

Revision and ecology of selected freshwater limpets (*Burnupia* spp.) in South Africa

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Abstract:

Freshwater limpets form part of the Burnupiidae family and there are 14 species belonging to the genus *Burnupia* in South Africa. Freshwater limpets has received little attention in modern molecular aquatic research. There are only three or four studies on the taxonomy of freshwater limpets, this shows how little information is known about these species. Molluscs and parasites interact in a variety of ways. Nematodes of molluscs are less researched than mollusc-trematode connections. Some parasites use molluscs as intermediate hosts, whereas other species conclude their life cycles in molluscs. The purpose of this study was to revise the taxonomy of South Africa's freshwater limpets and to investigate the presence and distribution of parasites in freshwater limpets, *Burnupia* spp. *Burnupia* specimens were collected from the Free State, North – West, Mpumalanga, Gauteng, and Limpopo. In total, two parasite species were collected from 77 *Burnupia* spp. A stereomicroscope was used to obtain photos of each of the *Burnupia* specimens that were collected. The photos were used to look for morphological characteristics that can be used to identify the species that were collected. A shedding method was used to induce the shedding of parasites from the *Burnupia* spp. The nematodes were fixed in 70% ethanol and studied with a light microscope. Photomicrographs of each nematode were taken to observe morphological characteristics that can be used to identify these parasites. For molecular analysis cytochrome c oxidase subunit 1 (cox1) and 18S were targeted. Phylogenetic analysis was done to determine the relationships between the limpet taxa. The sequences obtained in this study were analysed and compared to existing sequences on GenBank and BOLD. There were three *Burnupia* spp. obtained from this study, and they were identified and described. The nematodes that were collected were all female and were identified as *Daubaylia burnupiae*. The trematodes that were collected were identified and discovered through molecular analysis and this trematode was part of the *Hypoderaeum* genus. Future studies are needed to obtain more knowledge on the characteristics used to identify limpets because giving names to the species obtained in this study was problematic due to limited morphological features together with the limited number of available published sequences. Further studies are needed to provide more information on the taxonomy of the nematodes, the survival rate of the host, the transmission mode and if pollution has an effect of the reproduction behavior of parasites.

Key words: Molluscs, Burnupiidae, Freshwater limpets, Parasitic infections, Intermediate hosts, Nematodes, Trematodes.

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Chapter 1: General introduction

1.1 Mollusca

The phylum Mollusca displays remarkable diversity, with an estimated 80,000–100,000 described species and a potential total diversity reaching as high as 200,000 (Strong *et al.*, 2008). In terms of species richness, Mollusca ranks second only to arthropods (Strong *et al.*, 2008). Within the realm of freshwater molluscs, two primary groups emerge: Bivalvia and Gastropoda. Morphologically, the Gastropoda further subdivides into two informal groups, namely the pulmonates and prosobranchs (Seddon *et al.*, 2011). These aquatic invertebrates exhibit significant adaptive abilities, with representatives thriving in virtually all categories of inland water bodies (Vinarski *et al.*, 2021). Freshwater molluscs, in particular, exhibit their peak levels of diversity and endemism in lakes, and big river basins, as documented by Seddon (2000). Notably, these diverse habitats are distributed across various regions within the continent of Africa (Seddon *et al.*, 2011). Among the freshwater invertebrates, molluscs stand out as easily recognisable due to the presence of one or two shells (Appelton *et al.*, 2002). Gastropods exhibit snail and limpet-like forms, while bivalves, as indicated by their name, manifest clam- or mussel-like configurations with two shells (Appelton *et al.*, 2002).

Calcareous species, like molluscs, with a substantial fossil record can provide valuable information on oceanic changes and previous climate events, boosting our understanding of expected future changes (Fortunato, 2015). Numerous species are grown and harvested for aquaculture and commercial products, playing a significant role in today's economy (Fortunato, 2015). Historically, molluscs have held considerable importance to people in a variety of ways, and today they are a globally influential economic group (Fortunato, 2015). Molluscs have been utilised as food and used as decorations and adornments, such as pearls that are sold commercially (Cummings *et al.*, 2016). Some of these species are vital components of freshwater ecosystems that support inland fisheries by offering a valuable supply of food to humans (Brown, 1994; Moema *et al.*, 2008). They play a crucial role in ecosystem management, helping to shape aquatic bottom ecosystems, while also providing food, habitat, and protection for a variety of other taxa. In benthic ecosystems, snails play a significant role by providing complex and varied habitats that influence processes like the settlement and colonisation of some organisms and support a wide variety of different organisms (Fortunato, 2015).

Certain molluscs can be very sensitive to changes in the environment and thus they can be used as an indicator of the habitat they live in (Fortunato, 2015; Cummings *et al.*, 2016; Hanley, 2022). Due to their responsiveness to environmental changes and water quality, as well as their efficient water filtering capabilities, they are often metaphorically called "livers of the rivers" or "aquatic canaries in the coalmine" (Cummings *et al.*, 2016). Certain species are useful in biomedical research, whereas others are significant pests, invasives, vectors and intermediate hosts of harmful parasites (Brown, 1994; Fortunato, 2015).

1.2 Gastropoda

Gastropoda is the largest group in the phylum Mollusca, commonly referred to as snails and slugs. They are a diverse and abundant class, consisting of snails that have a shell (Solem, 2020). Freshwater gastropods are distributed across every continent except Antarctica, populating a wide array of aquatic habitats such as lakes, streams, rivers, and wetlands (Strong *et al.*, 2008). The majority of these gastropods thrive in submerged conditions and can be found on stones, vegetation, and in sediment (Strong *et al.*, 2008).

Despite their significance, there are very few current DNA-based systematic studies that have been done on the taxonomic status of these molluscs (Brown 1994; Appelton *et al.*, 2002; De Kock & Wolmarans, 2017; Raphalo *et al.*, 2021). In 1994, Brown (1994) stated that the molluscan catalogue is still incomplete and comparative morphology, molecular studies, and knowledge of their distribution are needed to understand these species as biological units. Brown (1994) also stated that more research is needed to determine the distribution and conservation of Gastropoda. The same statement was made by Appelton *et al.* (2002) when they stated that this family needs to be revised at both generic and species levels. Thus, it is likely that the taxonomic diversity of Mollusca, particularly of Gastropoda, has been underestimated and understudied despite their significance for ecosystem structure and functioning (Raphalo *et al.*, 2021).

Within the Gastropoda, most freshwater limpets belong to the subfamily Ancyliinae Rafinesque, 1815 (previously Ancyliidae Rafinesque, 1815) and to the family Burnupiidae Albrecht, 2017. These limpets are small organisms, with their shell typically no longer than 10 mm. They have a cap-like shell form, with little to no coiling and an obtuse or hooked apex. In streams, they are typically found on rocks or vegetation (Brown, 1994; De Kock & Wolmarans, 2017). They can attach to rocks because of the flexibility of the periostracal shell edges which enables a tight closing of the aperture (Albrecht *et al.*, 2003). Freshwater limpets serve very important ecological

functions where they play a critical role in the aquatic food chain by linking the flow of energy from the producers to consumers.

1.3 South African limpets

Africa is home to approximately 400 species of freshwater snails, which include limpets. In South Africa, an abundant and varied snail fauna can be found, occurring in a variety of terrestrial and aquatic habitats, and ranging in size from only a few millimetres to over 20 meters (Fortunato, 2015; Raphalo *et al.*, 2021). However, the taxonomy of the freshwater limpets in the Ancylinae and Burnupiidae is poorly known (Oberholzer & Van Eeden, 1963; Brown, 1994; Appleton, *et al.*, 2002; De Kock & Wolmarans, 2017; Raphalo *et al.*, 2021). Currently, only two freshwater limpet genera, *Burnupia* Walker, 1912 and *Pettancylus* Iredale, 1943 (previously *Ferrissia* Walker, 1903) have been identified from South Africa, under which numerous species have been identified and described (Day & Moor, 2002). De Kock and Wolmarans (2017) stated that out of the total number of species that are recorded in the South African National Freshwater Snail Collection (NFSC) database, only two species of *Burnupia* have a detailed account of their preferred habitat and distribution published, namely *Burnupia capensis* (Walker, 1912) and *Burnupia stenochorias* (Melvill & Ponsonby, 1903). Currently, 14 different species of *Burnupia* are documented in South Africa (Brown, 1994; Appleton, *et al.*, 2002; Griffiths *et al.*, 2015; De Kock & Wolmarans, 2017).

In the past ten years, a limited number of researchers have used molecular phylogenetic analyses and species delimitation techniques to clarify taxonomy and species complexity, in order to increase our understanding of the richness of both terrestrial and marine gastropod fauna and to describe new species (Raphalo *et al.*, 2021; Sands *et al.*, 2022). Likewise, there have only been a small number of molecular investigations on the gastropod fauna of South Africa (Raphalo *et al.*, 2021), especially the understudied freshwater limpets. The existing literature on limpet taxonomy is sparse, with only a few major publications available (Walker, 1912; Connolly, 1939; Oberholzer & van Eeden, 1969; Raphalo *et al.*, 2021; Sands *et al.*, 2022), reinforcing the fact that information on these limpets is limited and more studies on their taxonomy is needed. Proper morphological revisions and accurate descriptions, coupled with molecular characterisation, could lead to the possible discovery of new species, help identify endemic and invasive species, and assist in geographical distributions.

