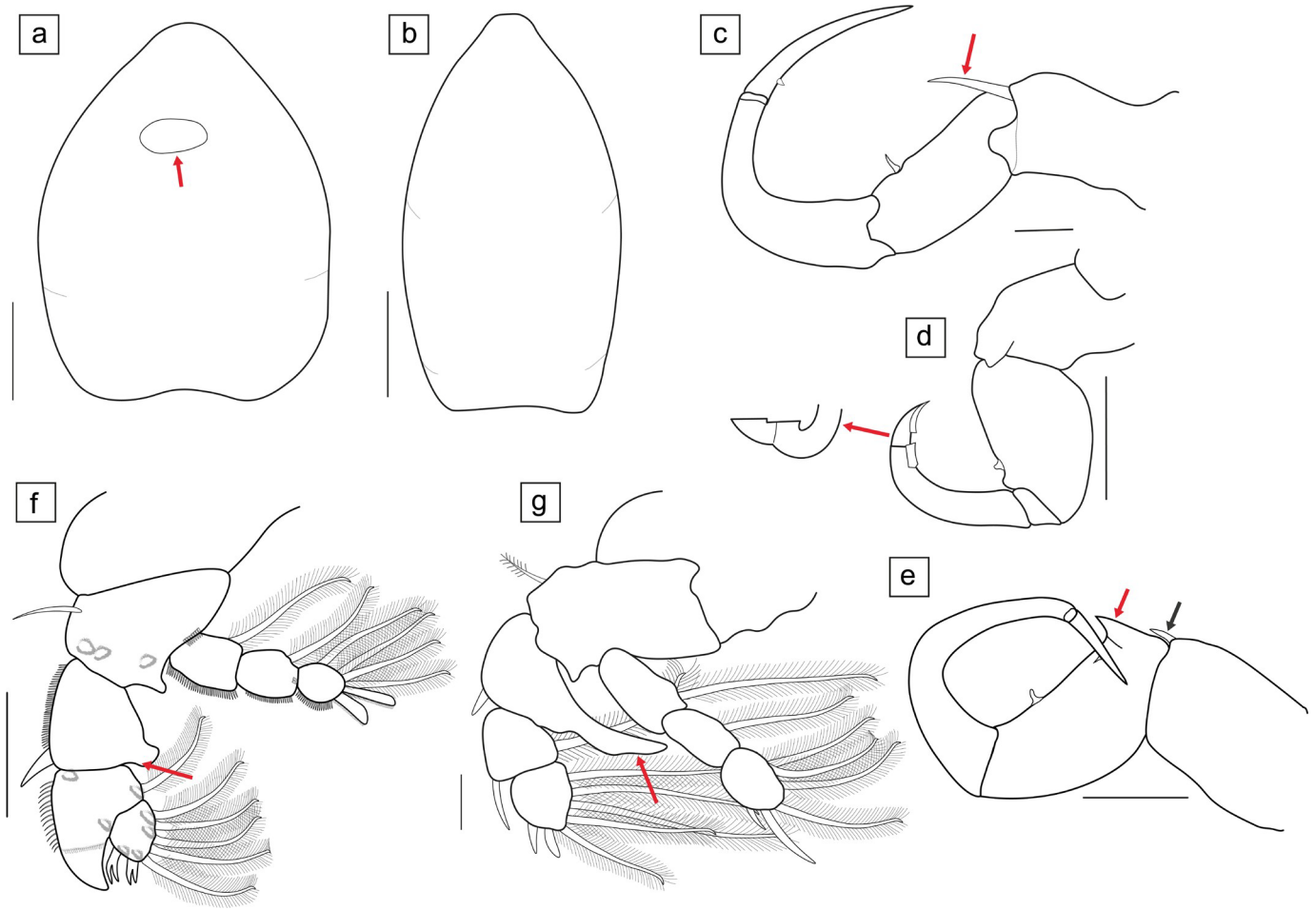


Figure 7.7: Illustrations of certain morphological characters used for keying out *Neoergasilus* Yin, 1956 species. (a) inflated cephalothorax with oval ornamentation (red arrow); (b) elongate cephalothorax without any ornamentation; (c) antenna with scalpel-like spine (red arrow); (d) antenna highlighting short and broad claw with tooth-like projection on inner margin (red arrow); (e) antenna with short elongate spine (black arrow) and stout cone-shaped spine (red arrow); (f) leg 1 with knob-like bulging process (red arrow) on inner margin of first exopodite; (g) leg 1 with blade-like process (red arrow) on inner margin of first exopodite. Scale bars: (a, b) 100 µm; (c, g) 20 µm; (d–f) 50 µm.

7.4.2. Molecular analysis

Seven novel sequences were generated from this study (three sequences for 18S, two sequences for 28S, and two sequences for COI). The phylogenetic analyses were done at family level since only one species from the genus, that is, *N. japonicus* has sequences deposited on GenBank (see Table 7.2). Tree topologies for Bayesian Inference and Maximum Likelihood analyses were similar for both 18S and 28S.

Phylogenetic alignments of 18S sequences yielded an alignment length of 1,387 bases. Newly generated sequences were 100% identical. Genetic divergence in percentage identity and base pair differences showed the highest similarity (97.70–97.87%) between newly generated sequences and *Sinergasilus* species (28–30 base pair difference). When compared with published *N. japonicus* sequences, there was a 97.48–97.71% range similarity (30–33 bp) (see Table 7.4). With the phylogenetic tree constructed (Fig. 7.8), the sequences generated from this study clustered with good BI support as a subclade to other genera in the family Ergasilidae, confirming its position within the family. The newly generated sequences clustered as a subclade to sequences of *Sinergasilus* species: *S. major* (Markevich, 1940), *S. polycolpus* (Markevich, 1940), and *S. undulatus* (Markevich, 1940); as well as some *Ergasilus* species: *E. anchoratus* Markevich, 1946 and *E. scalaris* Markevich, 1940. The genus *Ergasilus* is also seen forming several subclades with species from other genera in the family Ergasilidae: *Acusicola* and *Sinergasilus*. Sequences of *N. japonicus* also clustered with sequences of other *Ergasilus* species: *E. briani*, *E. hypomesi* Yamaguti, 1936, *E. parasiluri* Yamaguti, 1936, *E. peregrinus* Heller, 1865 and *E. tumidus* Markevich, 1940.

The phylogenetic alignment of the partial 28S gene region resulted in a length of 751 bases. Novel sequences were 100% identical. The closest Ergasilidae sequences with regards to percentage similarities were *E. hypomesi* (93.97–94.06%; 40–42 bp), *E. sieboldi* (92.89%; 46 bp), *E. peregrinus* (93.15–93.24%; 46–47 bp) and *E. briani* (92.95–93.02%; 47–49 bp). Newly generated sequences differed from *N. japonicus* sequences by 60–62 bases (90.29–91.08% similarity) (see Table 7.5). The phylogenetic trees for both BI and ML trees had similar topologies. Newly generated sequences clustered in a clade consisting of *Ergasilus* species (*E. anchoratus* and *E. peregrinus*) and *Sinergasilus* species (*S. major*, *S. polycolpus* and *S. undulatus*) (Fig. 7.9). As with the 18S, the phylogenetic analysis confirms that specimens from this study belong in the family Ergasilidae. From the 28S phylogenetic tree, the genus *Ergasilus* is seen in clades with *Sinergasilus* species; sequences of *N. japonicus* clustered in a clade with *Ergasilus* species: *E. briani*, *E. parasiluri*, *E. tumidus* and *E. scalaris.*

Table 7.4: Genetic divergences among aligned 18S sequences expressed as percentage similarity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from this study and The Czech Republic). Sequences from the present study are presented in bold and in grey shade, *Lernaea cyprinacea* (MH982204) was used as the outgroup. Abbreviations: AR – Argentina, BB – Babice, CN – China, CZ – Czech Republic, FR – The Great Fish River, HU – Hungary, HV – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, RH – Rohlik, ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	**P40R_13 FR *Neoergasilus* sp. nov. ZA**		**0**	**0**	**39**	**31**	**34**	**30**	**39**	**39**	**33**	**43**	**34**	**41**	**30**	**30**	**30**	**30**	**31**	**42**	**41**	**41**	**41**	**29**	**29**	**29**	**28**	**133**
2	**P47K_13 FR *N.* sp. nov. ZA**	**100**		**0**	**39**	**31**	**35**	**30**	**39**	**40**	**33**	**44**	**35**	**44**	**32**	**31**	**32**	**31**	**33**	**44**	**42**	**42**	**43**	**29**	**29**	**30**	**29**	**142**
3	**P59R_47 FR *N.* sp. nov. ZA**	**100**	**100**		**39**	**31**	**35**	**30**	**39**	**40**	**33**	**44**	**35**	**44**	**32**	**31**	**32**	**31**	**33**	**43**	**42**	**42**	**42**	**29**	**29**	**30**	**29**	**141**
4	MN852694 *Acusicola margulisae* NI	**96.95**	**97.13**	**97.01**		37	41	34	43	48	32	32	41	43	36	34	35	35	37	45	41	41	45	34	34	34	34	143
5	DQ107564 *Ergasilus anchoratus* CN	**97.57**	**97.72**	**97.63**	97.28		31	24	39	28	24	33	31	37	30	31	32	29	31	34	31	33	34	9	9	12	13	140
6	DQ107572 *Ergasilus briani* CN	**97.34**	**97.43**	**97.32**	96.98	97.72		14	37	41	21	37	4	36	12	13	14	11	13	37	34	34	20	31	31	32	34	143
7	DQ107573 *Ergasilus hypomesi* CN	**97.65**	**97.79**	**97.70**	97.50	98.23	98.97		31	35	19	31	14	34	12	13	14	11	13	35	32	32	20	23	23	24	26	141
8	DQ107577 *Ergasilus peregrinus* CN	**96.95**	**97.13**	**97.01**	96.84	97.13	97.28	97.72		50	33	43	36	39	33	34	35	32	34	39	36	36	42	39	39	38	38	148
9	DQ107565 *Ergasilus scalaris* CN	**96.95**	**97.06**	**96.94**	96.47	97.94	96.98	97.42	96.32		32	42	41	45	39	41	41	39	40	43	40	40	44	27	27	28	29	146
10	MW810238 *Ergasilus sieboldi* CZ	**97.42**	**97.57**	**97.47**	97.65	98.23	98.45	98.60	97.57	97.65		27	21	23	21	22	23	20	22	32	27	27	28	23	23	24	27	135
11	MT969345 *Ergasilus yandemontei* AR	**96.70**	**96.75**	**96.69**	97.65	97.57	97.27	97.72	96.82	96.89	98.02		37	34	31	31	31	31	32	37	33	33	37	30	30	33	36	141
12	DQ107569 *Ergasilus tumidus* CN	**97.34**	**97.43**	**97.32**	96.98	97.72	99.71	98.97	97.35	96.98	98.45	97.27		36	12	13	14	11	13	37	34	34	20	31	31	32	34	144
13	DQ107578 *Ergasilus yaluzangbus* CN	**96.79**	**96.77**	**96.63**	96.84	97.28	97.35	97.50	97.13	96.69	98.31	97.50	97.35		33	33	35	31	34	39	34	35	39	33	33	35	38	140
14	MW810236 *Neoergasilus japonicus* BB CZ	**97.65**	**97.64**	**97.55**	97.35	97.79	99.12	99.12	97.57	97.13	98.45	97.72	99.12	97.57		2	2	0	1	41	36	36	22	28	28	29	29	139
15	MH167969 *Neoergasilus japonicus* HV CZ	**97.65**	**97.71**	**97.62**	97.49	97.71	99.04	99.04	97.49	96.97	98.38	97.71	99.04	97.56	99.85		0	2	3	40	35	35	22	27	27	28	28	136
16	MW810237 *Neoergasilus japonicus* JZ CZ	**97.65**	**97.64**	**97.55**	97.42	97.64	98.97	98.97	97.42	96.98	98.31	97.72	98.97	97.42	99.85	100		2	3	41	36	36	22	28	28	29	29	139
17	MH167970 *Neoergasilus japonicus* RH CZ	**97.65**	**97.71**	**97.62**	97.42	97.86	99.19	99.19	97.64	97.12	98.52	97.71	99.19	97.71	100	99.85	99.85		1	40	35	35	22	27	27	28	28	136
18	KR048752 *Neoergasilus japonicus* KR (unpublished)	**97.58**	**97.58**	**97.48**	97.28	97.72	99.04	99.04	97.50	97.06	98.38	97.65	99.04	97.50	99.93	99.78	99.78	99.93		42	37	37	23	29	29	30	30	140
19	DQ107576 *Paraergasilus brevidigitus* CN	**96.71**	**96.76**	**96.71**	96.69	97.50	97.28	97.42	97.13	96.84	97.65	97.27	97.28	97.13	96.98	97.05	96.98	97.05	96.91		7	7	42	35	35	37	40	145
20	MW810239 *Paraergasilus longidigitus* CZ	**96.79**	**96.91**	**96.78**	96.98	97.72	97.50	97.64	97.35	97.05	98.01	97.57	97.50	97.50	97.35	97.42	97.35	97.42	97.28	99.48		2	39	33	33	35	38	141
21	DQ107574 *Paraergasilus medius* CN	**96.79**	**96.91**	**96.78**	96.98	97.57	97.50	97.65	97.35	97.06	98.01	97.57	97.50	97.43	97.35	97.42	97.35	97.42	97.28	99.48	99.85		39	33	33	35	38	142
22	DQ107567 *Ergasilus parasiluri* CN	**96.79**	**96.84**	**96.79**	96.69	97.5	98.53	98.53	96.91	96.76	97.94	97.27	98.53	97.13	98.38	98.38	98.38	98.38	98.31	96.91	97.13	97.13		34	34	35	37	152
23	MZ047814 *Sinergasilus major* HU	**97.73**	**97.87**	**97.78**	97.50	99.34	97.72	98.31	97.13	98.01	98.31	97.80	97.72	97.57	97.94	98.01	97.94	98.01	97.87	97.42	97.57	97.57	97.50		0	3	7	137
24	DQ107560 *Sinergasilus major* CN	**97.73**	**97.87**	**97.78**	97.50	99.34	97.72	98.31	97.13	98.01	98.31	97.80	97.72	97.57	97.94	98.01	97.94	98.01	97.87	97.43	97.57	97.57	97.50	100		3	7	137
25	DQ107563 *Sinergasilus polycolpus* CN	**97.73**	**97.8**	**97.70**	97.50	99.12	97.65	98.24	97.21	97.94	98.24	97.57	97.65	97.43	97.86	97.93	97.86	97.93	97.80	97.28	97.42	97.43	97.43	99.78	99.78		6	136
26	DQ107561 *Sinergasilus undulatus* CN	**97.81**	**97.87**	**97.78**	97.50	99.04	97.50	98.09	97.21	97.87	98.01	97.35	97.50	97.21	97.86	97.93	97.86	97.93	97.80	97.06	97.20	97.21	97.28	99.48	99.49	99.56		142
27	MH982195 *Lernaea cyprinacea* CN	**89.79**	**89.75**	**89.41**	89.68	89.89	89.68	89.82	89.31	89.46	90.25	89.63	89.60	89.89	89.95	90.14	89.95	90.14	89.90	89.53	89.81	89.75	89.03	90.10	90.11	90.18	89.75	