1.3.1 South African National Freshwater Snail Collection (NFSC) database

One of the research areas of the Institute (North-West University) that has figured most prominently over the years is snail research. The Snail Research Unit was officially established in 1956, with Prof JA van Eeden as director, and was initially funded by the CSIR and later by the SAMNR. In 1969, the funding was taken over by the Medical Research Council. The primary aim of the research was to learn more about freshwater snails that could possibly serve as intermediate hosts for medical and veterinary importance, referring to animal parasitic flatworms. Morphological investigations were carried out on snails and their geographical distribution in South Africa was mapped (Van Eeden, 2017).

To carry out this formidable task, the state ecologist, and several health inspectors from around the country were involved. Eventually and over many years, more than 20,000 specimens were documented. By 2015, this collection was still known as the National Freshwater Snail Collection of the North-West University and, in addition to medically and veterinary-important freshwater snails, also contained numerous other species collected by researchers. In order to explain each species' unique distribution pattern, time-consuming experimental investigations into their conditions of existence were undertaken, both in the laboratory and under the conditions in which they occur naturally. Over the years, reports of this research have appeared in various scholarly articles as well as in the in-house magazine *Indikator* (Van Eeden, 2017).

The peak of this research was in 1981 in the publication of the comprehensive *Atlas of Bilharzia in Southern Africa*. Prof JA van Eeden, Dr RJ Pitchford and Prof JHS Gear were the editors of this Atlas, which is the result of 50 years of research. The Atlas clearly shows that *Bilharzia* is not only found in east-flowing rivers, as was long assumed, but also in many other rivers. It gives a comprehensive account of the occurrence of parasites that cause intestinal and urinary *Bilharzia*, as well as of the intermediate host snails that play a role in their life course. Most likely, this Atlas is the most comprehensive geographical distribution document of any animal group in Africa (van Eeden et al., 1984). This NWU snail collection is now being used to look at the historical distributions of South African freshwater molluscs.

1.3.2 Morphological studies

Walker (1912) published the first extensive study of freshwater limpets in South Africa, but the presence of these molluscs in the country had been known for many years (Krauss, 1848).

Additionally, an important contribution to our understanding of this family originated from a monographic examination of non-marine Mollusca in South Africa (Connolly, 1939). Oberholzer and van Eeden (1969) stated that the taxonomy of these limpets is in a very poor state and that very little is known about their soft anatomy. They also mentioned that the simple description of the genus *Burnupia* makes the determination of different species difficult and sometimes impossible.

Most studies done on the taxonomy of gastropods are focused on the physical characteristics of the shell to identify these species. However, morphology-based taxonomy can be restricted, time-consuming, requires in-depth familiarity with a group, and can result in inaccurate species identification (Raphalo *et al.*, 2021). Accurate identification typically requires using original species descriptions and drawings of shells as a reference and taking their type localities into consideration, but this information is sometimes limited (Oberholzer & van Eeden, 1969). To understand speciation within the freshwater limpets, Brown (1994) suggested an assessment of the genetic diversity among limpet populations and taxa. This viewpoint was shared by Appleton *et al.* (2002), who believe that these molluscs need to be revised at both the generic and species levels.

1.3.3 Molecular studies

Recently, Raphalo *et al.* (2021) stated that there are difficulties regarding the taxonomy and distribution of limpets due to the lack of modern DNA-based systematics. There are only a few numbers of molecular studies done on freshwater limpets in contrast to terrestrial snail genera that have been thoroughly studied using DNA sequencing (Raphalo *et al.*, 2021). In a study that was done in 2022 by Sands *et al.* species were sampled in the Lesotho Highlands, and molecular analysis was used to identify these species and link them with their type localities. With this article, 47 species were sequenced and uploaded to GenBank, providing major contributions towards the building of a solid taxonomical database for limpets.

Most recently, Saadi *et al.* (2020) published a paper where they studied the molecular phylogeny of freshwater limpets and snails. In this study, they sequenced one species of the Burnupiidae family. The investigation reported the noticeable placement of *Burnupia* as a sister taxon to the family Planorbidae Rafinesque, 1815 in this paper's phylogenetic analysis (Saadi *et al.*, 2020). Bouchet *et al.* 2017, proposed the regrouping of *Burnupia* into a distinct family based on molecular

findings (Saadi *et al.*, 2020). Subsequently, *Burnupia* was formally assigned to a newly established family, Burnupiidae (Bouchet *et al.*, 2017; see chapter 2).

1.4 Conservation

Due to the genus *Burnupia* having a high sensitivity to dehydration and climate change, anthropogenic pressures could pose a significant threat to these taxa (De Kock & Wolmarans, 2017). The molluscs have experienced some of the most documented extinctions of any taxon, according to De Kock and Wolmarans (2017). Furthermore, the rate of extinction for freshwater species is thought to be as high as 4% per decade, and little is known about the conservation status of freshwater molluscs in South Africa (Dudgeon *et al.*, 2006; De Kock & Wolmarans, 2017). Information concerning the conservation of limpets is understudied and poorly understood (De Kock & Wolmarans, 2017).

Research on Gastropoda has previously been dominated by their distribution and the effects of the invasive molluscan species on native biota. It is important to update these literature gaps including morphological description and genetic analysis so that the necessary information on these species can be provided. These species need to be assessed in terms of genetic diversity both on a taxonomic level and between populations (Brown, 1994; Appleton *et al.*, (2002).

According to the International Union for the Conservation of Nature (IUCN) Red List of threatened species, molluscs (such as *Burnupia*) are data deficient and lacking information. Information on the species distribution and population is required to provide an accurate assessment of its extinction risk (IUCN, 2010). The lack of information on the genus *Burnupia* is particularly evident as the data available on the IUCN Red List indicates that the last assessment of this species was conducted in 2010.

1.5 Molluscs as intermediate hosts

As mentioned previously, molluscs can be vectors of neglected tropical diseases (NTDs) and can serve as intermediate hosts for several parasites including nematodes and trematodes (Hamburger *et al.*, 1998; Grewal *et al.*, 2003; Olson *et al.*, 2003; Morley *et al.*, 2010; Born-Torrijos *et al.*, 2014; Outa & Avenant-Oldewage, 2023).

1.5.1 Nematoda

Nematodes are non-segmented, multicellular organisms that can be anywhere from 0.1 millimetres to several meters long (Koppenhöfer, 2007), but most nematodes are tiny and need a microscope for examination (Bird & Bird, 2012). Due to their often cylindrical and elongated body shapes, nematodes are sometimes known as threadworms or roundworms (Koppenhöfer, 2007). The anatomy of nematodes is extremely constant (Bird & Bird, 2012), but the different species are incredibly diverse, complex, and of high significance (Lee, 2002). They do not have respiratory and circulatory systems, although they do have basic neurological, excretory, and muscular systems (Koppenhöfer, 2007). These organisms can use molluscs as their intermediate hosts, for the transmission of the juvenile nematodes to their final hosts.

Nematodes associate with molluscs in a wide variety of parasitic relationships (Grewal *et al.*, 2003), and mounting evidence suggests that they may play a significant role in controlling host population dynamics (Morand *et al.*, 2004). According to Morley (2010), a wide variety of species of terrestrial gastropods are frequently affected, but those nematodes with medical and veterinary significance are *Angiostrongylus cantonensis* (Chen, 1935), *Angiostrongylus vasorum* (Baillet, 1866), and *Muellerius capillaris* (Mueller, 1889).

The different relationships between nematodes and molluscs range from paratenic to parasitic and pathogenic relationships (Grewal *et al.*, 2003). A paratenic host is an organism that acts as an intermediary for the transmission of a parasite's larval stage or stages from one host to another, but in which little or no developmental activity occurs. Many nematode juveniles infect the mollusc hosts but do not grow further, whereas the adults parasitise vertebrate hosts. These molluscs thus serve as paratenic hosts of nematodes.

Molluscs can also serve as intermediate hosts for nematodes (see Figure 1.1). Typically, the first-stage nematode juvenile enters the mollusc host and matures into an infectious stage, which is subsequently acquired by the final vertebrate host by unintentionally or purposefully consuming the infected mollusc (Grewal *et al.*, 2003). Numerous kinds of snails can act as the nematodes' final, permanent hosts. Generally, the adult nematodes are free-living, whereas the juvenile nematode stages develop inside the body cavity or foot muscle of the host. In other instances, the nematode would complete its entire life cycle within one host and may kill its ultimate host (Grewal *et al.*, 2003).