Table 7.5: Genetic divergences among aligned 28S sequences expressed as percentage identities (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country (sites are included for sequences from this study and The Czech Republic). Sequences from the present study are presented in bold and in grey shade, *Lernaea cyprinacea* (MH982204) was used as the outgroup. Abbreviations: BB – Babice, CN – China, CZ – Czech Republic, EG – Egypt, FR – The Great Fish River, HU – Hungary, HV – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, RH – Rohlik, Unp. – Unpublished, ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	**P50Ra2_13FR *Neoergasilus* sp. nov. ZA**		**0**	**53**	**67**	**47**	**40**	**46**	**51**	**46**	**55**	**55**	**75**	**61**	**60**	**60**	**61**	**61**	**55**	**50**	**50**	**51**	**62**	**83**	**69**	**73**	**212**
2	**P59Ra2_47FR *Neoergasilus* sp. nov. ZA**	**100**		**53**	**68**	**49**	**42**	**47**	**52**	**46**	**57**	**57**	**77**	**61**	**60**	**60**	**61**	**62**	**55**	**52**	**52**	**53**	**62**	**85**	**71**	**75**	**212**
3	MN852851 *Acusicola margulisae* NI	**91.73**	**91.76**		71	50	43	49	51	54	59	53	79	64	62	62	63	63	49	45	49	49	67	82	71	71	203
4	DQ107528 *Ergasilus anchoratus* CN	**90.04**	**90.23**	88.89		74	63	64	76	73	80	78	100	81	80	80	81	83	72	72	71	78	9	25	12	11	228
5	DQ107532 *Ergasilus briani* CN	**93.02**	**92.96**	92.18	89.31		32	43	23	43	48	19	77	46	45	45	46	47	51	47	55	18	71	87	74	75	213
6	DQ107539 *Ergasilus hypomesi* CN	**94.06**	**93.97**	93.27	90.90	95.38		41	35	40	45	35	68	43	41	41	42	43	45	47	49	32	55	75	62	62	213
7	DQ107531 *Ergasilus peregrinus* CN	**93.15**	**93.24**	92.32	90.74	93.78	94.07		46	46	54	46	66	59	58	58	59	61	46	43	42	45	59	80	66	69	208
8	DQ107538 *Ergasilus scalaris* CN	**92.41**	**92.52**	92.01	89.00	96.67	94.93	93.33		46	51	30	73	40	36	38	37	41	56	55	59	9	74	91	78	79	205
9	MW810242 *Ergasilus sieboldi* CZ	**92.89**	**92.89**	91.25	88.68	93.33	93.80	92.86	92.86		14	44	66	53	51	51	52	52	49	49	50	46	71	90	76	79	218
10	OM812074 *Ergasilus sieboldi* EG	**91.63**	**91.45**	90.53	87.97	92.78	93.23	91.87	92.32	97.83		47	71	55	50	53	51	56	53	55	58	51	74	93	79	82	222
11	DQ107535 *Ergasilus tumidus* CN	**91.83**	**91.81**	91.71	88.73	97.25	94.94	93.34	95.66	93.18	92.93		74	43	41	41	42	43	52	52	58	25	75	91	78	77	213
12	DQ107540 *Ergasilus yaluzangbus* CN	**88.91**	**88.98**	87.73	85.65	88.95	90.24	90.52	89.51	89.81	89.37	89.38		78	75	77	76	79	74	71	73	74	94	111	98	97	214
13	MW810240 *Neoergasilus japonicus* BB CZ	**90.83**	**90.83**	90.00	87.75	93.04	93.57	91.14	94.02	91.82	91.59	93.57	88.29		2	2	1	1	60	55	55	40	80	92	81	79	215
14	MH167967 *Neoergasilus japonicus* HV CZ	**90.45**	**90.45**	89.92	87.18	92.79	93.43	90.69	94.22	91.84	92.01	93.43	88.08	99.76		0	1	1	58	54	54	38	79	91	80	78	211
15	MW810241 *Neoergasilus japonicus* JZ CZ	**90.98**	**90.98**	90.24	87.90	93.19	93.80	91.21	94.24	92.06	91.82	93.80	88.44	99.77	100		1	1	58	54	54	38	79	91	80	78	214
16	MH167968 *Neoergasilus japonicus* RH CZ	**90.29**	**90.29**	89.76	87.02	92.63	93.27	90.53	94.06	91.68	91.85	93.27	87.92	99.92	99.84	99.84		0	59	55	55	39	80	92	81	79	212
17	KR048823 *Neoergasilus japonicus* KR (Unp.)	**90.92**	**91.08**	90.13	87.99	93.20	93.78	91.16	94.06	91.93	91.57	93.78	88.65	99.92	99.84	99.85	100		59	56	56	40	80	93	82	80	215
18	MW810243 *Paraergasilus longidigitus* CZ	**91.68**	**91.68**	92.27	89.04	92.24	93.15	92.99	91.46	92.33	91.78	92.09	88.82	90.93	90.69	91.16	90.53	91.01		9	15	58	66	83	70	70	207
19	DQ107530 *Paraergasilus brevidigitus* CN	**92.56**	**92.52**	92.95	89.58	93.20	93.20	93.77	92.03	92.39	91.72	92.47	89.80	91.67	91.33	91.82	91.17	91.88	98.62		8	56	67	84	71	71	207
20	DQ107529 *Paraergasilus medius* CN	**92.56**	**92.52**	92.32	89.73	92.04	92.91	93.91	91.45	92.24	91.27	91.61	89.51	91.67	91.33	91.82	91.17	91.88	97.70	98.83		60	64	83	70	72	209
21	DQ107536 *Ergasilus parasiluri* CN	**92.41**	**92.37**	92.32	88.71	97.40	95.37	93.48	98.70	92.86	92.32	96.38	89.37	94.02	93.90	94.24	93.74	94.20	91.16	91.88	91.30		75	91	78	79	206
22	MZ047815 *Sinergasilus major* HU	**90.58**	**90.58**	89.38	98.62	89.14	91.59	90.96	88.67	88.91	88.54	88.53	85.74	87.75	87.34	87.90	87.18	87.75	89.89	89.74	90.20	88.51		21	8	11	228
23	DQ107524 *Sinergasilus major* CN	**87.67**	**87.79**	87.17	96.37	87.43	89.16	88.42	86.83	86.05	86.02	86.85	84.07	86.08	85.42	86.23	85.26	86.54	87.37	87.84	87.99	86.83	96.77		19	22	239
24	DQ107525 *Sinergasilus polycolpus* CN	**89.75**	**89.80**	88.89	98.26	89.31	91.04	90.45	88.71	88.22	88.12	88.73	85.94	87.75	87.18	87.90	87.02	88.13	89.35	89.73	89.87	88.71	98.77	97.24		9	227
25	DQ107526 *Sinergasilus undulatus* CN	**89.15**	**89.22**	88.89	98.40	89.16	91.04	90.01	88.57	87.75	87.67	88.87	86.08	88.05	87.50	88.20	87.34	88.42	89.35	89.73	89.58	88.57	98.31	96.80	98.69		229
26	MH982204 *Lernaea cyprinacea* CN	**70.10**	**70.10**	70.36	67.75	69.87	69.87	70.58	71.00	68.50	68.19	69.87	69.86	69.66	68.88	69.73	68.73	69.59	70.72	70.72	70.44	70.86	67.75	66.20	67.89	67.61	

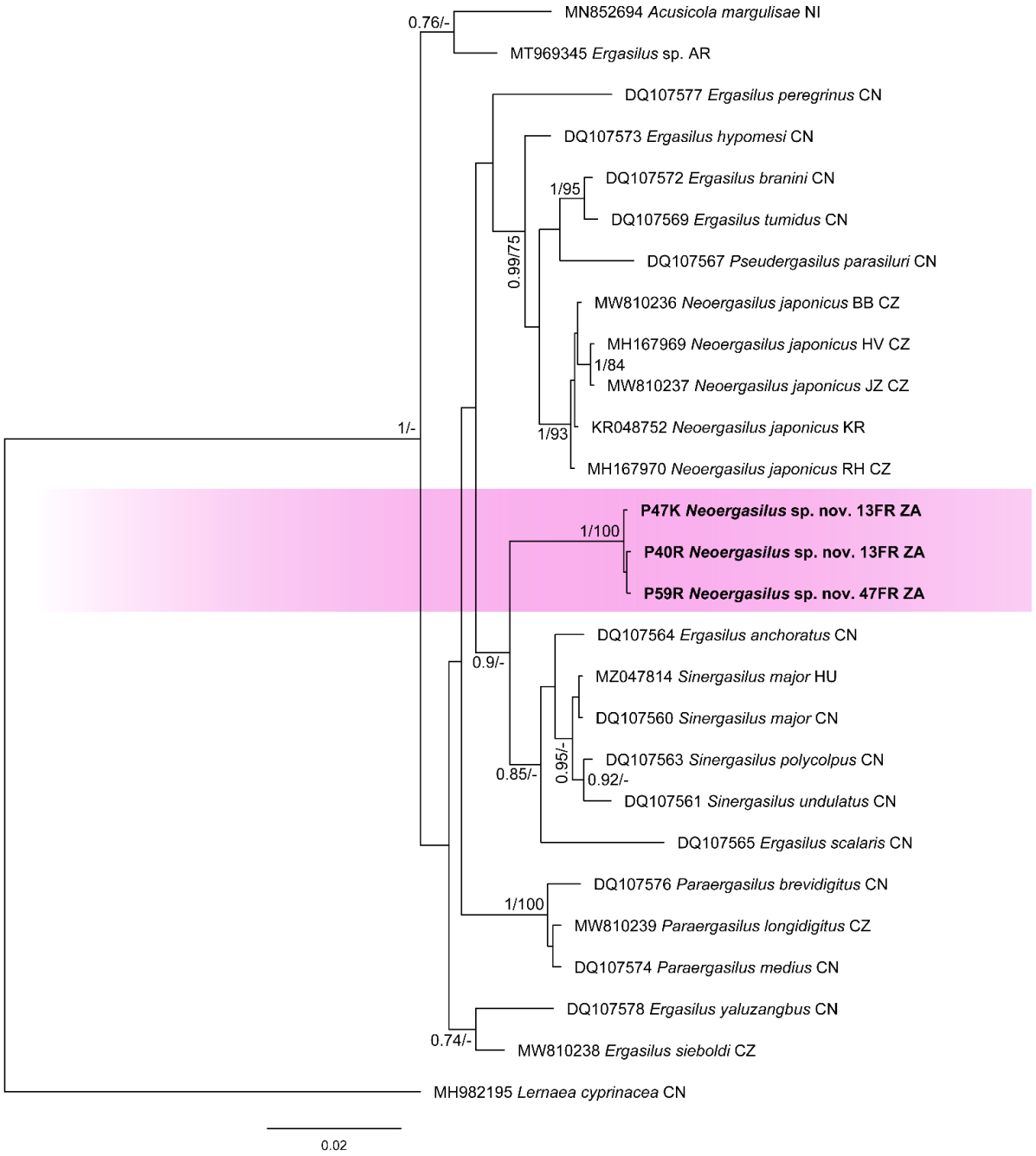


Figure 7.8: Phylogenetic tree of Ergasilidae copepods based on partial 18S rRNA gene alignments. Newly generated *Neoergasilus* sp. nov. sequences are bold in a gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: AR – Argentina, BB – Babice, CN – China, CZ – Czech Republic, FR – The Great Fish River, HU – Hungary, HV – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, RH – Rohlik, ZA – South Africa.

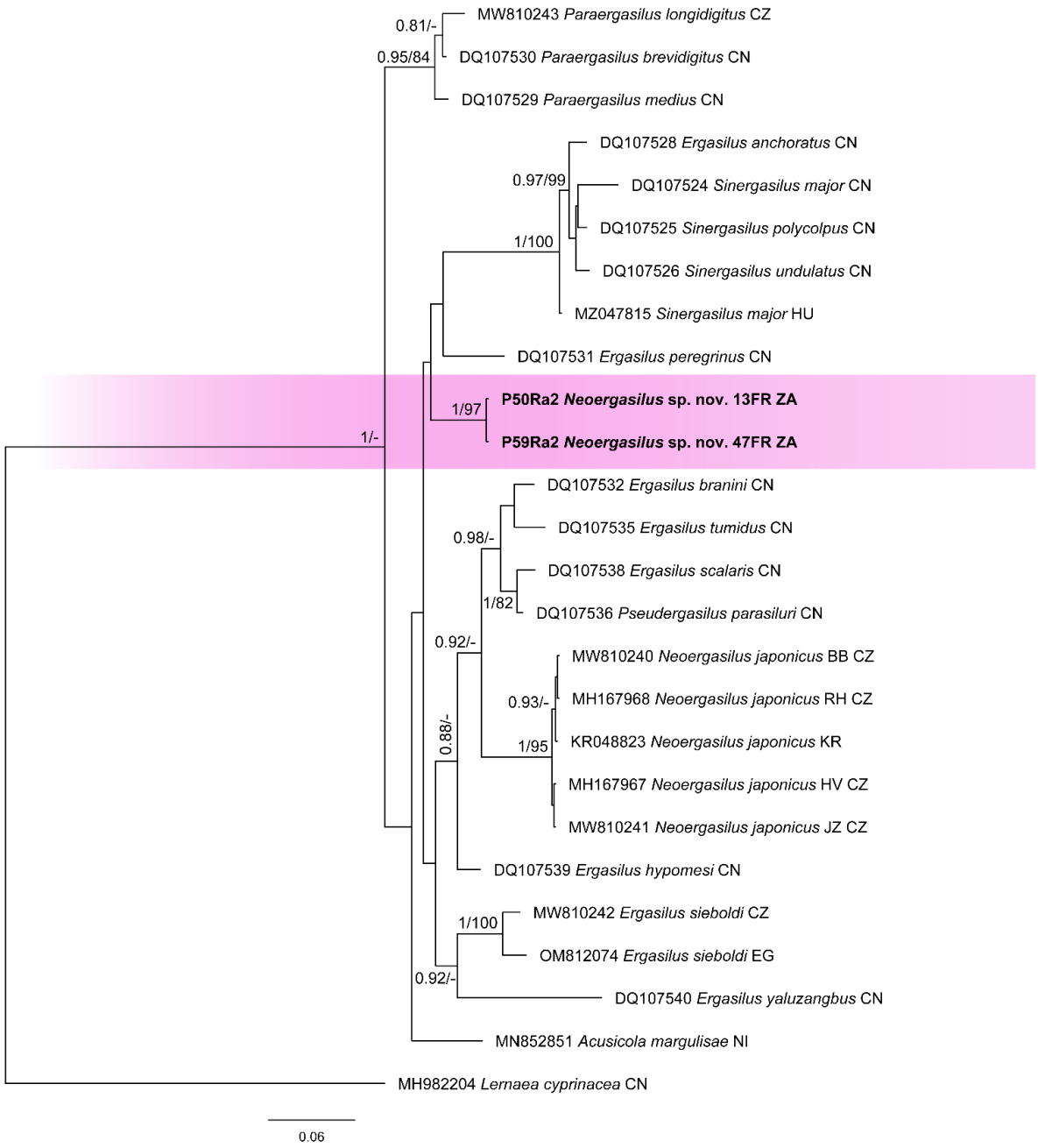


Figure 7.9: Phylogenetic tree of Ergasilidae copepods based on partial 28S rRNA gene alignments. Newly generated *Neoergasilus* sp. nov. sequences are bold in a gradient shade. Nodal support presented above or below branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Lernaea cyprinacea* Linnaeus, 1758 was used as the outgroup. Abbreviations: BB – Babice, CN – China, CZ – Czech Republic, EG – Egypt, FR – The Great Fish River, HU – Hungary, HV – Hvezda, JZ – Jezu, KR – South Korea, NI – Nicaragua, RH – Rohlik, ZA – South Africa.

No phylogenetic alignments are reported for the COI gene from this study. Preliminary alignment suggests that the primers used for DNA amplification of the specimens from this study amplified a fragment of the COI gene that is different from the fragments amplified for the other *Neoergasilus* and Ergasilidae sequences available on GenBank. These sequences are thus not compatible in the current form.

7.5. Discussion

The genus *Neoergasilus* is an Asian genus that was erected by Yin (1956) when he described two new species: *N. inflatus* and *N. longispinosus. Neoergasilus inflatus* has been synonymised with *N. squaliobarbi* (see Smirnova, 1971) because of its inflated cephalosome and the teeth on the inner margin of the antennal claw. It appears that the synonymisation of *N. inflatus is* not recognised by all authors or its status may still be unknown, as some authors have still used *N. inflatus* as a recognised species (see Kim & Choi, 2003).

The parasites collected from this study are morphologically different from the other three known Ergasilidae genera in Africa. They are placed in the genus *Neoergasilus* because of the unique morphology of the first swimming legs and the features of their antennae. Only the cosmopolitan species, *N. japonicus* has been reported from Africa (see Chapter 6). The specimens from this study are, however, morphologically differ from *N. japonicus* in a combination of features listed above.

As is common in taxonomy, within the genus *Neoergasilus,* certain morphological characters have been described differently by different authors. The antennae for instance, which is 4-segmented (Abdelhalim et al., 1993; El-Rashidy & Boxshall, 2002; Boxshall, 2016), is referred to as the second antenna by earlier authors and reported as 5-segmented by other authors. The description of the Ergasilidae antennae as given by El-Rashidy and Boxshall (2002) is 4-segmented (coxobasis and three endopodal segments) and a claw. The first antennal segment is the coxobasis, and the second to fourth antennal segments are the first to third endopodal segments (see Fig. 7.7c). It may be assumed that some authors have included the antennal claw as a segment (resulting in a 5-segmented antenna), rather than an attachment to the third endopodal segment. Kumari et al. (1988) describes the antenna of *N. ferozepurensis* as 5-semented which included the claw. The authors also referred to the coxobasis as the first and second antennal segments, thus excluding the third endopodal segment from their description. Others, on the other hand, report the antenna as 4-segmented, but might be referring to the claw as the fourth segment because the third endopodal segment is a greatly reduced segment and might easily be missed if not consciously inspected (see Kim & Choi, 2003).

Similarly, there has been confusion regarding the position of the generic spine(s) on the neoergasilid antenna. Many of the earlier authors had either described this spine to be on the second segment (Harada, 1930; Dogel' & Akhmerov, 1952; Vankara & Chikkam, 2010), at the margin between the second and third segment (Battish & Brar, 1989), or on the margin of the third segment (Kumari et al., 1988). However, not all spines described are the same. Two types of antennal spines have been reported on the first and second (or second and third according to other earlier authors) antennal segments. The first is a slender spine on the first segment, usually at the distal margin of the segment and may vary in length according to species, and the second is a slightly broader cone-shaped spine which is stout and pointed, almost extending from the base of the second segment. Three *Neoergasilus* species are described as having a slender spine on the first segment (*N. japonicus, N. longispinosus*, *N. squaliobarbi* (see Yin,1956; Kim & Choi, 2003), while *N. indicus* is described as having the cone-shaped stout spine. *Neoergasilus ferozepurensis* is described with a slender spine, and from the drawing, it is placed on the base of the second segment. The species from this study, *Neoergasilus* sp. nov., has two characteristic spines: the slender spine on the first antennular segment, similar to *N. squaliobarbi, N. japonicus*, and *N. longispinosus*; and also has a sharply pointed broad/stout cone-shaped spine extending from the basal end of the second antennal segment similar to *N. notopteri.* The antennae of *Neoergasilus* species may also be armed with other non-characteristic setae, spines, and papillae along the inner and outer margins of different segments.

Evolutionary analyses of the partial 18S and 28S rRNA genes of newly generated sequences confirm the position of this new species in the family Ergasilidae. The separation into clades seen with these genes might, however, be indicative of some form of polyphyly as seen in the genus *Ergasilus* (Song et al., 2008). With no other sequences from species in this genus, apart from *N. japonicus,* it is rather difficult to confirm this position. Although the COI sequences generated from this study may not be compatible in the current form, they will be essential for future evolutionary studies within the genus *Neoergasilus* and at family level.

The parasites from this study were collected from the gills of the sharptooth catfish, *C. gariepinus* in the Great Fish River, Eastern Cape South Africa. The other mention of a *Neoergasilus* species from this host in South Africa is an unpublished record for the invasive *N. japonicus* from Flag Boshielo Dam in the Olifants River, South Africa (Smit et al., 2018). The introduction of *Clarias gariepinus* was via translocation through the Orange-Fish River Inter-Basin Water Transfer (IBWT) (Cambray & Jubb, 1977). It cannot be affirmed at this point if *Neoergasilus* sp. nov. is a native species of catfish or if it is a product of spillback. Furthermore, as all of the other species described from this genus *Neoergasilus* have been from the northern hemisphere, it is unclear whether the species described in this study is an undescribed invasive species or if it is an undescribed native species from South Africa.

7.6. Conclusion

The specimens from this study are morphologically placed in the genus *Neoergasilus* based on the morphology of its first legs and antennae but differ from other nine species from this genus by a combination of morphological characteristics and have thus been described as new to science. These specimens were collected from the gills of the sharptooth catfish *Clarias gariepinus* sampled from the Great Fish River in the Eastern Cape Province, South Africa. This is the first species from this genus to be described from Africa and the southern hemisphere; and is the first *Neoergasilus* species to be described from the sharptooth catfish. Phylogenetic analyses of partial 18S and 28S genes confirm its placement in the family Ergasilidae, however, the sequences generated from this study do not clade with *N. japonicus* and may show a possibility of polyphyly within the genus, as seen in the genus *Ergasilus*. Apart from *N. japonicus*, there are no other *Neoergasilus* species with genetic data to confirm this relationship. There is, therefore, a need for more genetic data to be provided with species description records in order to understand the evolutionary relationship that exists within the genus *Neoergasilus* and the family Ergasilidae.

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CHAPTER 8

General Discussion

Fish parasites are an important component of the aquatic biodiversity in southern Africa (see Weyl et al., 2020 and the references therein). Numerous species have been reported to have significant impacts on both wild and farmed fish populations (Fryer, 1968; van As & Basson, 1984; Paperna, 1991; Piasecki et al., 2004; Hassanin et al., 2010; Shinn et al., 2023). Understanding the natural diversity of parasite species in a region can provide valuable insights into the species richness of the parasite communities, parasite-specific host populations, as well as the possibility of parasite spread to potential hosts (see Rowntree & Craig, 2019). The findings from this study have added valuable information on the diversity of fish parasitic crustaceans in southern Africa, with the identification of seven previously known parasite species and one species new to science, across several river systems (see Fig. 8.1.). The native species collected included *Ergasilus mirabilis* Oldewage & van As, 1987 (Ergasilidae), *Lamproglena clariae* Fryer, 1956 (Lernaeidae), *Lamproglena hepseti* Van As & Van As, 2007 (Lernaeidae), and *Lamproglena monodi* Capart, 1944 (Lernaeidae). Three known invasive species were also collected, namely, *Argulus japonicus* Thiele, 1900 (Argulidae), *Neoergasilus japonicus* (Harada, 1930) (Ergasilidae) and *Lernaea cyprinacea* Linnaeus, 1758 (Lernaeidae). Lastly, a new species *Neoergasilus* sp. nov. (Ergasilidae) was collected from the Great Fish River in South Africa.

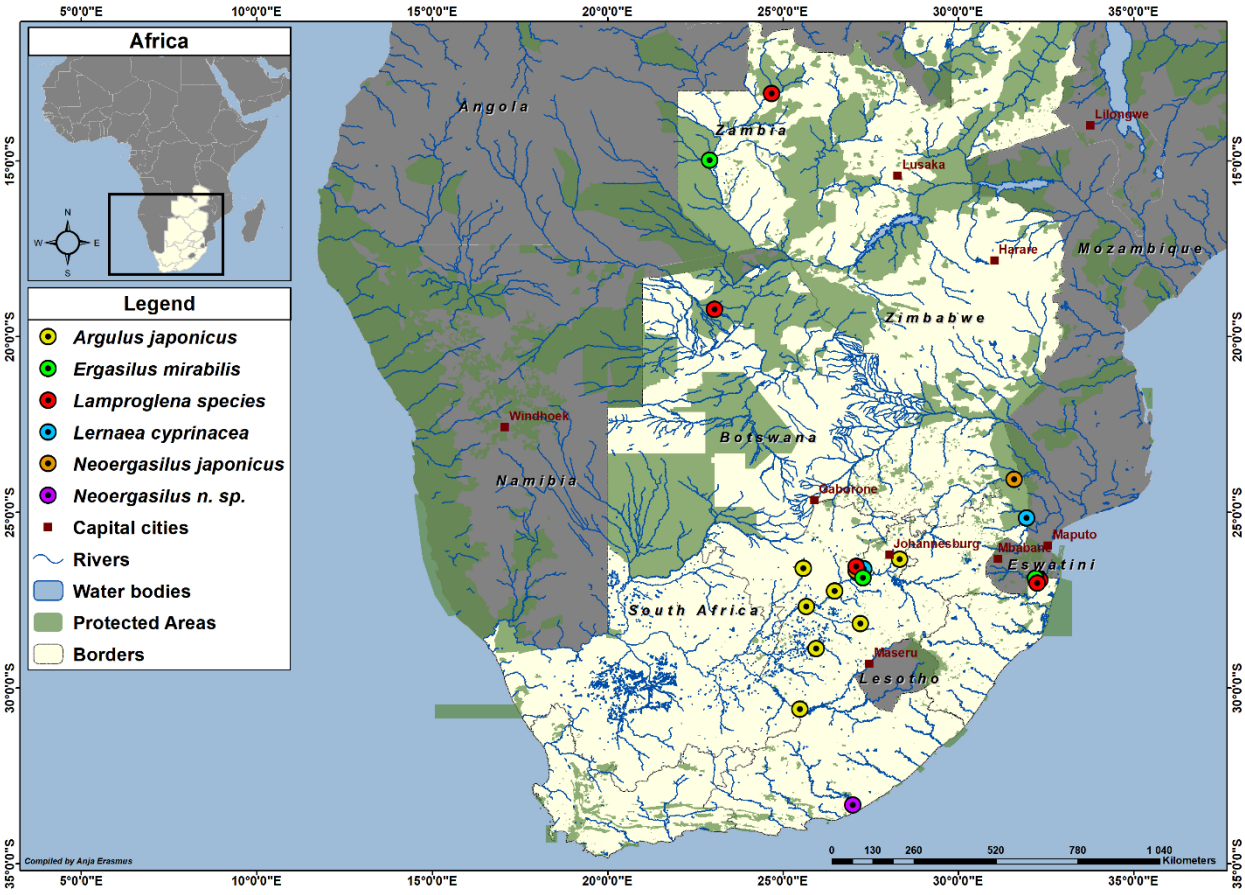


Figure 8.1: Map of all sampling localities in southern Africa where parasitic crustaceans were collected in the current study. Distribution of parasitic Crustacea represented as follows: *Argulus japonicus* Thiele, 1900 (yellow dots); *Ergasilus mirabilis* Oldewage & van As, 1987 (green dots); *Lamproglena* species von Nordmann, 1832 (red dots); *Lernaea cyprinacea* Linnaeus, 1758 (blue dots); *Neoergasilus japonicus* (Harada, 1930) (orange dots); *Neoergasilus* sp. nov. (purple dots).

8.1. Native copepod species of southern Africa

From this study, no representatives of native branchiurans were collected. The presence of four native parasitic copepod species reported from this study is consistent with reports within southern Africa (see van As, 2015).

Although the African ergasilid *E. mirabilis* is considered to be widely distributed across southern Africa (Oldewage & van As, 1988), there have been no recent reports of this parasite for almost four decades. From the current study, *E. mirabilis* was reported from major river systems in South Africa and Zambia (see Chapter 2), confirming its wide distribution in the southern African region. This present report, therefore, suggests that the absence of any records after 1994 (Douëllou & Erlwanger, 1994), might be because of a lack of research on this species, rather than the lack of parasites. In instances such as this, the lack of continuous reporting of parasitology research might greatly impact the perceived biodiversity

of these river systems. Further biodiversity research is encouraged across the other river systems of southern Africa, and findings on parasite distribution should be appropriately documented and published. More so, additional data not limited to the morphology (as was presented in this study) may be acquired from other parasitology and biodiversity-centered researchers.

Among the three *Lamproglena* species known, *L. clariae and L. monodi* have the widest distribution across several river systems within southern Africa (see checklist in Chapter 3). There are, therefore, extensive records on their morphology (see Marx & Avenant-Oldewage, 1996; Ibraheem & Izawa, 2000; Rindoria et al., 2022); distribution (Avenant-Oldewage & Oldewage, 1993; Hassan et al., 2013; Sinaré et al., 2016; Abdel-Gaber et al., 2017; M'bareck et al., 2019; Outa et al., 2021), and biology (Abdel-Gaber et al., 2017; Ibraheem et al., 2017; Moll & Avenant-Oldewage, 2017). These two species have also been utilised as target species in metal and stable isotope analysis (Gilbert et al., 2020; Latief et al., 2023; Pretorius & Avenant-Oldewage, 2023); microhabitat distributions research among hosts species (Gobbin et al., 2021); and as bioindicators of metal pollution (Pretorius & Avenant-Oldewage, 2022).

Compared to the other two *Lamproglena* species, *L. hepseti* has not been reported since its original description in 2007 (van As & van As, 2007) and, as such, no additional information is known. The high mean intensity of 20 reported from this study and the supplementary information provided on the maxilliped, antennae, and antennules, however, suggests that this species might be a potentially important species for morphological research. *Lamproglena hepseti* may also be appropriate for comparative histological studies that focus on the attachment and associated pathology of the parasite on host gills, as seen with *L. clariae and L. monodi*.