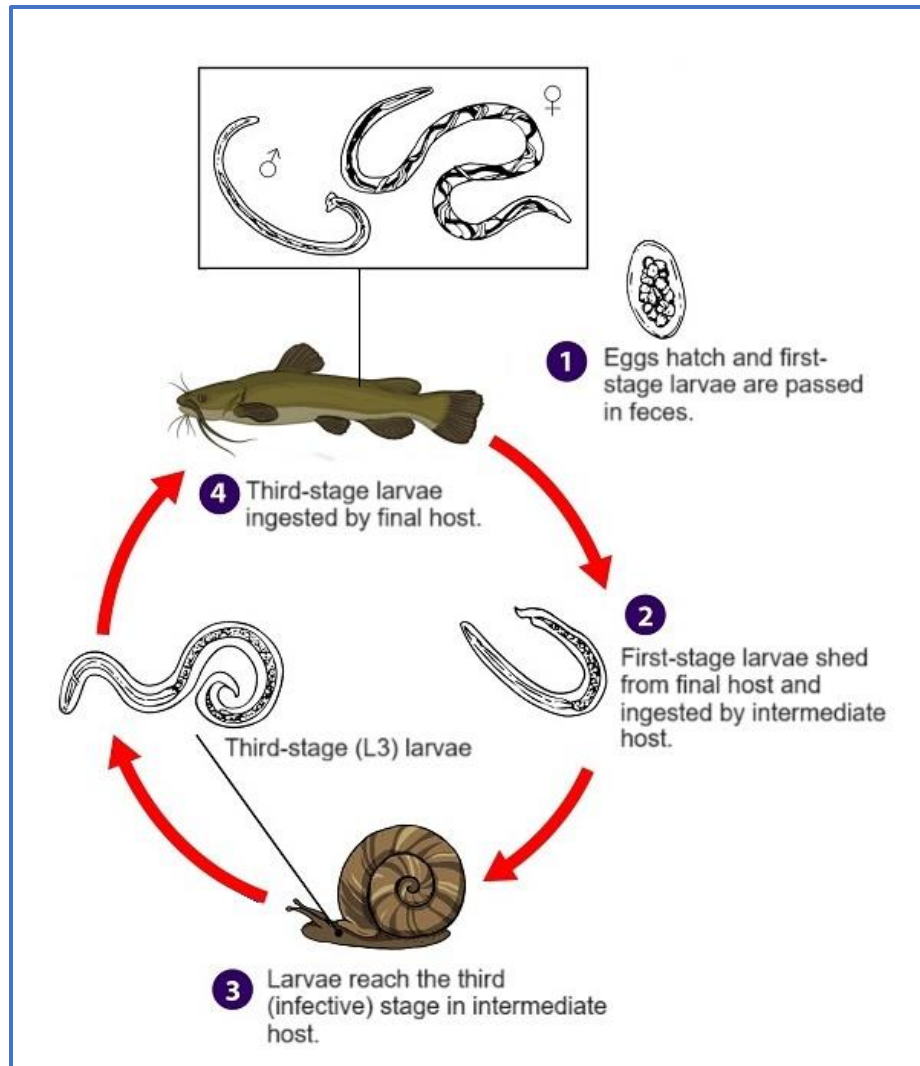


Figure 1.1: A generalised nematode life cycle utilising a snail as an intermediate host and fish as a final host (adapted from Global Health, Division of Parasitic Diseases, 2019).

The characteristics of nematode development in molluscs depend on their incredibly diverse life cycles (Morley *et al.*, 2010). Despite the large number of parasite species that have been reported, only two major groups—metastrongyloids and rhabditoids—dominate the fauna (Grewal *et al.*, 2003). Rhabditoids only need the mollusc to finish their life cycles, but metastrongyloids utilise the mollusc as an intermediate host and mature in a vertebrate (Grewal *et al.*, 2003; Morley *et al.*, 2010). Literature shows at least eleven rhabditid species complete their entire life cycles within freshwater gastropods (Grewal *et al.*, 2003; Zimmermann *et al.*, 2011a, 2011b; Outa & Avenant-Oldewage, 2023). Although nematode parasitism of molluscs is common, it is sometimes disregarded compared to trematode infections (Morley, 2010).

1.5.2 Trematoda

Digenean trematodes are the most prevalent among parasitic flatworms. Their hosts consist of a wide variety of organisms, ranging from all vertebrate categories to a majority of freshwater, marine and terrestrial invertebrates (Niewiadomska & Pojmanska, 2011). Digeneans exhibit a wide range of body types, but the majority have elongated, oval and dorsoventrally flattened bodies (Vermaak, 2021). However, exceptions exist, as some species can have spherical bodies or lengthy elongated shapes and others can be thick and wide. A distinctive characteristic of digeneans is the presence of an oral sucker encircling the mouth and a ventral sucker situated on more distant regions of the body (Dawes, 1946; Vermaak, 2021). Furthermore, the tegumental outer layers of trematodes can exhibit a variety of features such as certain vesicles, spines, sensory papillae, pits, and ridges (Cribb, 2005). Though they possess the ability to self-fertilise and are nearly all hermaphrodites, most digeneans prefer to locate and reproduce with other mature trematodes within their host (Vermaak, 2021).

Based on the characteristics of digenean trematodes, their life cycles, and their final hosts, they can be categorised into groups in marine and aquatic environments (Esch *et al.*, 1988). One category consists of autogenic organisms, this is where the life cycle of the digenean takes place entirely within a freshwater or marine environment, where both the intermediate and final host are permanently inhabiting this habitat. The second category is allogenic organisms, where the parasite finishes their life cycle in hosts that are transient or temporary visitors to the environment (Esch & Fernandez, 1994).

The digenean life cycle (see Figure 1.2) occurs in one of three environments: first in aquatic habitats, where all hosts are required to be aquatic animals or to live near water, second in semi-aquatic habitats where organisms are parasitised by parthenogenic reproductions and metacercaria with terrestrial organisms serving as the ultimate host, and lastly in terrestrial habitats where terrestrial animals are used throughout the life cycle. Digeneans were able to finish their intricate life cycle in each of these environments by developing a variety of strategies (Niewiadomska & Pojmanska, 2011).

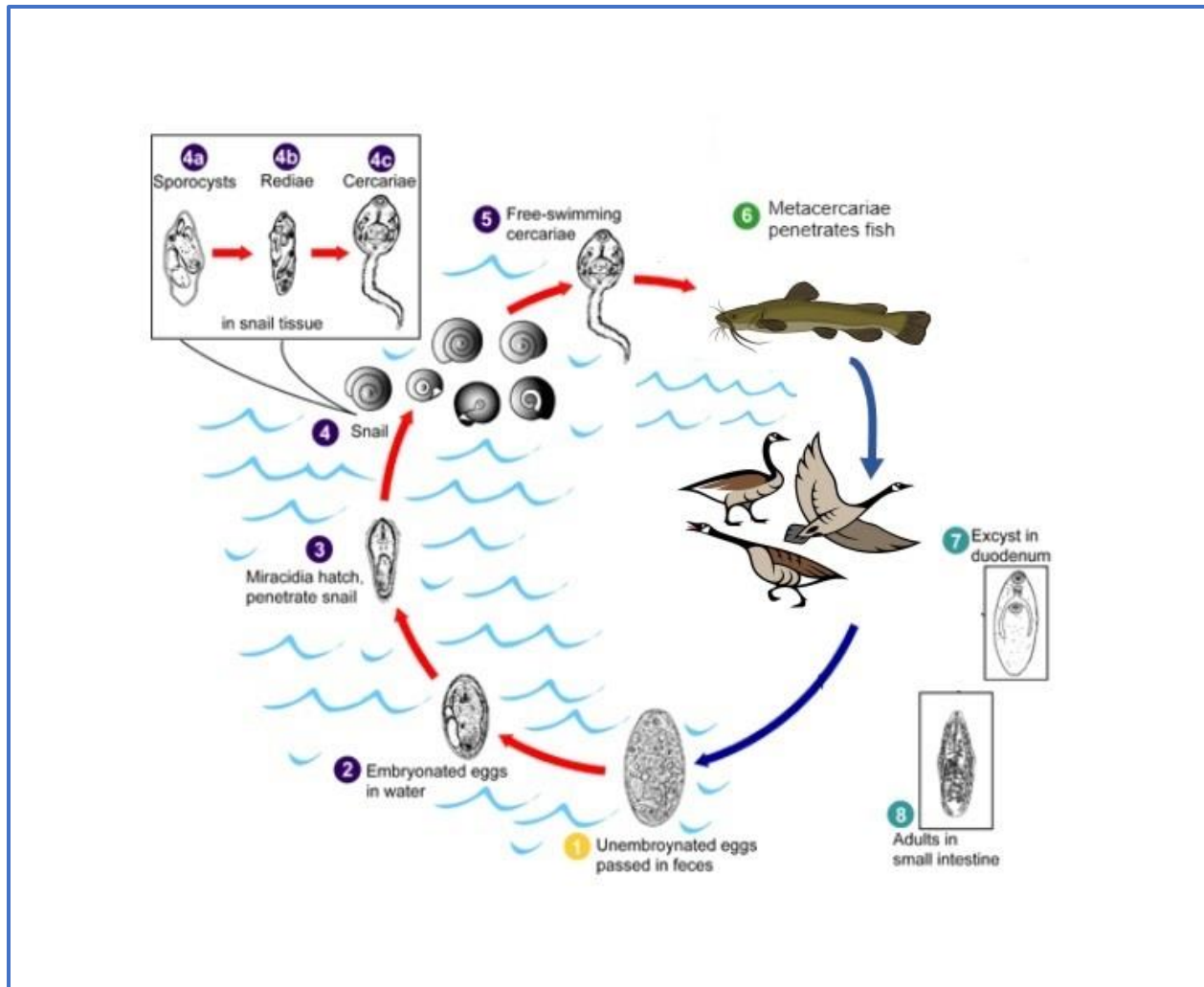


Figure 1.2: A generalised trematode life cycle utilising a snail as an intermediate host and fish (and other vertebrates) as a final host (adapted from Global Health, Division of Parasitic Diseases, 2019).

Digeneans possess a distinct and intricate life cycle that is characterised by the following features: the presence of free-swimming cercariae that consists of a tail, the miracidium that consists of layers of ectodermal cells, the absence of a digestive system in both the mother sporocyst and the miracidium obtaining a host as the final step in their life cycle, switching between asexual and sexual reproductive processes, and a sequence of asexual reproduction taking place in the first intermediate host (usually a mollusc) (Kostadinova, & Olmo, 2014).

The intricate life cycle of digeneans features a single hermaphroditic reproduction stage and a limited number of parthenogenic reproduction stage. The parthenogenic reproduction is typically associated with molluscan hosts which are the first intermediate hosts and hermaphroditic reproduction is usually associated with certain invertebrates which are usually the second

intermediate hosts, and all vertebrate groups which function as the final hosts (Niewiadomska & Pojmanska, 2011).

These digeneans also adapted the ability to manipulate the host and ultimately causing behavioural changes in the host. This includes prompting the infected host to exhibit unwise behaviour by causing it to move closer to potential predators, which can result in the host being captured and eaten by the predator. Additionally, this can cause the host to be easily spotted by a predator and increase the likelihood of the host being hunted and consumed (Niewiadomska & Pojmanska, 2011). The ultimate purpose of these manipulative behaviours is to facilitate the transmission of the parasite to its final host.

In South Africa, trematodes have infected different molluscs and used them for intermediate hosts. Cawston (1924) has consistently demonstrated that *Radix natalensis* (Krauss, 1848) typically serves as the host for *Fasciola* Linnaeus, 1758 parasites. Additionally, *Bulinus africanus* (Krauss, 1848) is an intermittent carrier of *Fasciola* and the primary carrier of diverse schistosomes in South Africa. Amphistomes (stomach or rumen flukes), and occasionally schistosomes, are known to infect *Physa tropica* (Krauss, 1848) and *Biomphalaria pfeifferi* (Krauss, 1848) (see Cawston, 1924).

1.6. Problem identification

As mentioned above, the morphology and molecular analysis of freshwater limpets is lacking. Proper morphological revisions and accurate descriptions, coupled with molecular characterisation, could lead to the possible discovery of new species, help identify endemic and invasive species, assist in geographical distributions, and fill the knowledge gaps of freshwater limpets.

Research on nematodes that parasitise aquatic molluscs is lacking in comparison to that of their terrestrial counterparts (Outa & Avenant-Oldewage, 2023). This study hopes to address this lack of information and follow methods that will give information on the abundance and prevalence of parasites in selected freshwater limpet species. The main aim of this study was to address the gaps in the current database and follow methods that would provide more information on the abundance and prevalence of parasites in selected freshwater limpet species.

Thus, it was hypothesised that the molecular and morphological analysis of freshwater limpets will lead to a more refined taxonomy of the selected freshwater limpets and that freshwater limpets are an intermediate host for digenean trematodes in South Africa.

1.6.1 Research Aims:

The following research aims were established:

Determine the biodiversity and systematics of selected *Burnupia* spp. from South Africa.

- Update, if possible, the distribution and conservation status of selected freshwater limpets in South Africa.
- Investigate the distribution and presence of parasites associated with freshwater limpets of South Africa.

1.6.2 Objectives

To accomplish the three aims, the following objectives were set:

- Collect *Burnupia* specimens from selected sites in the Free State, Gauteng, Limpopo, Mpumalanga and North West provinces.
- Use morphological studies and molecular analyses to identify the collected limpets and to determine their current biodiversity.
- Determine the genetic diversity of the *Burnupia* spp.
- Investigate the presence of parasites in the selected freshwater limpets with morphological and molecular analysis as well as shedding experiments.

1.7. Chapter layout

Chapter 1: Provides background information on freshwater limpets, the importance of these organisms and how they can be utilised as parasite hosts. The research aims, objectives and hypotheses are outlined and explained.

Chapter 2: Focuses on the revision of selected *Burnupia* spp. It provides information on the current and historical distribution of freshwater limpets in South Africa, achieved through molecular studies and morphological analysis.

Chapter 3: Focuses on the possible parasite infection in freshwater limpets. Infections of parasites were determined by shedding experiments. The parasites were morphologically studied, and molecular analyses were done.

Chapter 4: Provides a summative discussion, in which the project's findings are addressed, concluding remarks are made on the key research findings regarding the study's aims, and concludes with recommendations for future research.

Chapter 5: Provides the references that were used in this dissertation.

1.8. Project conference contributions

The outputs of this dissertation include two national conference contributions:

- Ungerer A, Hadfield KA, Malherbe W (2023) Revision and distribution of freshwater limpets (*Burnupia* sp.) in South Africa. 60th Southern African Society of Aquatic Scientists (SASAqS), 25–29 June 2023, Lord Charles Hotel, South Africa.
- Ungerer A, Hadfield KA, Malherbe W (2022) Revision and distribution of freshwater limpets (*Burnupia* sp.) in South Africa. 59th Southern African Society of Aquatic Scientists (SASAqS), 26 – 30 June 2022, Brandfort, South Africa.

Chapter 2: Revision and distribution of freshwater limpets (*Burnupia* spp.) in South Africa

2.1. Introduction

Freshwater limpets are small organisms, and their shell is not bigger than 10mm (Brown 1994). The identification guide used in Brown (1994) states that the shell of a limpet has a cap-like shape, with little to no coiling, and an obtuse or hooked apex, which typically has radial rows of tiny pits. They usually live on rocks, stones, and vegetation in fast-flowing water of rivers and streams (Brown, 1994; De Kock & Wolmarans, 2017).

The identification of limpets can be problematic, predominately for two reasons. Firstly, these molluscs have limited morphological structures, and secondly, these morphological features can be influenced by environmental factors (Sands *et al.*, 2022). Morphological structures such as the shell size can be altered by factors such as the location of the limpet; if the limpet is present on plants the shell shape can be elongated (Oberholzer & van Eeden, 1963) and the shell can be rounder if present on rocks. This can complicate limpet identification even further.

Thus, DNA sequence analysis has become essential for supporting taxonomic revisions and determining the state of species conservation (Raphalo *et al.*, 2021). The precision of genetic analysis, combined with an awareness of critical factors such as inbreeding and genetic diversity loss, has heightened the significance of population studies in conservation biology (Mortiz, 1994). The variations in DNA sequences serve as a measure for overall genetic differentiation, emphasising the necessity of their determination before establishing conservation priorities. (Mortiz, 1994).

Over the past two decades, DNA barcoding of the maternally inherited cytochrome oxidase c subunit I (COI) has been adopted for species-level identification when morphology-based classification is known to be difficult (Raphalo *et al.*, 2021). This molecular approach has been effectively applied to the identification of specimens in molluscs, building a database of references for identifying species (Raphalo *et al.*, 2021) and distinguishing between closely related species to assess phylogenetic relationships (Weigand *et al.*, 2011). Freshwater limpets have received

little attention in modern molecular aquatic research, and there are not many studies that can even begin to tackle these issues in freshwater biota (Sands *et al.*, 2022).

Freshwater limpets in South Africa were first thoroughly researched by Walker (1912) who provided descriptions, type localities, and morphological characteristics to identify the species. There is a scarcity of significant publications addressing the taxonomy of freshwater limpets in South Africa, underscoring the limited knowledge available about these molluscs. The few studies available provided type localities, taxonomic keys, and features on several freshwater molluscs, but not much information on environmental preferences or regional distribution (Connolly, 1936).

Freshwater limpets form part of the family Burnupiidae Albrecht, 2017 and the subfamily Ancyliinae Rafinesque, 1815 (previous family Ancyliidae Rafinesque, 1815). The genus *Burnupia* Walker, 1912 was part of the Ancyliinae, however, in 2017 Albrecht established a new monotypic family, Burnupiidae, based on the *Burnupia*. The Burnupiidae family was formed due to the number of unique characteristics it has. The two salivary glands of *Burnupia* are distinct from those of other ancyliid species in that they each have a duct with an epithelium that has remarkably long cilia. Additionally, Burnupiidae is differentiated from other freshwater limpets by the presence of cilia in the caecum and unique sperm morphology, including features like the acrosomal pedestal and the presence of glycogen granules in nine shallow depressions in the nuclear fossa (Oberholzer & van Eeden, 1969; Hodgson & Healy, 1998). Compared to other families, Planorbidae Rafinesque, 1815 and Bulinidae Fischer & Crosse, 1880, *Burnupia* also has fewer chromosomes ($n=17$). Another distinctive molecular feature unique to the Burnupiidae (autapomorphy) is the 35-base pair insert in the nuclear 18S rRNA (Albrecht *et al.*, 2004).

In 2005, Bouchet and Rocroi (2005) highlighted that Starobogatov (1967) gave relative precedence to the name Planorbidae over Ancyliidae, leading to the exclusion of Ancyliidae as a family. Albrecht *et al.* (2007) stated that the traditional understanding of Ancyliidae, including most freshwater limpet gastropods, is paraphyletic. This is because the genera *Burnupia* and *Protancyclus* Sarasin & Sarasin, 1897 were found to be phylogenetically outside the Ancylini. Bouchet *et al.* (2017) further emphasised that the classification of Planorbidae relies on molecular phylogenetic results from Morgan *et al.* (2002) and Albrecht *et al.* (2007), with additional insights from Hubendick (1978). Ancyliinae is now ranked following the findings of Albrecht *et al.* (2007).