In southern Africa, the distribution of *L. hepseti* seems to be within the Okavango River and Delta. As is consistent with the genus, *Lamproglena*, *L. hepseti* is very host specific and seems to be confined to hosts from the family Hepsetidae (African pikes). This fish family was initially considered a monotypic genus (Decru et al., 2012; Skelton, 2016), but now has six species described, including the "recently rehabilitated" *Hepsetus cuvieri* (Castelnau, 1861) (Decru et al., 2012; Skelton, 2016). At the time of writing, *L. hepseti* has not been reported from any other African pikes except *H. odoe* (original description) and *H. cuvieri* (current study). More sampling across regions where the African pikes are naturally occurring might give more information on the distribution of this native *Lamproglena* species.

8.2. Invasive crustacean species of southern Africa

The three known invasive species collected during this study belong to the Branchiura (*A. japonicus*) and Copepoda (*L. cyprinacea* and *N. japonicus*). Several studies have documented the presence of *A. japonicus* and *L. cyprinacea* in southern Africa (see Kruger et al., 1983; van As, 2015; Smit et al., 2017; Welicky et al., 2017; Truter et al., 2023). However, for *N. japonicus*, there are no published reports at the time of writing (only an unpublished dissertation and conference abstract), except for Shinn et al. (2023) who referenced the conference abstract of Smit et al. (2018) in their paper of parasites affecting tilapia culture. The introduction of these invasive species has been suggested to be via translocation or transportation of host species for the purposes of aquaculture, angling and sports fishing, or as food and trade (Ellender & Weyl, 2014; van As, 2015; Smit et al., 2017; Weyl et al., 2020). In South Africa, several invasive parasite species have been co-introduced with their exotic fish hosts, some of which have become established in native fish populations (see Smit et al., 2017; Weyl et al., 2020; Truter et al., 2023). As native hosts and introduced parasite communities have not co-evolved, the hosts have not adapted the necessary immune responses to these parasites, which can lead to severe consequences in the fish populations (Gozlan, 2008). Furthermore, there are possibilities of parasite spillback and spillover between native and introduced hosts, as highlighted in Truter et al. (2023). The exploration of such possibilities is increasingly relying on phylogenetic studies as a tool to uncover these interactions. These findings carry significant implications for the epizootiology of parasites across the continent.

The common carp and goldfish are the two most implicated fishes regarding the introduction of non-native parasite species. According to Smit et al. (2017), the common carp was responsible for the majority of the co-introduced and co-invaded fish parasites in South Africa, serving as hosts to seven of the thirteen invasive parasite species known from South African rivers, including *A. japonicus* and *L. cyprinacea* (van As, 2015; Smit et al., 2017; Weyl et al., 2020). The introduction of *N. japonicus* into South Africa may also have been from the transportation of the alien common carp or even the goldfish, which have been reported in other regions globally (see checklist in Chapter 6). Results from this study further highlighted possibilities of multiple events of introduction for these invasives (see discussion in Chapters 4–6). This, therefore, emphasises the potential impact of human-mediated introductions of non-native species on local ecosystems. Considering the current global drive towards increasing freshwater aquaculture, by the South African Government, it is important to monitor the spread of the parasites as possible pathogens (Bastos Gomes et al., 2017).

The chapter on the invasive *A. japonicus* reported on adult males, females, and larval stages, all of which are parasitic, as well as a possible new larval stage not previously described (see Chapter 4). The description of a possible additional larval stage not previously reported adds to our knowledge of the life cycle of this species.

8.3. New hosts and locality reports

This study provided new locality records for both invasive and native parasitic crustacean species (Fig. 8.1). *Argulus japonicus* was reported for the first time in the Orange River (Gariep Dam) with a few new localities recorded from the Vaal River System (see Chapter 4). *Lernaea cyprinacea* was also reported from the Phongolo Floodplain and the Vaal River, and a notable record is the first report of this invasive species in the Incomati System (see Chapter 5). Additionally, a substantial range expansion and host distribution for *N. japonicus* was recorded from the Vaal and Olifants rivers, with the parasite infesting the invasive largemouth bass and the native African tigerfish (see Chapter 6). These records highlight the successful establishment of these invasive species in the various systems, as well as their success in utilising native and non-native host species. However, the extent to which these parasites have been established in the river systems remains largely unknown.

Ergasilus mirabilis was reported for the first time in the Vaal River (Takwasa) with another new locality record in the Phongolo Floodplain (Kushokwe Pan) (Chapter 2). The report from the Vaal River follows more than four decades since the original description from the Phongolo River System, and thus suggests that the geographical distribution of *E. mirabilis* is much broader than originally thought. *Lamproglena clariae* was also collected from the Nyamiti Pan and Phongolo River (South Africa), and from the Kabompo River (Zambia), for the first time. The description of a new ergasilid species (Chapter 7) from the sharptooth catfish is also a new host record for the genus *Neoergasilus*.

8.4. Impact of fish introduction on parasite diversity

Since South Africa suffers from water scarcity, several inter-basin water transfer schemes (IBWTs) have been constructed to help reconcile this problem (Snaddon et al., 1998). These schemes help with the supply of water for human use and for irrigation (Snaddon et al.,1999). The Orange River Project was one of such schemes that had water transferred from the Gariep Dam of the Orange River to the Great Fish River and the Sundays River in the Eastern Cape for irrigation (Cambray & Jubb, 1977; Snaddon et al., 1998; 1999; Truter et al., 2023). While this project was reported to have increased water levels significantly, converting the recipient rivers from seasonal to perennial rivers (Snaddon et al.,1999), there have also been

significant changes to aquatic biodiversity (see Weyl et al., 2020). The Orange/Fish River IBWT resulted in the transfer of five fish species to the Great Fish River, one of which was the sharptooth catfish (Snaddon et al.,1999; Ellender & Weyl, 2014). The translocation of the sharptooth catfish was suggested to have been as far back as the 1970s (Cambray & Jubb, 1977). Only recently did Truter et al. (2023) provide the first report of parasite communities of the sharptooth catfish that have been co-introduced from the Gariep Dam to the Great Fish River. These authors also confirmed events of enemy releases within the native communities of the catfish in the Great Fish River. From the current study, the invasive fish species *A. japonicus* and the native *E. mirabilis* were reported from the Gariep Dam, but none at this time have been collected from the Great Fish River. The absence of these widely distributed species in the catfish population from the Great Fish River may suggest an event of enemy release, as reported by Truter et al. (2023), but this claim requires more sampling from the regions and so cannot be confirmed at this time. Furthermore, there is also the possibility that these parasites may be present in the Great Fish River but not observed in the present study, especially considering their wide distribution and host ranges globally, and in southern Africa, respectively.

As a result of the scarcity of records across river systems in southern Africa, the impact of the invasive parasite species on native fish species as well as their habitats is largely unknown. Continuous sampling is therefore encouraged as the study did not simultaneously target all parasitic crustacean groups. Additionally, advocating for collaboration among researchers across the continent and ensuring proper specimen curation, sharing of voucher specimens, and provision of adequate funding, especially for molecular research, is essential. Some parts of the project from where materials were collected targeted certain host species and localities; it is acknowledged, therefore, that the report from the current study may only represent a fragment of the biodiversity of parasitic Crustacea fauna in southern Africa. The absence of, for instance, *E. mirabilis* in the Great Fish River during these surveys does not mean it may not already exist there, and the same could be said for every other region in southern Africa as well as every other potential host species. It is also possible that parasite species may have been missed, so further sampling is encouraged.

The status of the newly described *Neoergasilus* species from the study is still unclear as to whether *Neoergasilus* sp. nov. is an undescribed invasive species or an undescribed African *Neoergasilus* species. More studies on this will need to be done in the future.

8.5. Integrative taxonomy with molecular analyses

The incorporation of molecular studies into taxonomical analyses creates several new possibilities and challenges in taxonomy. For instance, the resolution of cryptic species based solely on morphological data has proven rather difficult (Schander & Willassen, 2005). With

the inclusion of molecular analyses, these species can be identified and provided with a genetic barcode. Additionally, damaged, or degraded tissues may result in the loss of specific diagnostic characteristics. In such cases, molecular analyses may be applied in an attempt to generate a genetic identity (barcode) for the samples (Schander & Willassen, 2005). This genetic identity can further be compared with other available information in the databases to see which species they are most closely related to evolutionarily; however, this is all depended on the credibility of the genetic data available on databases such as GenBank.

Molecular analyses may either be used as a baseline guide for morphological studies or as a confirmatory and supporting tool after morphology. According to Dayrat (2005), an important part of integrative taxonomy is that it helps to, or tries to, “prevent the overabundance of both synonyms and names of doubtful applications from worsening”. Thus, a combination of morphology and molecular techniques (termed integrative taxonomy) for species identification proves to be a more efficient approach to systematics (see Hua et al., 2019).

Considering an integrative approach to parasite characterisation, combinations of morphological, taxonomical, and molecular techniques were used to characterise parasite species during the current study. Light microscopy (LM) included the use of stereomicroscopes as well as the bright field and phase-contrast functions of the differential interference contrast (DIC) microscope. This study highlighted the importance of scanning electron microscopy (SEM) as an invaluable tool in morphological studies, as certain characters not observed using LM were seen with the aid of SEM.

All parasite species collected during this study have been successfully analysed molecularly using fragments of three target genes: two ribosomal RNA genes 18S and 28S genes, and one mitochondrial DNA COI gene. This study generated a total of 31, 34, and 18 sequences (used in the final alignments) for 18S, 28S, and COI, respectively (see Table 8.1). The successful sequencing and amplification of all three target genes is a significant achievement and a notable addition to future evolutionary studies of parasitic Crustacea. The availability of genetic data for COI is particularly noteworthy, as this gene is commonly used in DNA barcoding and species identification for a wide range of organisms, including parasites. The genetic data for COI obtained for *Lamproglena* species is an important contribution to the field since this information was not previously available for the genus.

The utilisation of eggs, larvae, and adult stages for molecular analyses in this study, will enable continued parasite characterisation, especially in cases where there is a loss of specific diagnostic characters as a result of damaged or degraded tissues (Schander & Willassen, 2005). Additionally, the success reported with linking the genetic data for eggs, larval and adult stages, adds reliable information to the parasitic Crustacea database for future integrative and evolutionary studies. This database now consists of an additional 83 parasitic crustacean sequences (18S, 28S, and COI) generated from this study, excluding the COI sequences for the new species. This addition significantly increases the range of possibilities

for future biodiversity studies, including the detection of these parasite species using environmental DNA samples.

8.6. Evolutionary relationship of parasitic crustaceans using molecular data

The subclasses Branchiura and Copepoda are generally accepted as monophyletic (Regier et al., 2008; Møller, 2009; see Richter et al., 2009). The phylogenetic trees constructed for all datasets (18S, 28S, and COI) and species from this study, yielded two major clades, Branchiura and Copepoda, which is consistent with the monophyletic categorisation of these subclasses (see Ho, 1998; Møller et al., 2008 and preceding references). The Branchiura clade had all species from the families Argulidae, while the Copepoda clade formed two sub-clades of the families Ergasilidae and Lernaeidae (for 18S and 28S) (see Figs. 8.2–8.4) (with *Lamproglena* and *Lernaea* forming sister clades to Ergasilidae, for COI).

Within the Branchiura, all *Argulus japonicus* sequences clustered in a sister clade, but the 18S tree also included an *Argulus siamensis* Wilson, 1926 (see Patra et al., 2016) sequence (see Fig. 8.2). As mentioned in Chapter 4, this might be as a result of misidentification of the species. Compared to the copepods, there is very scanty genetic data available for the branchiurans, furthermore, the majority of sequences submitted and the identity of some of the sequences do not correspond with the identities reported in the respective literature (see Feroz-Khan et al., 2014). The sequences from the current study are therefore a substantial addition, as they have been verified with corresponding morphological analysis. From the phylogenetic analyses, it may be hypothesised that the genus *Argulus* is monophyletic (see Figs. 8.2–8.4), but this study had limited data as it only included sequences from the sister genus *Chonopeltis.* Future phylogenetic studies should aim to analyse more species from the other genera of the Argulidae to better understand its evolutionary position in the family.

Table 8.1: List of all parasitic Crustacea sequences generated from this study and selected GenBank and Bold sequences included in the phylogenetic analyses. The taxa in boldface are newly generated sequences. *Levisunguis subaequalis* Curran, Overstreet, Collins & Benz, 2014 was used as the outgroup.

Taxon	Host	Locality	18S	28S	COI	Reference
Argulus americanus	*Amia calva*	USA	-	MN688128	-	Andres et al. (2019)
Argulus americanus	-	(Maybe USA)	-	-	NC_005935	Lavrov et al. (2004)
Argulus bicolor	*Hypanus sabinus*	USA	-	MN688129	-	Andres et al. (2019)
Argulus bengalensis	*Labeo rohita*	India	KM016969	-	-	Patra et al. (2016)
Argulus flavescens	*Acipenser oxyrinchus*	USA	-	MN688125	-	Andres et al. (2019)
Argulus foliaceus	-	-	-	EU370442	-	von Reumont et al. (2009)
Argulus foliaceus	*Carassius auratus*	India	-	-	KF723419	Feroz-Khan et al. (2014)
Argulus indicus	*Channa striata*	Indonesia	MT538304	-	-	Amriana et al. (2021)
Argulus japonicus	*Schizothorax richardsonii*	India	MW857091	-	-	Tandel et al. (2021)
Argulus japonicus	*Schizothorax richardsonii*	India	MW857090	-	-	Tandel et al. (2021)
Argulus japonicus	*Labeo rohita*	India	-	-	OM836429	Unpublished
Argulus japonicus	*Cyprinus carpio*	Japan	-	-	LC588400	Unpublished
Argulus japonicus	Egg clusters	India	-	-	KF713320	Feroz-Khan et al. (2014)
Argulus japonicus	*Carassius auratus*	India	-	-	JX025025	Feroz-Khan et al. (2014)
Argulus japonicus	*Micropterus salmoides*	China	MW866569	-	-	Yang & Gu (2021)
Argulus japonicus	***Labeobarbus aeneus***	**South Africa**	**P03; P05; P08–P011; P14–P16**	**P03–P06; P08–P011; P14–P16**	**P03; P09–P011; P14–P16**	**Present Study**
Argulus nobilis	*Lepisosteus osseus*	USA	M27187	-	-	Abele et al. (1989)
Argulus pugettensis	*Oncorhynchus clarkia*	USA	MH915670	-	-	Losee et al. (2020)
Argulus siamensis	*Cyprinus carpio*	India	KF583879	-	-	Patra et al. (2016)
Argulus siamensis	Egg clusters	India	-	-	KF713315	Feroz-Khan et al. (2014)