The species within Burnupiidae are predominately distributed across sub-Saharan Africa with a notable concentration in the eastern and southern regions spanning from the highlands in Ethiopia to the Cape regions. Additionally, a diminutive species has been documented from South America,

especially in Brazil. Africa hosts 21 normal species within this family, however, their taxonomic validity remains unexplored (Brown, 1994; Bouchet *et al.*, 2017).

In South Africa, only two freshwater limpet genera have been identified, namely *Burnupia* and *Pettancylus* Iredale, 1943 (see Appelton *et al.*, 2002; Jørgensen *et al.*, 2004). Worldwide there are 22 species in the genus *Burnupia* and 37 species belonging to the genus *Pettancylus* (MolluscaBase 2023). In Table 2.1, all the known species of *Burnupia* are listed, with their type localities, synonyms, conservation status, and if there are available sequences for the species.

Table 2.1: All of the currently known *Burnupia* species (n=22) with their synonyms, type localities, available sequence information, and IUCN red list category. The stars (*) indicate the species that are present in South Africa.

Species name	Synonyms	Type locality	Sequences	IUCN Red List category
<i>Burnupia alta</i> Pilsbry & Bequaert, 1927.	None.	Congo: Kisanga River near Elisabethville (Pilsbry & Bequaert, 1927)	None.	Data Deficient.
*<i>Burnupia brunnea</i> Walker, 1924.	None.	South Africa: Zoutpansberg, Limpopo (Walker, 1924).	None.	Data Deficient.
*<i>Burnupia caffra</i> (Krauss, 1848).	- <i>Ancylus caffer</i> Krauss, 1848, (original name). - <i>Ancylus (Burnupia) caffer</i> Krauss, 1848 (superseded combination). - <i>Ancylus gaulus</i> Gould, 1859, (junior synonym).	South Africa: Cape of Good Hope and Pietermaritzburg (Krauss, 1848; Gould, 1859). .	Two sequences on GenBank (18s and CO1) and one sequence present on Barcode of life data system (BOLD) (CO1).	Least Concern.
*<i>Burnupia capensis</i> (Walker, 1912).	- <i>Ancylus (Burnupia) caffer</i> var. <i>capensis</i> Walker, 1912, (original name). - <i>Ancylus (Burnupia) caffer capensis</i> Walker, 1912, (superseded combination).	South Africa: Lakeside, Cape Peninsula (Walker, 1924).	None.	Data Deficient.
<i>Burnupia crassistriata</i> (Preston, 1911).	<i>Ancylus crassistriatus</i> Preston, 1911 (original name).	Kenya: between Rumuruti and Mount Kenya (Preston, 1911).	None.	Near Threatened.
<i>Burnupia edwardiana</i> Pilsbry & Bequaert, 1927.	None.	Congo: Kabare, Lake Edward (Pilsbry & Bequaert, 1927).	None.	Data Deficient.

Species name	Synonyms	Type locality	Sequences	IUCN Red List category
*Burnupia farquhari (Walker, 1912).	- <i>Ancylus (Burnupia) caffer</i> var. <i>farquhari</i> Walker, 1912 (original name).	South Africa: KwaZulu Natal, East Griqualand (Walker, 1912).	None.	Data Deficient.
*Burnupia gordonensis (Melvill & Posonby, 1903).	<i>Ancylus (Ferrissia) gordonensis</i> Melvill & Ponsonby, 1903 (original name).	South Africa: KwaZulu Natal (Melvill & Posonby, 1903).	None.	Data Deficient.
<i>Burnupia ingae</i> Lanzer, 1991	None.	Brazil: Sombrio lake, Santa Catarina state (Santos, 2003)	None.	No information.
<i>Burnupia kempfi</i> (Preston, 1912).	<i>Ancylus kempfi</i> Preston, 1912 (original name).	Uganda: Kigezi (Preston, 1912).	Three sequences on GenBank (16S, 18S and CO1) and one sequence present on BOLD (CO1).	Data Deficient.
<i>Burnupia kimiloloensis</i> Pilsbry & Bequaert 1927.	None.	Congo: Kimilolo River near Elisabethville (Pilsbry & Bequaert 1927).	None.	Data Deficient.
*Burnupia mooiensis (Walker, 1912).	<i>Ancylus (Burnupia) mooiensis</i> Walker, 1912(original name).	South Africa: Mooi River, Potchefstroom (Walker, 1924).	None.	Data Deficient.
*Burnupia nana (Walker, 1912).	<i>Ancylus (Burnupia) caffer</i> var. <i>nanus</i> B. Walker, 1912 (original name).	South Africa: Karkloof stream, Kwazulu-Natal (Walker, 1912).	None.	Data Deficient.
*Burnupia obtusata Walker, 1926.	None.	South Africa: Pietermaritzburg, Kwazulu-Natal (Brown, 1994).	None.	Data Deficient.
*Burnupia ponsonbyi Walker, 1924.	None.	South Africa: Umgeni River, Kwazulu-Natal (Brown, 1994).	None.	Data Deficient.
*Burnupia stenochorias (Melvill & Ponsonby, 1903).	<i>Ancylus (Ferrissia) stenochorias</i> Melvill & Ponsonby, 1903 (original name).	South Africa: Eastern Cape (Melvill & Ponsonby, 1903).	Two sequences on GenBank (18s and CO1) and one sequence present on BOLD (CO1).	Data Deficient.
<i>Burnupia stuhlmanni</i> (von Martens, 1897).	<i>Ancylus stuhlmanni</i> E. von Martens, 1897(original name).	Tanzania: Lake Victoria, Busisi (von Martens, 1897).	Three sequences on GenBank (16S, 18S and CO1) and one sequence present on BOLD (CO1).	Near Threatened.
*Burnupia transvaalensis (Craven, 1881).	<i>Ancylus transvaalensis</i> Craven, 1881(original name).	South Africa: Mooi River (Craven, 1881).	None.	Data Deficient.
*Burnupia trapezoidea (Boettger, 1910).	<i>Ancylus trapezoideus</i> Boettger, 1910 (original name).	South Africa: Witkop (De Kock & Wolmarans, 2017).	None.	Data Deficient.

Species name	Synonyms	Type locality	Sequences	IUCN Red List category
*<i>Burnupia verreauxi</i> (Bourguignat, 1853).	<i>Ancylus verreauxii</i> Bourguignat, 1853 (original name).	South Africa: Cape of Good Hope, Constance (Walker, 1924)..	None.	No information.
*<i>Burnupia vulcanus</i> Walker, 1924.	None.	South Africa: Mooi River, Potchefstroom (Walker, 1924).	None.	Data Deficient.
<i>Burnupia walkeri</i> Pilsbry & Bequaert 1927.	None.	Congo: Kalengwe, in the Lualaba River (Pilsbry & Bequaert 1927).	None.	Data Deficient.

According to Lacerda *et al.* (2011), in the ecology and taxonomy of molluscs, shell morphometry serves as a valuable and helpful tool. In investigations of freshwater snails, it has proven effective in distinguishing between species, identifying intraspecific morphological variations, and linking shell differences to geographic distribution and environmental factors.

The freshwater limpets, in particular, require taxonomic revision because they have gotten far less attention than the other molluscs (Jørgensen *et al.*, 2004; Sands *et al.*, 2022). To fully understand their taxonomy and geographical preference, proper morphological identification (revising all available taxa) with molecular analysis is needed. The aims of this study were to revise the taxonomy and distribution of the *Burnupia* species that are present in South Africa using morphological identification and molecular analysis.

2.2 Materials and methods

2.2.1 Study areas:

The sampling sites that were chosen were based on limpet species type localities and on other existing data (Walker, 1912; Walker, 1924; Walker, 1926; Connolly, 1936; Brown, 1994). The study sites that were chosen for this project were usually shallow systems where rocks and vegetation are present. The sites were usually in slow-flowing water but in some instances standing water was also used for sampling and these sites were rich in vegetation. Species were mostly collected from stones and vegetation. Samples were collected from 10 sites. There were 14 species collected from the Elands River, five from the Vaal River, 23 from the Mooi River, 10 from the Suid-Kaap River, four from the Klein Olifants River, three from Mac Mac River, five from the Sabie River, four from the Magalies River, six from Bronkhorstspuit and eight from the Crocodile River. The sample sites are indicated on the below map (Figure 2.1). Samples were

collected from March 2022 to March 2023 (see Table 2.2 for details). This study was seen as a low-risk study and provided the ethics number NWU-01353-22-A9.

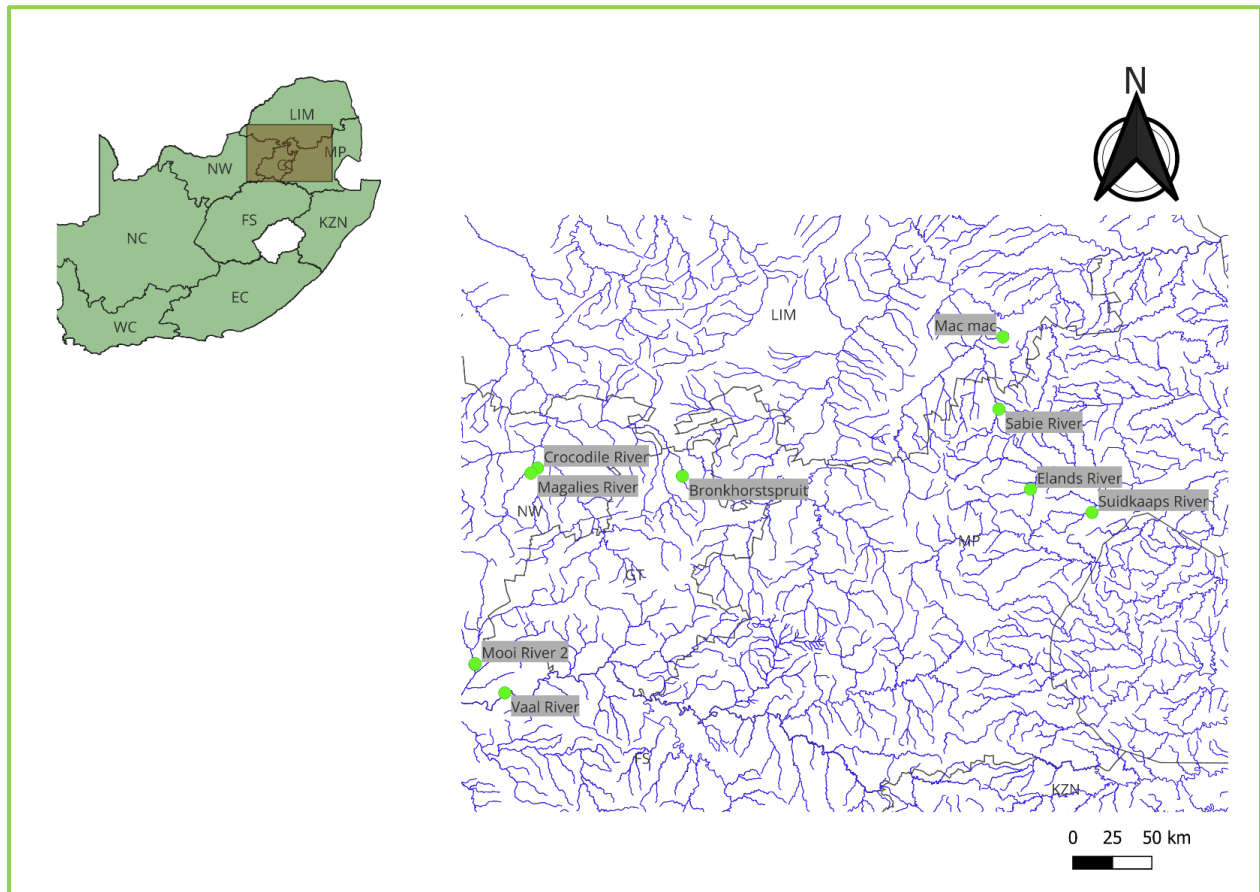


Figure 2.1: Map with locations or rivers where the sampling was done in 2022 and 2023 in the Free State, Gauteng, Limpopo, Mpumalanga and North-West.

Table 2.2: Name of the sites, provinces, GPS coordinates and photos of the sites used for sampling during the course of this study, and date.

Name of site	Province	Sampling date	GPS coordinates	Photo
Bronkhorstspuit	Gauteng	27/03/2023	-25.482185, 28.432468	
Crocodile river	North-West	30/03/2023	-25.430233, 27.503587	
Elands River	Mpumalanga	9/11/2022	-25.56584404 30.66366983	

Name of site	Province	Sampling date	GPS coordinates	Photo
Klein Olifants River	Mpumalanga	27/03/2023	-25.86620, 29.61373	
Mac Mac River	Limpopo	28/03//2023	-24.591054, 30.485608	
Magalies River	North-West	27/03/2023	-25.462812, 27.461334	

Name of site	Province	Sampling date	GPS coordinates	Photo
Mooi River 1	North West	22/10/2022	-26.68634607, 27.10230189	
Mooi River 2	North West	24/06/2022	-26.68209369, 27.10442808	
Vaal Rivier	Free State	21/03/2022	-26.86919840, 27.29365898	

Name of site	Province	Sampling date	GPS coordinates	Photo
Sabie River	Mpumalanga	27/03/2023	-25.053580, 30.460831	

2.2.2 Collection of limpets:

There are three ways to collect limpets: with a SASS net, a snail sampler, or with a hand-picking method. At the selected sites, mainly hand-picking methods were used that included lifting loose rocks and collecting limpets from the underside, or from vegetation. The limpets that were collected were fixed in 96% ethanol for further analysis.

2.2.3 Morphological analysis:

The freshwater limpets that were collected were taken to the North-West University (NWU) laboratories for identification with the help of identification guides such as “Guide to the freshwater invertebrates of Southern Africa” (Appelton *et al.*, 2002), as well as any other appropriate literature, such as Melvill & Ponsonby (1903), Walker (1912), Connolly (1939), and Brown (1994).

Each of the limpets were cleaned using an ultrasonic bath and sonicated with a mixture consisting of ammonium, water, and sunlight liquid dish soap (1:2:1). Limpets were studied with the aid of a dissection microscope (Zeiss, AxioCam ERc 5s). Photomicrographs were taken of each limpet using a Nikon AZ100M stereomicroscope. Specimens were photographed using the Z-stack function on the Nikon Eclipse Ni microscope (Nikon Instruments, Tokyo, Japan). These photos were used to determine the limpet morphological characteristics and compared to existing literature and taxonomic keys. The identification key described by Brown (1994) was used to identify the species to family level. All shells were illustrated using a camera lucida or photographed and categorised based on the shape of the shell's aperture, the presence of radial lines, and the position of the apex in relation to the shell margin.

Measurements of each limpet species were taken. In total, 29 limpet shells were measured with the aid of a calliper and dissection microscope (Zeiss). Figure 2.2 indicates where and how the shells were measured. The total length (L), anterior width (W1), posterior width (W2) and the height of the apex's (H) measurements were obtained. To analyse the difference in morphometrics of the shell, the following ratios were used: $W1/L$, $W2/L$, H/L , $H/W1$ and $W2/W1$.

Descriptive statistical analysis of the morphometric variations and differences between species and the different sample sites were completed in Graphpad Prism v. 5. Both the actual measurements and the ratios were used in the statistical analysis. Box and whisker plots were constructed using the minimum, maximum, median and mean values of each morphometric value. The data was tested for normality and homogeneity of variance using the Shapiro-Wilk normality test, if distributed normally, a one-way ANOVA was completed with a Tukey's Multiple Comparison Test as posthoc analysis (Erasmus *et al.*, 2020). These analyses were completed to determine if there were possible relationships between the morphometrics of each species or sampling site to allow for a better integrative morphological identification.

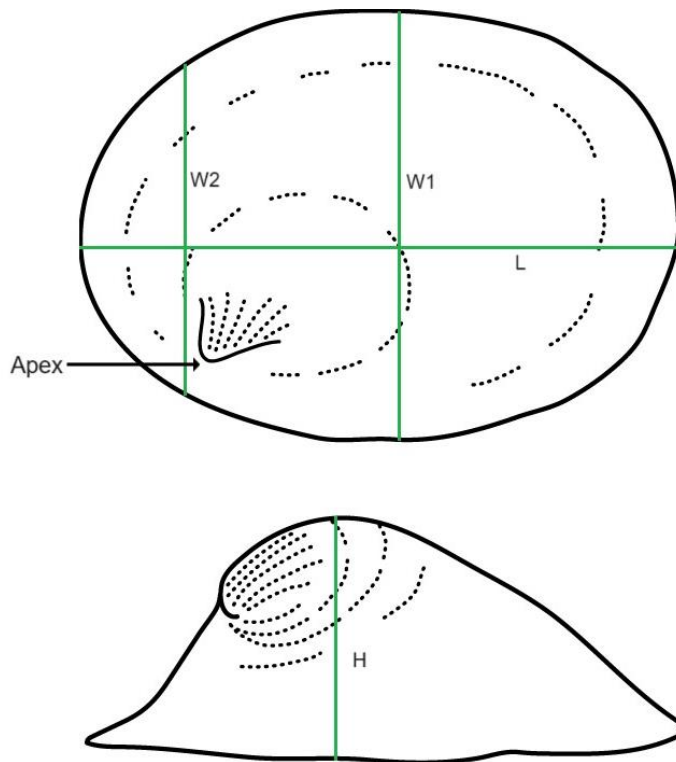


Figure 2.2: Drawing illustrating the measurements that were taken of each limpet. Abbreviations: L – Total length, W1 – anterior width, W2 – Posterior width, H – Height of the apex.

2.2.4 Molecular analysis:

For DNA extraction and processing, limpets were kept in 96% ethanol. The soft tissue from the limpets were removed from its shell using a scalpel, tweezers, and a dissection microscope. The soft tissue was placed in molecular grade ethanol (Carolus *et al.*, 2019). After each limpet was dissected, the equipment was sterilised with 70% ethanol to avoid cross-contamination between the specimens (Carolus *et al.*, 2019).

The PCR BIO Rapid Extraction PCR Kit (PCR biosystems; Analytical Solutions) and the Chelex buffer (Merck) was used to extract limpet genomic DNA from the specimen. Universal invertebrate COI primers, LCO1490 and HCO2198, and a combination of LCO1490 with a different reverse primer, COR 722b, were used to amplify the DNA fragments (see Table 2.3). Additionally, a third set of primers, ASMIT 1 and ASMIT 2, were used for samples that were problematic. The primers that were used were based on previous literature from Wilke & Davis (2000), Jørgensen *et al.* (2004), Raphalo *et al.* (2012), Geller *et al.* (2013), Sands *et al.* (2022), and Pearson (2022). DNA amplifications of the partial cytochrome c oxidase subunit 1 (COI) were done using a SimpliAmp Thermal Cycler in volumes of 25 µl, which includes 1.25 µl of each primer, 8 µl deionised water, 2 µl DNA, and 12.5 µl master mix (KAPA HotStart & Dream Taq).