Taxon	Host	Locality	18S	28S	COI	Reference
Argulus siamensis	*Carassius auratus*	India	-	-	KF723415	Feroz-Khan et al. (2014)
Argulus sp.	-	USA	-	AF363322	-	Oakley et al. (2002)
Argulus sp.	-	USA	-	AY210804	-	Mallat et al. (2004)
Argulus sp.	-	Mexico	-	-	MZ518075	Valdez-Moreno et al. (2021)
Chonopeltis lisikili	*Synodontis zambezensis*	South Africa	MW678625	-	MW679715	Van As et al. (2021)
Acusicola margulisae	*Amphilophus citrinellus, Parachromis managuensis, Oreochromis sp., Poecilia exicana*	Nicaragua	MN852694	MN852851	MN854870	Santacruz et al. (2020)
Ergasilus anchoratus	*Pseudobagrus fulvidraco*	China	DQ107564	DQ107528	-	Song et al. (2008)
Ergasilus briani	*Misgurnus anguillicaudatus*	China	DQ107572	DQ107532	-	Song et al. (2008)
Ergasilus caparti	*Neolamprologus brichardi*	Burundi	OQ407469	OQ407474	-	Míč et al. (2023)
Ergasilus hypomesi	*Acanthogobius hasta*	China	DQ107573	DQ107539	-	Song et al. (2008)
Ergasius lizae	*Fundulus diaphanous*	Canada	-	-	ECTCR024-14	BOLD, 2023
Ergasilus macrodactylus	*Gnathochromis permaxillaris*	Burundi	OQ407465	OQ407470	-	Míč et al. (2023)
Ergasilus megacheir	*Simochromis diagramma*	Burundi	OQ407466	OQ407471	-	Míč et al. (2023)
Ergasilus mirabilis	***Clarias gariepinus***	**Vaal River, South Africa**	**P38K_38VR**	**P38Ka_38VR**	**P38Kb_38VR**	**Present study**
Ergasilus mirabilis	***Clarias gariepinus***	**Zambezi River, Zambia**	**P49K_72ZR**	**P49Ka_72ZR**	**P49Kb_72ZR**	**Present study**
Ergasilus parasarsi	*Simochromis diagramma*	Burundi	OQ407467	OQ407473	-	Míč et al. (2023)
Ergasilus parvus	*Spathodus erythrodon*	Burundi	OQ407468	OQ407472	-	Míč et al. (2023)
***Ergasilus parasiluri*	*Tachysurus fulvidraco*	China	DQ107567	DQ107536	-	Song et al. (2008)
Ergasilus peregrinus	*Siniperca chuatsi*	China	DQ107577	DQ107531	-	Song et al. (2008)

Taxon	Host	Locality	18S	28S	COI	Reference
Ergasilus scalaris	*Tachysurus dumerili*	China	DQ107565	DQ107538	-	Song et al. (2008)
Ergasilus sieboldi	*Perca fluviatilis*	Czech Republic	MW810238	MW810242	-	Kvach et al. (2021)
Ergasilus sieboldi	*Sparus aurata*	Egypt	-	OM812074	-	Abdel-Radi et al. (2022)
Ergasilus sp.	Free-living	South Korea	-	-	KR049035	Baek et al. (2016)
Ergasilus sp.	*Mugil liza*	Argentina	-	-	KU557411	Castro-Romero et al. (2016)
Ergasilus tumidus	*Acanthorhodeus taenianalis*	China	DQ107569	DQ107535	-	Song et al. (2008)
Ergasilus wilsoni	Free-living	South Korea	-	-	KR049036	Baek et al. (2016)
Ergasilus yaluzangbus	*Gymnocypris stewartia*	China	DQ107578	DQ107540	-	Song et al. (2008)
***Ergasilus yandemontei*	*Odontesthes hatcheri*	Argentina	MT969345	-	-	Waicheim et al. (2021)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MH167969	MH167967	-	Ondračková et al. (2019)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MH167970	MH167968	-	Ondračková et al. (2019)
Neoergasilus japonicus	*Lepomis gibbosus*	Czech Republic	MW810236	MW810240	-	Kvach et al. (2021)
Neoergasilus japonicus	*Lepomis gibbosus, Scardinius erythrophthalmus*	Czech Republic	MW810237	MW810241	-	Kvach et al. (2021)
****Neoergasilus japonicus*	*Collected by plankton net*	USA	-	-	MZ964935	Vasquez et al. (2021)
Neoergasilus japonicus	*Free-living*	South Korea	-	-	KR049037	Baek et al. (2016)
Paraergasilus brevidigitus	*Cyprinus carpio*	China	DQ107576	DQ107530	-	Song et al. (2008)
Paraergasilus longidigitus	*Abramis brama, Perca fluviatilis, Scardinius erythrophthalmus*	Czech Republic	MW810239	MW810243	-	Kvach et al. (2021)
Paraergasilus medius	*Ctenopharyngodon idellus*	China	DQ107574	DQ107529	-	Song et al. (2008)
Sinergasilus major	*Ctenopharyngodon Idella*	China	DQ107560	DQ107524	-	Song et al. (2008)
Sinergasilus major	*Silurus glanis*	Hungary	MZ047814	MZ047815	-	Dos Santos et al. (2021)

Taxon	Host	Locality	18S	28S	COI	Reference
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	China	DQ107563	DQ107525	-	Song et al. (2008)
Sinergasilus polycolpus	*Hypophthalmichthys molitrix*	China	-	-	KR263117	Feng et al. (2016)
Sinergasilus undulatus	*Cyprinus carpio*	China	DQ107561	DQ107526	-	Song et al. (2008)
Sinergasilus undulatus	*Cyprinus carpio*	China	-	-	MW080644	Hua et al. (2021)
Lamproglena chinensis	*Channa argus*	Dangjiangkou, China	DQ107553	DQ107545	-	Song et al. (2008)
Lamproglena clariae	***Clarias gariepinus***	**West Lunga, Zambia**	**P19K**	**P19Ka**	**P19Ki**	**Present study**
Lamproglena clariae	***Clarias gariepinus***	**Pongola River, South Africa**	**P20R**	**P20Ra**	**-**	**Present study**
Lamproglena clariae	***Clarias gariepinus***	**Nyamiti Pan, South Africa**	**P23R**	**P23Ra, P24Ka**	**-**	**Present study**
Lamproglena clariae	***Clarias gariepinus***	**Vaal River, South Africa**	**-**	**P25Ka, P26Ka, P27Ka**	**-**	**Present study**
Lamproglena clariae	***Clarias gariepinus***	**Barberspan, Harts River, South Africa**	**P28K**	**P28Ka**	**P28Ki**	**Present study**
Lamproglena hemprichii	*Hydrocynus vittatus*	Lake Kariba, Zimbabwe	OP277526	OP277527	-	Mabika et al. (2022)
Lamproglena hepseti	***Hepsetus cuvieri***	**Okavango River, Botswana**	**P42R**	**P42Ra**	**P42Rb**	**Present study**
Lamproglena monodi	*Oreochromis niloticus*	El-Minia, Egypt	ON419448	ON419432	-	Rindoria et al. (2022)
Lamproglena monodi	*Oreochromis niloticus*	Sharqia, Egypt	ON419450	ON419435	-	Rindoria et al. (2022)
Lamproglena monodi	*Oreochromis niloticus*	Kibos Fish Farm, Kenya	ON419439	ON419422	-	Rindoria et al. (2022)
Lamproglena monodi	***Serranochromis angusticeps***	**Okavango River, Botswana**	**P18K**	**P18Ka**	**P18Kb**	**Present study**
Lamproglena orientalis	*Chanodichthys dabryi*	Tangxun Lake, China	DQ107549	DQ107542	-	Song et al. (2008)
Lamproglena orientalis	*Chanodichthys dabryi*	E-zhou farm, China	DQ107550	DQ107543	-	Song et al. (2008)
Lamproglena orientalis	*Squaliobarbus curriculus*	Dangjiangkou, China	DQ107552	DQ107544	-	Song et al. (2008)

Taxon	Host	Locality	18S	28S	COI	Reference
Lernaea cruciata	*Gambusia holbrooki*	China	MH982203	MH982215	MH982226 ; MH982227	Hua et al. (2019)
Lernaea cyprinacea	***Larbeobarbus aeneus***	**TW, South Africa**	**P44K, P45K,**	**P44Ka, P45Ka**	**P44Kh**	**Present study**
Lernaea cyprinacea	***Oreochromis mossambicus***	**NP, South Africa**	**P61K, P64K, P65K**	**P61Ka, P64Ka, P65Ka**	**P64Kh**	**Present study**
Lernaea cyprinacea	***Labeo umbratus***	**BP, South Africa**	**P69K**	**P69Ka**	**P69Ki**	**Present study**
Lernaea cyprinacea	***Oreochromis mossambicus***	**LSR, South Africa**	**P70K, P72K**	**P70Ka, P72Ka**	-	**Present study**
Lernaea cyprinacea	Several (host not specified per sequence)	Australia	MT367695	MT371347	MT371895	Zhu et al. (2021)
Lernaea cyprinacea	Several (host not specified per sequence)	Australia	MT367704	MT371367	MT371896	Zhu et al. (2021)
Lernaea cyprinacea	*Sandelia bainsii*	South Africa	MN243682	-	-	Chakona et al. (2019)
Lernaea cyprinacea	*Odontesthes bonariensis*	Argentina	MH198054	-	-	Waicheim et al. (2019)
Lernaea cyprinacea	*Percichthys trucha*	Argentina	MH198053	-	-	Waicheim et al. (2019)
Lernaea cyprinacea	*Oreochromis mossambicus*	South Africa	KY435939	-	-	Welicky et al. (2017)
Lernaea cyprinacea	-	China	-	-	KM235194	Su et al. (2016)
Lernaea cyprinacea	*Pseudorasbora parva*	Romania	-	KF751649	-	Stavrescu-Bedivan et al. (2014)
Lernaea cyprinacea	*Lepomis gibbosus*	Romania	-	KF751648	-	Stavrescu-Bedivan et al. (2014)
Lernaea cyprinacea	*Carassius auratus*	China	MH982195	MH982210	MH982221	Hua et al. (2019)
Lernaea cyprinacea	*Chanodichthys erythropterus*	China	MH982197	MH982208	MH982220	Hua et al. (2019)
Lernaea cyprinacea	*Squaliobarbus curriculus*	China	MH982198	-	MH982219	Hua et al. (2019)
Lernaea cyprinacea	*Cyprinus carpio*	China	MH982196	MH982204	MH982216	Hua et al. (2019)
Lernaea cyprinacea	*Odontesthes bonariensis*	Argentina	-	KX908211	-	Soares et al. (2018)
Lernaea cyprinacea	*Hemiculter leucisculus*	China	DQ107554	DQ107546	-	Song et al. (2008)

Taxon	Host	Locality	18S	28S	COI	Reference
Lernaea cyprinacea	*Chanodichthys erythropterus*	China	DQ107555	DQ107547	-	Song et al. (2008)
Lernaea cyprinacea	*Cyprinus carpio*	China	DQ107556	-	-	Song et al. (2008)
Lernaea cyprinacea	*Opsariichthys bidens*	China	DQ107557	DQ107548	-	Song et al. (2008)
Lernaea cyprinacea	*Plecoglossus altivelis*	Japan	KP235363	KP235364	-	Yoshimine et al. (2015)
Lernaea cyprinacea	*Carassius auratus*	Australia	-	KY346866	-	McCredden, (2016)
Neoergasilus japonicus	***Hydrocynus vittatus***	**Olifants River, South Africa**	**P36R**	**P36Ra**	**-**	**Present study**
Neoergasilus japonicus	***Hydrocynus vittatus***	**Olifants River, South Africa**	**P46K**	**P46Ka**	**P46Kg**	**Present study**
Neoergasilus japonicus	***Micropterus salmoides***	**Boskop Dam, South Africa**	**P43R**	**P43Ra**	**P43Rb**	**Present study**
Neoergasilus sp. nov.	***Clarias gariepinus***	**Great Fish River, South Africa**	**P40R, P47K, P59R**	**P50Ra2, P59Ra2**	**P50RJ, P59RJ**	**Present study**
Levisunguis subaequalis	*Gambusia affinis*	USA	MN065569	MN065508	MN062096	Woodyard et al. (2019)

References for the sequences in this table (except the outgroup) are not included in this chapter as they have been given in the other respective result chapters.
** *Ergasilus yandemontei* (Published on GenBank as *Ergasilus* sp.); *Ergasilus parasiluri* (published on GenBank as its synonym *Pseudergasilus parasiluri*).
*** Only able to access abstract and sequences that were published as supplementary data

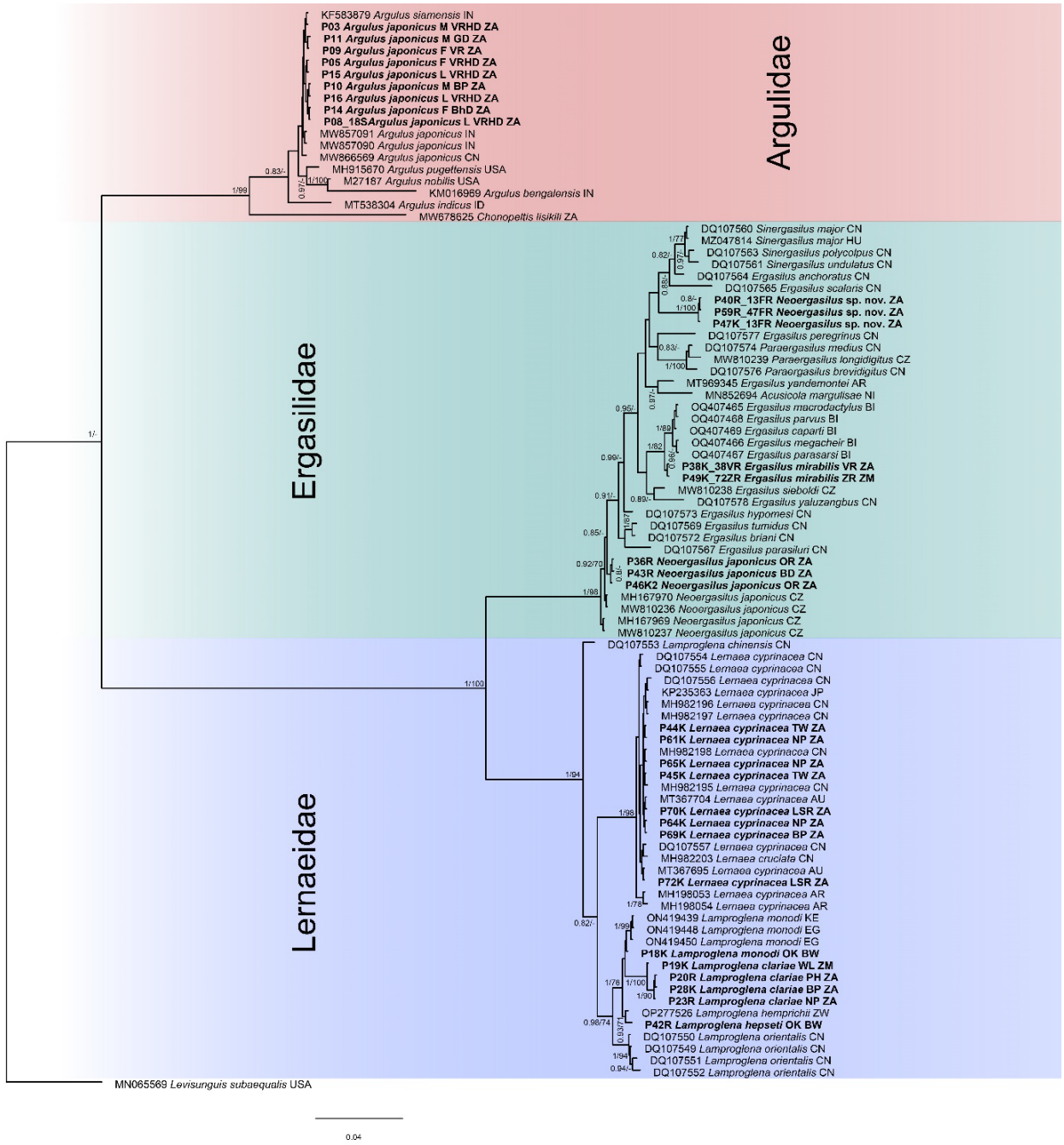


Figure 8.2: Phylogenetic tree of all parasitic Crustacea sequences generated from the current study and selected GenBank sequences based on partial 18S rRNA gene alignments. Newly generated sequences are bold in gradient shade: Argulidae (orange); Ergasilidae (green); Lernaeidae (blue). Nodal support presented close to branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Levisunguis subaequalis* was used as the outgroup. Abbreviations: AR – Argentina, AU – Australia, BD – Boskop Dam, BhD – Bloemhof Dam, BI – Burundi, BP – Barberspan, BW – Botswana, CN – China, CZ – Czech Republic, EG – Egypt, F – Female, FR – The Great Fish River, GD – Gariep Dam, HU – Hungary, ID – Indonesia, IN – India, JP – Japan, KE – Kenya, L – Larva, LSR – Lower part of Sabie River, M – Male, NI – Nicaragua, NP – Nyamiti Pan, OK – Okavango, OR – Olifants River, PH – Pongola River, TW – Takwasa, USA – The United States of America, VR – Vaal River, VRHD – Vaal River Holding Dam, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZR – Zambezi River, ZW – Zimbabwe.