For the universal COI invertebrate primers (LCO1490 and HCO2198), the Polymerase chain reaction (PCR) conditions followed are shown in Figure 2.3a. The PCR conditions for LCO1490 and COR722b used in Sands *et al.* (2022) were followed and are shown in Figure 2.3b and for ASMIT 1 and ASMIT the PCR conditions are indicated in Figure 2.3c.

Table 2.3: Primers used for amplification of DNA and their references.

Genetic maker	Primer	Sequence	Reference
COI	LCO1490	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	Folmer <i>et al.</i> (1994)
	HCO2198	5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'	Folmer <i>et al.</i> (1994)
	LCO1490	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	Sands <i>et al.</i> (2022)
	COR722b	5'-TAAACTTCAGGGTGACCAAAAAATYA-3'	Sands <i>et al.</i> (2022)
	ASMIT 1	5'-TTT TTG GGC ATC CTG AGG TTT AT-3'	Jørgensen <i>et al.</i> (2004)
	ASMIT 2	5' -TAA AGA AAG AAC ATA ATG AAA ATG-3'	Jørgensen <i>et al.</i> (2004)

The PCR products were examined by 1% agarose gel electrophoresis run for 30 min at 90V, after which they were visualised under ultraviolet light. Samples that were a success were then sent to a commercial sequencing company, Inqaba Biotechnical Industries (Pretoria, South Africa), who purified and sequenced the PCR amplicons. Newly generated sequences were aligned, trimmed, and assembled using Geneious Prime bioinformatics software v 2.1. The Basic Local Alignment Tool (BLAST) was used to identify similar sequences for phylogenetic analysis (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

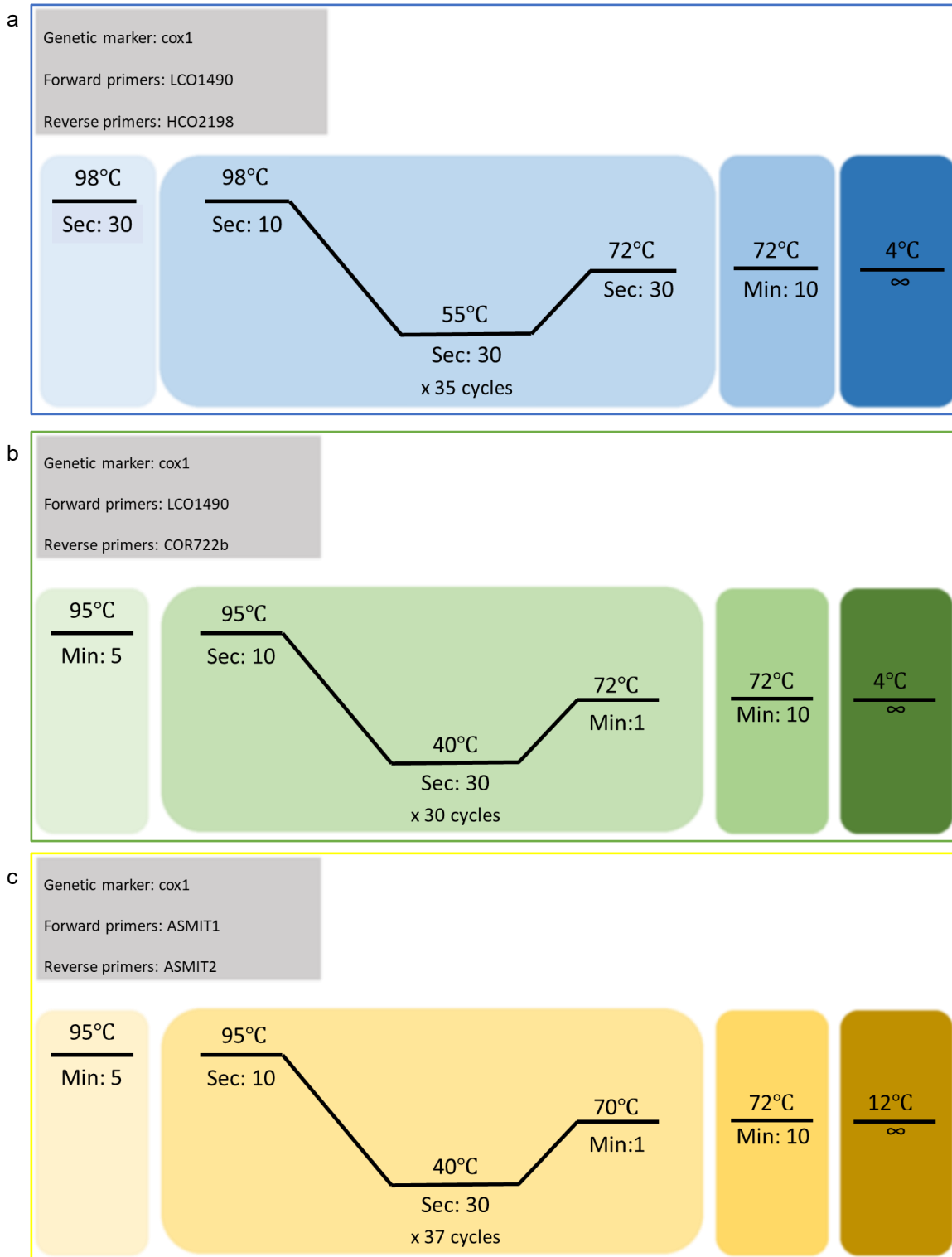


Figure 2.3: a) PCR conditions for LCO1490 and HCO2198; b) PCR conditions for LCO1490 and COR722b, c) PCR conditions for ASMIT1 and 2.

According to the Akaike information criterion, the best nucleotide models for the alignment was established with jModelTest2.1. For the phylogenetic analysis, Bayesian inference (BI) and maximum likelihood (ML) analyses were done. The ML analysis was made with PhyML v. 3.0 (1000 repetitions) (available at <http://www.atgc-montpellier.fr/phyml/>) and the BI was carried out with MrBayes v3.2.6 software and run through CIPRES science Gateway v.3.3. (available at <https://www.phylo.org/>). FigTree v. 1.4.3 software was used to visualise the tree and MEGA v. 7 was used to calculate the p-distance and the difference in the numbers of base pairs were generated on Geneious Prime bioinformatics software v 2.1. Sequences used in the phylogenetic analysis are provided in Table 2.4.

Table 2.4: Sequences used from GenBank with species names, accession numbers (or sample code for new sequences), length of sequences, and corresponding reference. The outgroup sequence is shaded in green and newly generated sequences from this study are in bold.

Species name	Accession number (GenBank) or sample code	Base pair length	Reference
<i>Burnupia</i> sp.1	ON256521	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.1	ON256525	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.1	ON256531	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.1	ON256537	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.2	ON256527	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.2	ON256543	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.3	ON256517	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.3	ON256520	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.4	ON256550	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.5	ON256547	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.6	ON256546	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.7	ON256548	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.8	ON256429	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.8	ON256539	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.8	ON256540	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.8	ON256542	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.8	ON256553	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.9	ON256557	655	Sands <i>et al.</i> (2022)
<i>Burnupia</i> sp.9	ON256555	655	Sands <i>et al.</i> (2022)

Species name	Accession number (GenBank) or sample code	Base pair length	Reference
<i>Burnupia</i> sp.10	ON256545	655	Sands <i>et al.</i> (2022)
<i>Burnupia caffra</i>	AY282584	959	Albrecht <i>et al.</i> (2007)
<i>Burnupia stenochorias</i>	AY282585	603	Albrecht <i>et al.</i> (2007)
<i>Burnupia</i> sp.	BEISA111-19.COI-5P	655	Sequence from Albany Museum present on BOLD
<i>Burnupia</i> sp.	BEISA176-19.COI-5P	655	Sequence from Albany Museum present on BOLD
<i>Burnupia</i> sp.	BEISA166-19.COI-5P	655	Sequence from Albany Museum present on BOLD
Vaal River <i>Burnupia</i> sp.	V1A1	713	Present study
Vaal River <i>Burnupia</i> sp.	V1A2	784	Present study
Vaal River <i>Burnupia</i> sp.	V1A3	708	Present study
Elands River <i>Burnupia</i> sp.	ER1A4	710	Present study
Elands River <i>Burnupia</i> sp.	ER1A10	791	Present study
Klein Olifants River <i>Burnupia</i> sp.	KOA1	708	Present study
Mooi River <i>Burnupia</i> sp.	MR1A1	711	Present study
Mooi River <i>Burnupia</i> sp.	MR1A4	710	Present study
Mooi River <i>Burnupia</i> sp.	MR2A1	712	Present study
Mooi River <i>Burnupia</i> sp.	MR2A5	708	Present study
Mooi River <i>Burnupia</i> sp.	MR2A6	708	Present study
Mooi River <i>Burnupia</i> sp.	MR3A1	723	Present study
Mooi River <i>Burnupia</i> sp.	MR3A5	719	Present study
Magalies River <i>Burnupia</i> sp.	NWA2	686	Present study
Magalies River <i>Burnupia</i> sp.	NWA3	787	Present study
Crocodile River <i>Burnupia</i> sp.	CDA1	714	Present study
Crocodile River <i>Burnupia</i> sp.	CDA2	711	Present study
Bronkhorstspuit <i>Burnupia</i> sp.	BSA1	710	Present study
Bronkhorstspuit <i>Burnupia</i> sp.	BSA2	708	Present study
Mac Mac <i>Burnupia</i> sp.	MMA2	713	Present study
Sabie River <i>Burnupia</i> sp.	SRA2	714	Present study
Sabie River <i>Burnupia</i> sp.	SRA3	710	Present study
Suid-Kaap River <i>Burnupia</i> sp.	SK1A2	714	Present study
Suid-Kaap River <i>Burnupia</i> sp.	SK1A4	708	Present study
Suid-Kaap River <i>Burnupia</i> sp.	SK1A6	709	Present study
<i>Ferrissia rivularis</i> (OG)	AY282586	959	Albrecht <i>et al.</i> (2007)