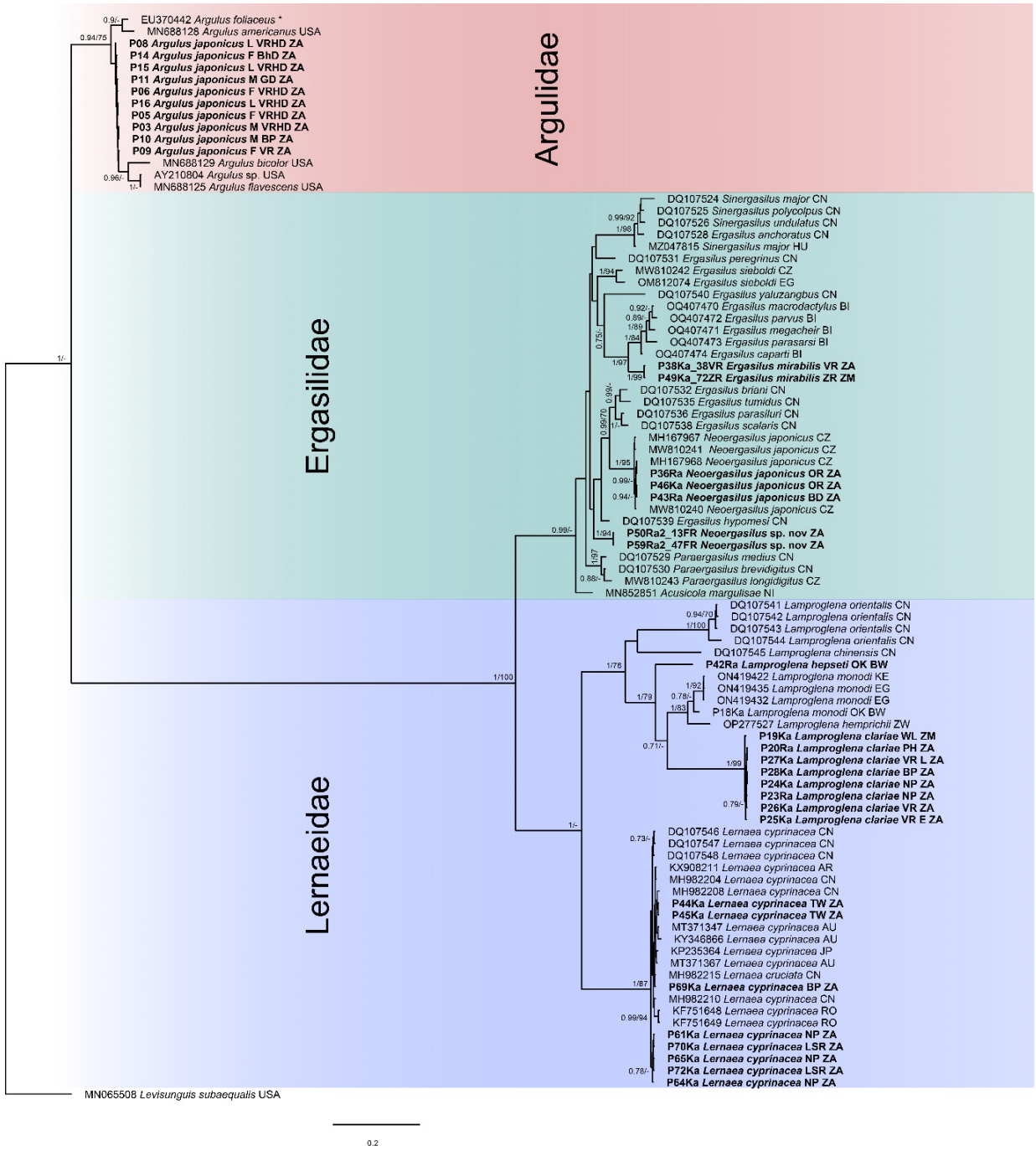


Figure 8.3: Phylogenetic tree of all parasitic Crustacea sequences generated from the current study and selected GenBank sequences based on partial 28S rRNA gene alignments. Newly generated sequences are bold in gradient shade: Argulidae (orange); Ergasilidae (green); Lernaeidae (blue). Nodal support presented close to branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Levisunguis subaequalis* was used as the outgroup. Abbreviations: AR – Argentina, AU – Australia, BD – Boskop Dam, BhD – Bloemhof Dam, BI – Burundi, BP – Barberspan, BW – Botswana, CN – China, CZ – Czech Republic, E – Eggs, EG – Egypt, F – Female, FR – The Great Fish River, GD – Gariep Dam, HU – Hungary, JP – Japan, KE – Kenya, L – Larva, LSR – Lower part of Sabie River, M – Male, NI – Nicaragua, NP – Nyamiti Pan, OK – Okavango, OR – Olifants River, PH – Pongola River, RO – Romania, TW – Takwasa, USA – The United States of America, VR – Vaal River, VRHD – Vaal River Holding Dam, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZR – Zambezi River, ZW – Zimbabwe.

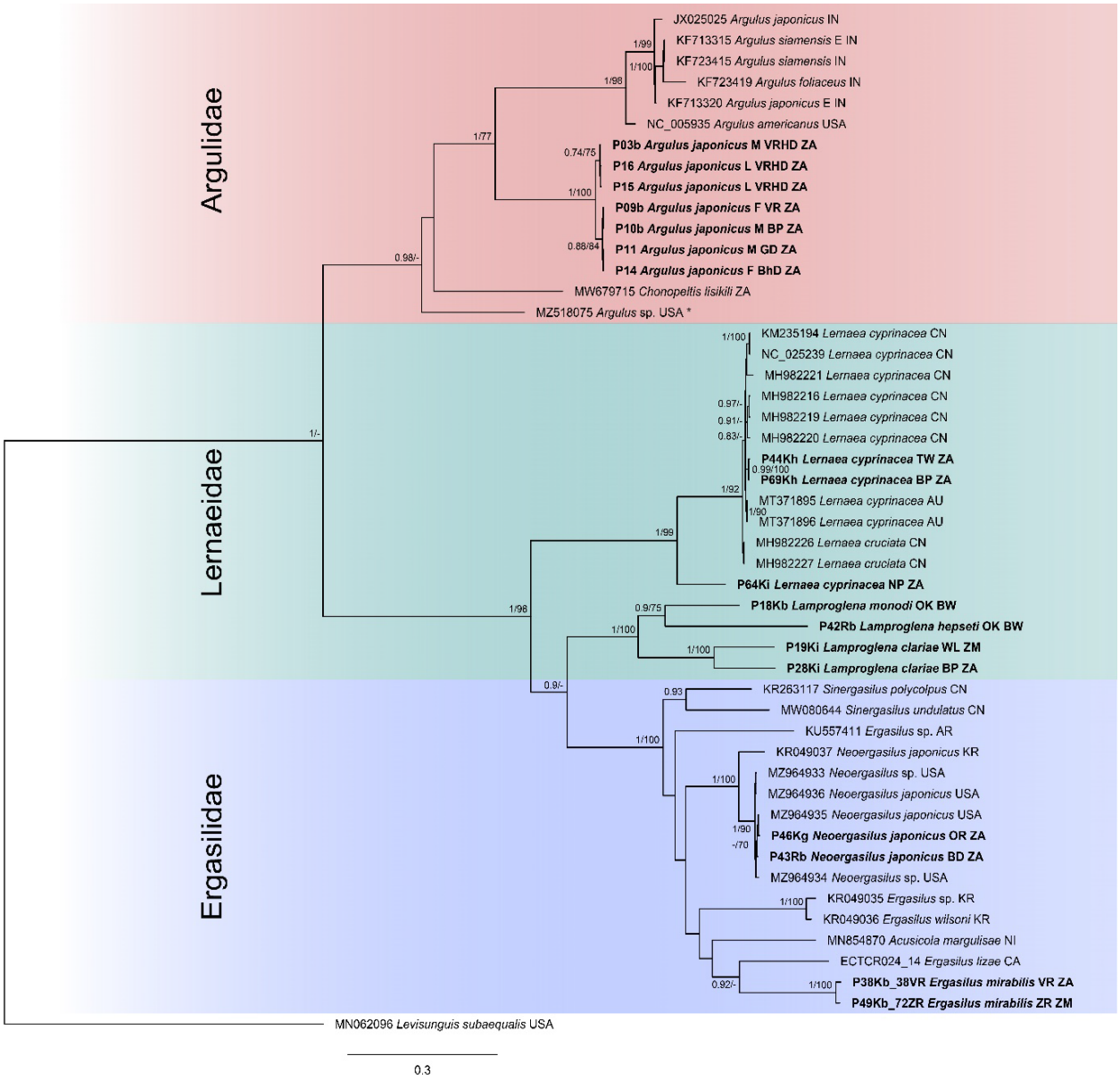


Figure 8.4: Phylogenetic tree of all parasitic Crustacea sequences generated from the current study and selected GenBank and Bold sequences based on partial COI mitochondrial gene alignments. Newly generated sequences are bold in gradient shade: Argulidae (orange); Ergasilidae (green); Lernaeidae (blue). Nodal support presented close to branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Levisunguis subaequalis* was used as the outgroup. Abbreviations: AR – Argentina, AU – Australia, BD – Boskop Dam, BhD – Bloemhof Dam, BP – Barberspan, BW – Botswana, CA – Canada, CN – China, E – Eggs, F – Female, GD – Gariep Dam, IN – India, KR – South Korea, L – Larva, M – Male, NI – Nicaragua, NP – Nyamiti Pan, OK – Okavango, OR – Olifants River, TW – Takwasa, USA – United States of America, VR – Vaal River, VRHD – Vaal River Holding Dam, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZR – Zambezi River.

With the ribosomal genes alone, it may be suggested that the Lernaeidae is a monophyletic group, however, the separation, in the COI tree, of the Copepoda clade into the *Lamproglena* and *Lernaea* sister clades may suggest otherwise. Other than the sequences generated from this study, there are no *Lamproglena* sequences available for COI. Additionally, only two species from the genus *Lernaea* were represented in the tree (*L. cyprinacea* and *Lernaea cruciata*), the latter of which has been synonymised with *L. cyprinacea* (Hua et al., 2019).

For the family Ergasilidae, phylogenetic studies by Song et al. (2008) using fragments of the 18S and 28S genes categorised the genera *Sinergasilus* and *Paraergasilus* as monophyletic genera and gave the evolutionary status of *Ergasilus* as a polyphyletic genus in the family. Song et al. (2008) further proposed that *Pseudergasilus* and *Sinergasilus* species may have evolved from *Ergasilus*. In 2003, Kim and Choi synonymised the genus *Pseudergasilus* with *Ergasilus* (Kim & Choi, 2003) and all previously described *Pseudergasilus* species have been renamed as either *Ergasilus* or *Sinergasilus* (see Walter & Boxshall, 2023). It is possible that Song et al. (2008) were unaware of the synonymisation by Kim and Choi (2003), however, molecularly arrived at the same conclusion.

The results from all datasets (18S, 28S, and COI) of this study, corroborate Song et al. (2008). *Paraergasilus* and *Sinergasilus* are monophyletic, although *E. anchoratus* was observed in the *Sinergasilus* clade of the 28S analyses (Figs. 8.2, 8.3). The evolutionary position of *Ergasilus* is polyphyletic as individuals from the genus are seen in clusters with other genera. This might suggest the need for a revision of the whole *Ergasilus* genus for future studies. At the time, Song et al. (2008) reported *Ergasilus* as the only polyphyletic genus in the family. The phylogenetic analyses from the current study, however, suggest that the genus *Neoergasilus* may also be categorised as a polyphyletic genus, as the sequences from the newly described species, *Neoergasilus* sp. nov., clustered separately from the available *N. japonicus* sequences (see Figs. 8.2, 8.3). It is, however, difficult to affirm this position because, apart from *N. japonicus,* no other *Neoergasilus* species have been molecularly characterised. More sequences from *Neoergasilus* and related genera are needed to discuss the evolutionary hypothesis for the genus.

The COI sequences generated for *Neoergasilus* sp. nov. were not included in the phylogenetic alignment of Fig. 8.4 because they have been amplified with a different primer set. This primer template amplified a different fragment of the COI gene thereby making these sequences incompatible for the time being until further studies can be conducted. The sequences for *Neoergasilus* sp. nov., however, clustered with other ergasilids that were amplified with the same primers (see blue block in Appendix Fig. A.1). Throughout the study, several primer templates were used in combination to amplify the target genes. For future research, these primers (listed in all respective chapters) could be used for DNA amplifications and combined as required.

8.7. Outcomes

The overarching aim of this study was to adopt an integrative approach to parasitology research, which specifically included linking distribution data of selected freshwater fish parasitic crustaceans in southern Africa, with proper characterisation, using morphology, taxonomy, and genetic data/techniques. It was therefore hypothesised that the knowledge of native and invasive parasitic Crustacea species in southern Africa would increase and there would be new host and locality records, with the possibility of new species. Furthermore, with the use of integrated taxonomy (morphology and molecular techniques), novel information on known parasites would be generated (such as previously undocumented characteristics and DNA sequences). These hypotheses were confirmed in this study, and all aims were achieved.

The outcomes from this study are as follows:

- Positive identification/characterisation of seven previously known parasite species:
    - Three invasive species: *Argulus japonicus*, *Neoergasilus japonicus*, and *Lernaea cyprinacea;*
    - Four were native species: *Ergasilus mirabilis*, *Lamproglena clariae*, *L. hepseti*, and *L. monodi*; and
    - Description of a new species, *Neoergasilus* sp. nov.
- A new taxonomic key for the genus *Neoergasilus.*
- New locality records for invasive and native parasite species, highlighting their establishment and success in utilising native and non-native host species.
- Reports of parasitic Crustacea distribution from the Orange-Vaal River System, the Phongolo River and Floodplain, the Incomati System, Olifants River, the Okavango River and Delta, and the Zambezi River System, all across southern Africa.
- Eighty-three newly generated sequences for 18S, 28S and COI gene regions with emphasis on the COI gene, as a significant contribution to the DNA barcoding database.
- First reports of molecular and morphological characterisation of the following:

  Globally:
    - *Ergasilus mirabilis*
    - *Lamproglena clariae*
    - *Lamproglena hepseti*

  In Africa:
    - *Argulus japonicus*
    - *Neoergasilus japonicus*

Molecular studies with all three genes:

- All species listed globally and in Africa, *Lamproglena monodi* and *Lernaea cyprinacea* from Africa.

- Support for the monophyletic evolutionary position of subclasses Branchiura and Copepoda and report on the phylogenetic relationships among the parasite Crustacea species as follows:
  - Supports monophyletic status of *Sinergasilus* and *Paraergasilus*, respectively.
  - Supports polyphyletic status of *Ergasilus.*
  - Suggests polyphyletic status *Neoergasilus.*
  - Suggests monophyletic status for *Lamproglena* and *Lernaea*, respectively.

8.8. Recommendations

Even though the current study provided valuable information on the diversity of parasitic crustaceans in southern Africa, described a new species, and morphologically and molecularly characterised seven other known species, while providing descriptions and redescriptions of these parasitic crustaceans from southern Africa, several knowledge gaps still remain. These areas were not reached because of the scope of the current project, but this creates a very elaborate foundation for future biodiversity and evolutionary studies of parasitic Crustacea in Africa and beyond. Some of the possible areas of future research have been suggested in the discussion earlier and are given in this section under the two major anchor points of this study, which are biodiversity and integrative taxonomy.

8.8.1. Biodiversity of parasitic Crustacea

The study underlines the importance of increased sampling and comprehensive documentation of parasitic crustaceans in southern Africa. While the study covered various river systems in the region, there are numerous locations that remain unexplored, which could verify the findings presented in this study. These additional samplings could specifically target the hosts identified in this study, enabling a better understanding of the parasite communities within native, introduced, and extralimital distributions of these hosts throughout southern Africa. Where research groups are limited by funding or permit access, collaborative projects are highly recommended, as was done in this study. This will help to further broaden the scope of the research and regions covered, and more species not previously known to science may be described from these studies.

The current study focused on eight parasite species from four parasitic Crustacea genera. Future studies can consider expanding sampling efforts to encompass a wider range of parasite species from different hosts and regions of southern Africa and beyond, for biogeographical comparisons.

8.8.2. Integrative studies using morphology and molecular techniques

The other link throughout this project was the use of morphology and molecular techniques to characterise parasite species. Adequate morphological characterisation of a species validated the credibility of the molecular data presented, and molecular analyses support the morphology of a species, as well as enable evolutionary studies within taxa and among higher taxa. For the efficiency of integrative studies, the use of LM and SEM techniques in the description of each species is highly recommended as certain features often missed during light microscopy are visible with the higher magnifications of the scanning electron microscope. It should be emphasised that field collectors of parasite species, as well as collaborators, should try to clean specimens as much as possible before fixing the parasites to enable easier microscopy. Furthermore, parasitic crustacean species collected must be fixed appropriately (70–96% ethanol) in view of both morphological and molecular analyses. Even though genetic data can be extracted from specimens fixed in 70% ethanol, a higher concentration of 96% is recommended. For molecular analyses, it is also advised that fresh specimens be used for DNA extraction as it had been a limitation that was overcome during this study.

It may also be suggested that future evolutionary studies test a combination of primer sets for DNA amplification of various extractions, especially for fragments of the mitochondrial gene region, COI. These may shed light on the impact that primer templates have in parasitic Crustacea studies. Sequences which have been successfully amplified should be used as a “positive control” when trying to re-amplify failed amplifications, as was done in this study. Additionally, researchers could opt for whole mitochondrial genome amplifications instead, as this will amplify several fragments, which could aid future molecular studies within this family. The use of DNA barcoding genes in the study of parasitic crustaceans is still very scanty compared to the other ribosomal RNA genes. It is possible that results with these “problematic sequences” may have contributed to this scarcity. Some of the primer sets reported throughout this study may be helpful to other researchers and could be used in the combinations reported from this study. On the other hand, researchers could test new primer templates and/or combinations with them. In addition to primers, remarkable success was achieved in this study by exploring and investigating a range of Polymerase Chain Reaction conditions and concentrations during DNA amplifications and is thus highly recommended for curious researchers carrying out molecular studies.

As most protocols used in this study were novel, or adaptations from literature, future research may also follow said protocols to test the reproducibility of these protocols. Ultimately, the goal would be to create a database of parasitic crustaceans amplified with the same or similar primers that are comparable in molecular studies.

8.8.3. Future studies

Biodiversity studies could be a useful tool in the biosecurity of aquatic systems. The assessment of parasite species from biodiversity studies may be used to inform government policies and regulations on the introduction and translocation of host species. This is specifically to curb the co-introduction of invasive species into non-native populations, as these introductions not only impact the parasite fauna but may also impact the overall health of native hosts due to parasitisation. Some of such regulations can also help retain what may be left of the native fauna. Additionally, for potential aquaculture projects, reports from biodiversity studies can inform adequate planning with the inclusion of targeted prevention and control measures of parasite species, especially in areas where they have been established.

Recommendations for future species-specific projects include:

- A complete revision of the genus *Ergasilus* with supporting molecular characterisation, in a bit to understand/review the current polyphyletic evolutionary status of the genus within the family Ergasilidae. Further proposing a taxonomic key for the genus as it may become a helpful tool to most researchers who may not have access to molecular tools.
- More in-depth studies on the co-evolutionary relationships that may exist between African *Lamproglena* species and the hosts they parasitise, in relation to host distribution (introduction and translocation). As well as potential impact of less host species-specific parasites like *L. monodi* on the fast-growing Tilapia aquaculture in southern Africa.
- Further molecular and morphological studies to confirm the evolutionary relationship and intraspecific variability among *Lernaea* species, using sampling localities and hosts as possible factors. Also, a more specific study to molecularly compare *L. cyprinacea* specimens of varying morphotypes.
- It is also recommended that in-depth morphological analysis be carried out to possibly characterise the species of hypersymbionts usually associated with lernaeids.
- Additional sequences for other species in the genus *Neoergasilus* are needed to investigate the possible polyphyletic evolutionary status observed with the new species described from this study.

8.9. Conclusion

In conclusion, this study reveals the presence of diverse parasitic Crustacea species in southern Africa, including four native, three known invasive, and one newly described species. These findings highlight the significance of an integrative approach in parasite characterisation. By linking distribution data with morphological, taxonomical, and molecular data, this study provides valuable information that contribute to current and future evolutionary and biodiversity studies throughout southern Africa. Additionally, the study reports the establishment of invasive parasitic crustacean species in southern African river systems, along with new host and locality records, and the identification of a potentially previously undescribed *Argulus japonicus* larval stage.

The detailed morphological notes and redescriptions of native and invasive species provided in this study will add to our understanding of these organisms. Notably, this study represents the first molecular characterisation of parasitic crustacean species using ribosomal RNA and mitochondrial DNA genes, yielding 83 new gene sequences (for 18S, 28S, and COI gene regions). These findings significantly advance the field of fish parasitology and establish a notable base for future evolutionary investigations. While certain limitations were encountered in scope and techniques, recommendations for future research have been proposed. The study emphasises that the biodiversity of parasitic crustaceans in southern African systems surpasses the extent documented in this research, pointing to a wider range of diversity than what has been recorded. To comprehensively evaluate the biodiversity of native and invasive species, it is vital to sustain research endeavours and promote collaboration among scientists.

Some of these future studies could include the potential impact of parasitic crustaceans in aquaculture, as well as expanded distribution range and possible new host utilisations, with a focus on potential spillover to endemic host species. Future evolutionary studies could target host-parasite co-evolution as this was beyond the scope of the current studies. Additionally, in the light of recent advancements in molecular biology and bioinformatics, new projects could be set up to carry out whole genome sequencing for these parasitic crustacean species.

8.10. References

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APPENDIX

Table A1: Sites of amino acid variation in the alignment of partial COI *Ergasilus mirabilis* Oldewage & van As, 1987 sequences from the Vaal River (VR), South Africa and the Zambezi River (ZR), Zambia from this study, using invertebrate mitochondrion translation and stating what amino acids the codons translate.

	Sequence ID	Locality	Position in alignment	Nucleotide substitutions	Codons	Codon positions	3-letter Amino acid	Amino acid
1	P38Kb_38VR	VR	18	A	GG**A**	16–18	Gly	Glycine
	P49Kb_72ZR	ZR		G	GG**G**			
2	P38Kb_38VR	VR	72	A	AG**A**	70–72	Ser	Serine
	P49Kb_72ZR	ZR		G	AG**G**			
3	P38Kb_38VR	VR	183	A	AT**A**	181–183	Met	Methionine
	P49Kb_72ZR	ZR		G	AT**G**			
4	P38Kb_38VR	VR	339	G	GG**G**	337–339	Gly	Glycine
	P49Kb_72ZR	ZR		A	GG**A**			
5	P38Kb_38VR	VR	354	A	GT**A**	352–354	Val	Valine
	P49Kb_72ZR	ZR		G	GT**G**			
6	P38Kb_38VR	VR	387	G	TC**G**	385–387	Ser	Serine
	P49Kb_72ZR	ZR		A	TC**A**			
7	P38Kb_38VR	VR	468	C	AC**C**	466–468	Ser	Serine
	P49Kb_72ZR	ZR		T	AG**T**			
8	P38Kb_38VR	VR	570	T	CC**T**	568–570	Pro	proline
	P49Kb_72ZR	ZR		G	CC**G**			
9	P38Kb_38VR	VR	600	C	CT**C**	598–600	Leu	Leucine
	P49Kb_72ZR	ZR		T	CT**T**			
10	P38Kb_38VR	VR	670	C	**C**TA	670–672	Leu	Leucine
	P49Kb_72ZR	ZR		T	**T**TA			

Table A2: Genetic divergence of COI sequences alignment including *Lernaea cyprinacea* Linnaeus, 1758 sequence amplified with a different primer set; expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). Represented as GenBank/Sequence ID, Taxon and Country. Sequences from the present study are represented in bold and grey shade, *Sinergasilus polycolpus* was used as the outgroup. Abbreviations: AU – Australia; BP – Barberspan; CN – China; NY – Nyamiti Pan; TW – Takwasa; ZA – South Africa.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	**P44Kh *Lernaea cyprinacea* TW ZA**		**62**	**1**	**8**	**8**	**12**	**14**	**15**	**14**	**14**	**14**	**22**	**318**
2	**P64Ki *Lernaea cyprinacea* NY ZA**	**85.55**		**62**	**75**	**75**	**90**	**79**	**78**	**80**	**77**	**77**	**81**	**164**
3	**P69Kh *Lernaea cyprinacea* BP ZA**	**99.91**	**85.55**		**7**	**7**	**11**	**13**	**14**	**13**	**13**	**13**	**21**	**319**
4	MT371896 *Lernaea cyprinacea* AU	**99.20**	**85.32**	**99.30**		0	8	10	10	10	8	8	17	286
5	MT371895 *Lernaea cyprinacea* AU	**99.20**	**85.32**	**99.30**	100		8	10	10	10	8	8	17	286
6	KM235194 *Lernaea cyprinacea* CN	**98.96**	**85.44**	**99.05**	99.26	99.26		15	15	15	14	14	18	373
7	MH982216 *Lernaea cyprinacea* CN	**98.76**	**85.45**	**98.85**	99.07	99.07	98.79		2	8	15	15	19	339
8	MH982219 *Lernaea cyprinacea* CN	**98.67**	**85.64**	**98.76**	99.07	99.07	98.79	99.84		8	15	15	19	339
9	MH982220 *Lernaea cyprinacea* CN	**98.76**	**85.27**	**98.85**	99.07	99.07	98.79	99.36	99.36		15	15	19	338
10	MH982226 *Lernaea cruciata* CN	**98.76**	**85.82**	**98.85**	99.26	99.26	98.87	98.79	98.79	98.79		0	20	334
11	MH982227 *Lernaea cruciata* CN	**98.76**	**85.82**	**98.85**	99.26	99.26	98.87	98.79	98.79	98.79	100		20	334
12	MH982221 *Lernaea cyprinacea* CN	**98.05**	**85.08**	**98.14**	98.42	98.42	98.55	98.47	98.47	98.47	98.39	98.39		339
13	KR263117 *Sinergasilus polycolpus* CN	**72.44**	**73.46**	**72.36**	73.47	73.47	72.23	72.68	72.68	72.76	73.09	73.09	72.68	

Table A3: Sites of amino acid variation in the alignment of partial COI *Neoergasilus* sequences from this study using invertebrate mitochondrion translation and stating what amino acids the codons translate. Abbreviations: BD – Boskop Dam (from the Mooi River); KR – South Korea; OR – Olifants River (in the Kruger National Park); US – The United States of America; ZA – South Africa.

	Sequence ID	Locality	Position in alignment	Nucleotide substitutions	Codons	Codon positions	3-letter Amino acid	Amino acid
1	KR049037	KR	66	T	GC**T**	64–66	Ala	Alanine
	Other *Neoergasilus* sequences			C	GC**C**			
2	KR049037	KR	69	C	CT**C**	67–69	Leu	Leucine
	Other *Neoergasilus* sequences			T	CT**T**			
3	KR049037	KR	81	T	AT**T**	78–81	Ile	Isoleucine
	Other *Neoergasilus* sequences			C	AT**C**			
4	KR049037	KR	85	T	**T**TA	85–87	Leu	Leucine
	Other *Neoergasilus* sequences			C	**C**TA			
5	KR049037	KR	90	G	GA**G**	88–90	Glu	Glutamic acid
	Other *Neoergasilus* sequences			A	GA**A**			
6	KR049037	KR	120	C	GA**C**	118–120	Asp	Aspartic acid
	Other *Neoergasilus* sequences			T	GA**T**			
7	KR049037	KR	150	C	GC**C**	148–150	Ala	Alanine
	Other *Neoergasilus* sequences			T	GC**T**			
8	KR049037	KR	165	G	**AT**G	163–165	Met	Methionine
	Other *Neoergasilus* sequences			A	AT**A**			
9	KR049037	KR	210	C	AA**C**	208–210	Asn	Asparagine
	Other *Neoergasilus* sequences			T	AA**T**			
10	KR049037	KR	228	A	AT**A**	226–228	Met	Methionine
	Other *Neoergasilus* sequences			G	AT**G**			
11	KR049037	KR	249	C	GC**C**	247–249	Ala	Alanine
	Other *Neoergasilus* sequences			T	GC**T**			
12	KR049037	KR	255	G	CC**G**	253–255	Pro	Proline
	Other *Neoergasilus* sequences			C	CC**C**			

	Sequence ID	Locality	Position in alignment	Nucleotide substitutions	Codons	Codon positions	3-letter Amino acid	Amino acid
13	KR049037	KR	270	A	AT**A**	268–270	Met	Methionine
	Other *Neoergasilus* sequences			G	AT**G**			
14	*KR049037	KR	275 & 276	TC	T**TC**	274–276	Phe	Phenylalanine
	Other *Neoergasilus* sequences			AT	T**AT**		Tyr	Tyrosine
15	KR049037	KR	280	G	TG**G**	277–280	Trp	Tryptophan
	Other *Neoergasilus* sequences			A	TG**A**			
16	KR049037	KR	285	G	TT**G**	283–285	Leu	Leucine
	Other *Neoergasilus* sequences			A	TT**A**			
17	KR049037	KR	288	T	GT**T**	286–288	Val	Valine
	Other *Neoergasilus* sequences			C	GT**C**			
18	KR049037	KR	324	G	GT**G**	323–324	Val	Valine
	Other *Neoergasilus* sequences			A	GT**A**			
19	KR049037	KR	342	T	AC**T**	340–342	Thr	Threonine
	Other *Neoergasilus* sequences			C	AC**C**			
20	KR049037	KR	345	T	GG**T**	343–345	Gly	Glycine
	Other *Neoergasilus* sequences			C	GG**C**			
21	KR049037	KR	351	A	AC**A**	349–351	Thr	Threonine
	Other *Neoergasilus* sequences			G	AC**G**			
22	KR049037	KR	354	A	GT**A**	351–354	Val	Valine
	Other *Neoergasilus* sequences			G	GT**G**			
23	KR049037	KR	360	C	CC**C**	358–360	Pro	Proline
	Other *Neoergasilus* sequences			T	CC**T**			
24	KR049037	KR	363	T	CC**T**	361–363	Pro	Proline
	Other *Neoergasilus* sequences			A	CC**A**			
25	KR049037	KR	423	T	CA**T**	421–423	His	Histidine
	Other *Neoergasilus* sequences			C	CA**C**			
26	*KR049037	KR	433	G	**G**TT	433–435	Val	Valine

	Sequence ID	Locality	Position in alignment	Nucleotide substitutions	Codons	Codon positions	3-letter Amino acid	Amino acid
	Other *Neoergasilus* sequences			A	**A**TT		Ile	Isoleucine
	KR049037	KR		T	**T**TA			
27	Other *Neoergasilus* sequences		445	C	**C**TA	445–447	Leu	Leucine
	KR049037	KR		G	AG**G**			
28	Other *Neoergasilus* sequences		468	A	AG**A**	466–468	Ser	Serine
	KR049037	KR		T	GG**T**			
29	Other *Neoergasilus* sequences		495	A	GG**A**	493–495	Gly	Glycine
	KR049037	KR		A	AT**A**			
30	Other *Neoergasilus* sequences		513	G	AT**G**	511–513	Met	Methionine
	KR049037	KR		C	TT**C**			
31	Other *Neoergasilus* sequences		522	T	TT**T**	520–522	Phe	Phenylalanine
	KR049037	KR		G	TG**G**			
32	Other *Neoergasilus* sequences		528	A	TG**A**	526–528	Trp	Tryptophan
	KR049037	KR		C	AT**C**			
33	Other *Neoergasilus* sequences		540	G	AT**G**	538–540	Ile	Isoleucine
	KR049037	KR		A	AC**A**			
34	Other *Neoergasilus* sequences		543	G	AC**G**	541–543	Thr	Threonine
	KR049037	KR		C	**C**TA			
35	Other *Neoergasilus* sequences		553	T	**T**TA	553–555	Leu	Leucine
	KR049037	KR		A	TC**A**			
36	Other *Neoergasilus* sequences		564	C	TC**C**	562–564	Ser	Serine
	P43Rb; P46Kg; MZ964935	BD, ZA; OR, ZA; US		C	**C**TA		Leu	Leucine
37	KR049037; MZ964933; MZ964936; ZOOPS113-18	KR; US	574	T	**T**TA	574–576	Leu	Leucine
	*MZ964934	US		A	**A**TA		Met	Methionine
	MZ964934	US		C	AC**C**			
38	Other *Neoergasilus* sequences		591	T	AC**T**	589–591	Thr	Threonine

	Sequence ID	Locality	Position in alignment	Nucleotide substitutions	Codons	Codon positions	3-letter Amino acid	Amino acid
39	KR049037	KR	600	T	CT**T**	598–600	Leu	Leucine
	Other *Neoergasilus* sequences			G	CT**G**			
40	*MZ964934	US	604	T	**T**AT	604–606	Tyr	Tyrosine
	Other *Neoergasilus* sequences			G	**G**AT		Asp	Aspartic acid
41	KR049037	KR	613	C	**C**TA	613–615	Leu	Leucine
	Other *Neoergasilus* sequences			T	**T**TA			
42	P43Rb	BD, ZA	690	C	CA**C**	688–690	His	Histidine
	P46Kg	OR, ZA		T	CA**T**			
43	P43Rb	BD, ZA	693	T	CC**T**	691–693	Pro	Proline
	P46Kg	OR, ZA		A	CC**A**			

*Nucleotide substitutions that led to missense mutations.

Table A4: Adult female morphological characters of the ten *Neoergasilus* species used in generating the new taxonomic key.

Characters	*Neoergasilus* sp. nov.	*Neoergasilus bullatus* Kim I.H. & Choi, 2003	*Neoergasilus squaliobarbi* (Dogiel & Akhmerov, 1952) (syn. *Neoergasilus inflatus* Yin, 1956)	*Neoergasilus kherai* Batish & Brar, 1989	*Neoergasilus indicus* Vankara & Chikkam, 2010
Shape / size of cephalothorax	inflated	inflated	inflated	elongate	elongate
Spines / setae on antennae	elongate spine on the proximal margin of first segment; stout cone-shaped spine emerging from the base of second segment	one large, scalpel-like inner distal seta; second segment with one papillate seta on distal third of inner margin; terminal claw with one proximal seta	basal segment does not have a disk of visible expansion, and there is a spine on the inner side of the antennae.	antenna basal segment devoid of any armature, second segment bears a small spine near the joint between the 2nd and 3rd segment; third segment longest and bears a long spine at some distance from the base	antenna basal segment naked; second segment with cone-like basal process and a small median spine; third segment sub-chelate
Ornamentation on the cephalothorax	one oval structure present	absent / not mentioned	absent / not mentioned	absent / not mentioned	absent / not mentioned
Leg 1: processes on first exopodite	knob-like process on inner distal margin	elongate blade process on inner distal margin	absent / not mentioned	elongate blade process on outer distal margin	knob-like process on inner distal margin
Leg 4: exopod and endopod	2-segmented exopod and 3-segmented endopod	2-segmented exopod and 3-segmented endopod	1-segmented exopod and 2-segmented endopod	2-segmented exopod and 3-segmented endopod	2-segmented exopod and 3-segmented endopod
Legs 2–4: length of first exopodal spines on outer margins	less than half of second segment	less than half of second segment	less than half of second segment	less than half of second segment	less than half of second segment
Leg 5: unsegmented or one-segmented	uniramous, single segmented	uniramous, single segmented	not mentioned	uniramous, single segmented	uniramous, two segmented
Leg 5: setation	one seta extending from fifth pedigerous somite and three unequal setae attached to free segment	one dorsolateral seta of fifth pedigerous somite, free segment with one minute inner and one large, distally plumose terminal seta	not mentioned	two long terminal setae, one short lateral seta	one short seta on proximal segment, three unequal setae on distal segment

Table A4 cont.

Characters	*Neoergasilus ferozepurensis* Kumari, Khera & Gupta, 1988	*Neoergasilus notopteri* Kumari, Khera & Gupta, 1988	*Neoergasilus angustus* Kim I.H. & Choi, 2003	*Neoergasilus longispinosus* Yin, 1956	*Neoergasilus japonicus* (Harada 1930)
Shape / size of cephalothorax	elongate	elongate	elongate	elongate	elongate
Spines / setae on antennae	third segment bears a long spine at base	third segment curved around centre, forming a blunt short spine at base	first segment with one large, scalpel-like distal seta; second segment with one small seta at distal 1/4 of inner margin; terminal claw with one small proximal seta	long thorn at the junction of the distal inner side of the first segment and the third segment	first segment with a spine at its distal end, second segment with a minute spine at almost the middle of the inner border.
Ornamentation on the cephalothorax	absent / not mentioned	absent / not mentioned	one oval structure present (drawing)	absent / not mentioned	Absent / not mentioned (except drawing of Kim & Choi, 2003)
Leg 1: processes on first exopodite	absent / not mentioned	Knob-like process on inner distal margin	absent / not mentioned	absent / not mentioned	absent / not mentioned
Leg 4: exopod and endopod	2-segmented endopod and exopod	2-segmented exopod and 3-segmented endopod	2-segmented endopod and exopod	2-segmented exopod and 3-segmented endopod	1-segmented exopod and endopod
Legs 2–4: length of first exopodal spines on outer margins	longer than second segment	less than half of second segment	longer than second segment	longer than third segment	less than half of second segment
Leg 5: unsegmented or one-segmented	uniramous, single segmented	uniramous, single segmented	uniramous, single segmented	uniramous, single segmented	uniramous, single segmented
Leg 5: setation	two setae	three setae	one dorsolateral seta of fifth pedigerous somite and three unequal setae on free segment	three equal setae	two setae

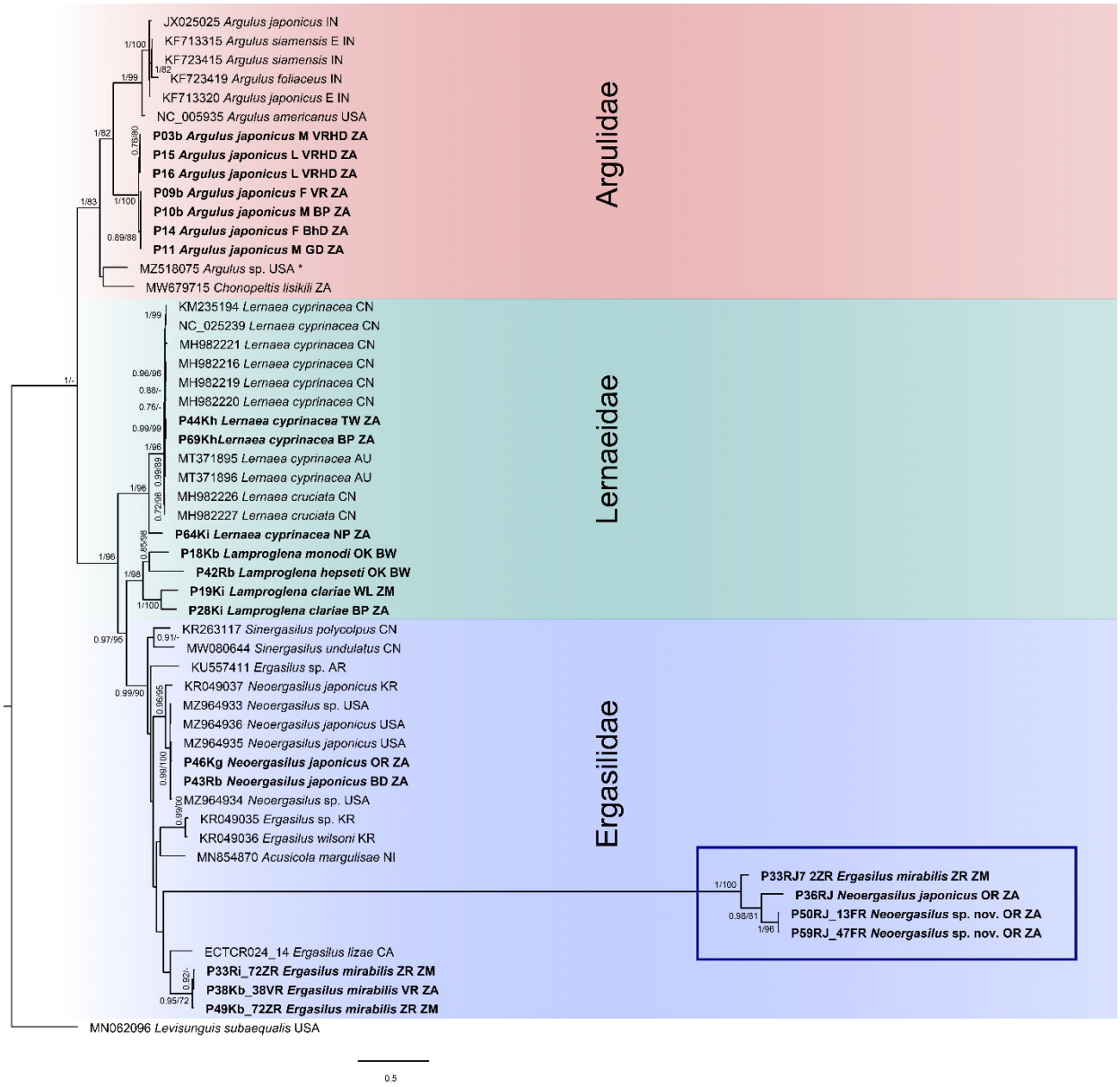


Figure A1: Phylogenetic tree of all parasitic Crustacea sequences generated from the current study and selected GenBank and Bold sequences based on partial COI mitochondrial gene alignments. Newly generated sequences are bold in gradient shade: Argulidae (orange); Ergasilidae (green); Lernaeidae (blue). Sequences in blue block represent the newly described species *Neoergasilus* sp. nov. and other Ergasilidae sequences amplified with the same primers. Nodal support presented close to branches for Bayesian Inference (>0.7) and Maximum Likelihood (>70%) analyses (BI/ML). *Levisunguis subaequalis* was used as the outgroup. Abbreviations: Abbreviations: AR – Argentina, AU – Australia, BD – Boskop Dam, BhD – Bloemhof Dam, BP – Barberspan, BW – Botswana, CA – Canada, CN – China, E – Eggs, F – Female, FR – The Great Fish River, GD – Gariep Dam, IN – India, KR – South Korea, L – Larva, M – Male, NI – Nicaragua, NP – Nyamiti Pan, OK – Okavango, OR – Olifants River, TW – Takwasa, USA – United States of America, VR – Vaal River, VRHD – Vaal River Holding Dam, WL – West Lunga, ZA – South Africa, ZM – Zambia, ZR – Zambezi River.