2.3. Results

2.3.1 Morphological results:

2.3.1.1 *Burnupia* taxonomy:

Phylum Mollusca

Class Gastropoda

Superfamily Lymnaeoidea

Family Burnupiidae

Genus *Burnupia* Walker, 1912

Type species: *Burnupia caffra* (Krauss, 1848) (type by original designation) (described as *Ancylus caffer* Krauss, 1848).

Generic remarks:

The shell type of Burnupiidae usually shows phenotype variations (Bouchet *et al.*, 2017). Brown (1994) and Bouchet *et al.* (2017) gave the following characteristics for *Burnupia*: the shell size can be between 5mm and 10mm in length, the apex is typically prominent and turned to the right, (if viewed from above) and the apex usually has radial rows of small pits. As can be seen from Figure 2.4, the apex (indicated by red arrows) is quite prominent and slightly to the side. In Figure 2.5, the radial rows of small pits on the apex can be observed with a white circle. Based on these distinctive traits, these limpets were morphologically analysed and identified as *Burnupia* spp. Each species collected was subsequently reviewed (see below).

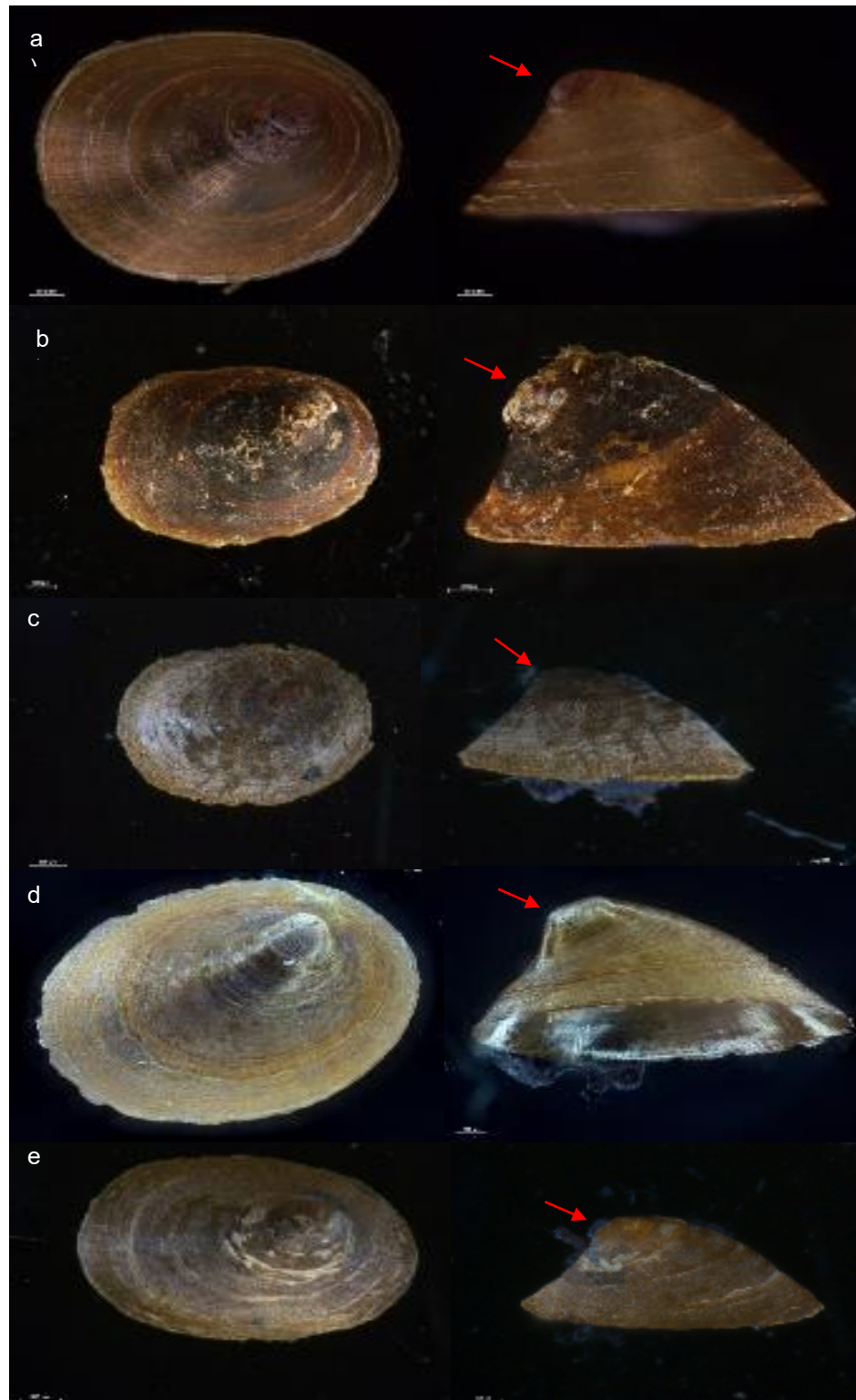


Figure 2.4: Photomicrographs of *Burnupia* Walker, 1912 limpet specimens from a) Vaal River, b) Elands River, c) Klein Olifants River, d) Mooi River, and e) Bronkhorstspuit. The red arrows show the prominent apex, slightly to the side.

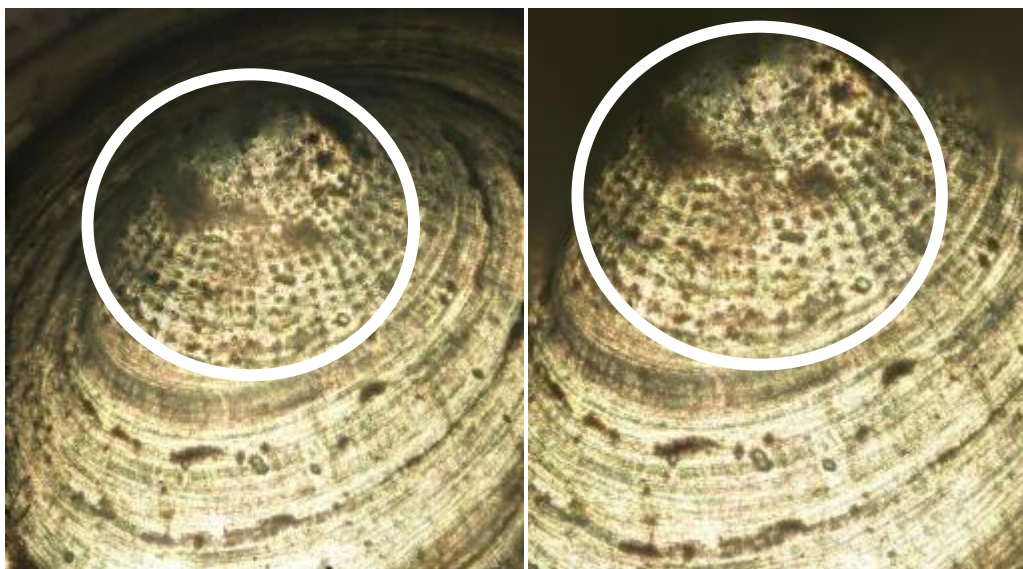


Figure 2.5: Z-stack photos of the apex of *Burnupia transvaalensis* (Craven, 1881). The white circle indicates the radial rows of small pits on the apex.

2.3.1.2 *Burnupia caffra* (Krauss, 1848)

Type locality: South Africa – Cape of Good Hope and Pietermaritzburg (Krauss, 1848; Brown, 1994).

Burnupia caffra was first described by Kraus in 1848 as *Ancylus (Burnupia) caffer*. Brown (1994) stated that this species occupies most of the range that is known for the *Burnupia* genus. The shell is oval shaped, and the apex is noticeable and is so far to the right when viewed from above that it reaches the basal margin (Brown, 1994) (Figure 2.6). The apex is not sharp and somewhat short. The anterior part of the shell has a high slope, and the posterior part is short and steep. This species can sometimes have vertical radial lines (Connolly, 1939).

Specimens from the current study that conformed to this description were collected from the Suid-Kaap, Mac Mac and Sabie rivers. The distribution of *B. caffra* is indicated in Figure 2.7, where the green dots indicate the data found in GBIF (Global Biodiversity Information Facility) and the pink dots indicate the data from the South African National Freshwater Snail Collection (NFSC) database (also known as the NWU snail database).

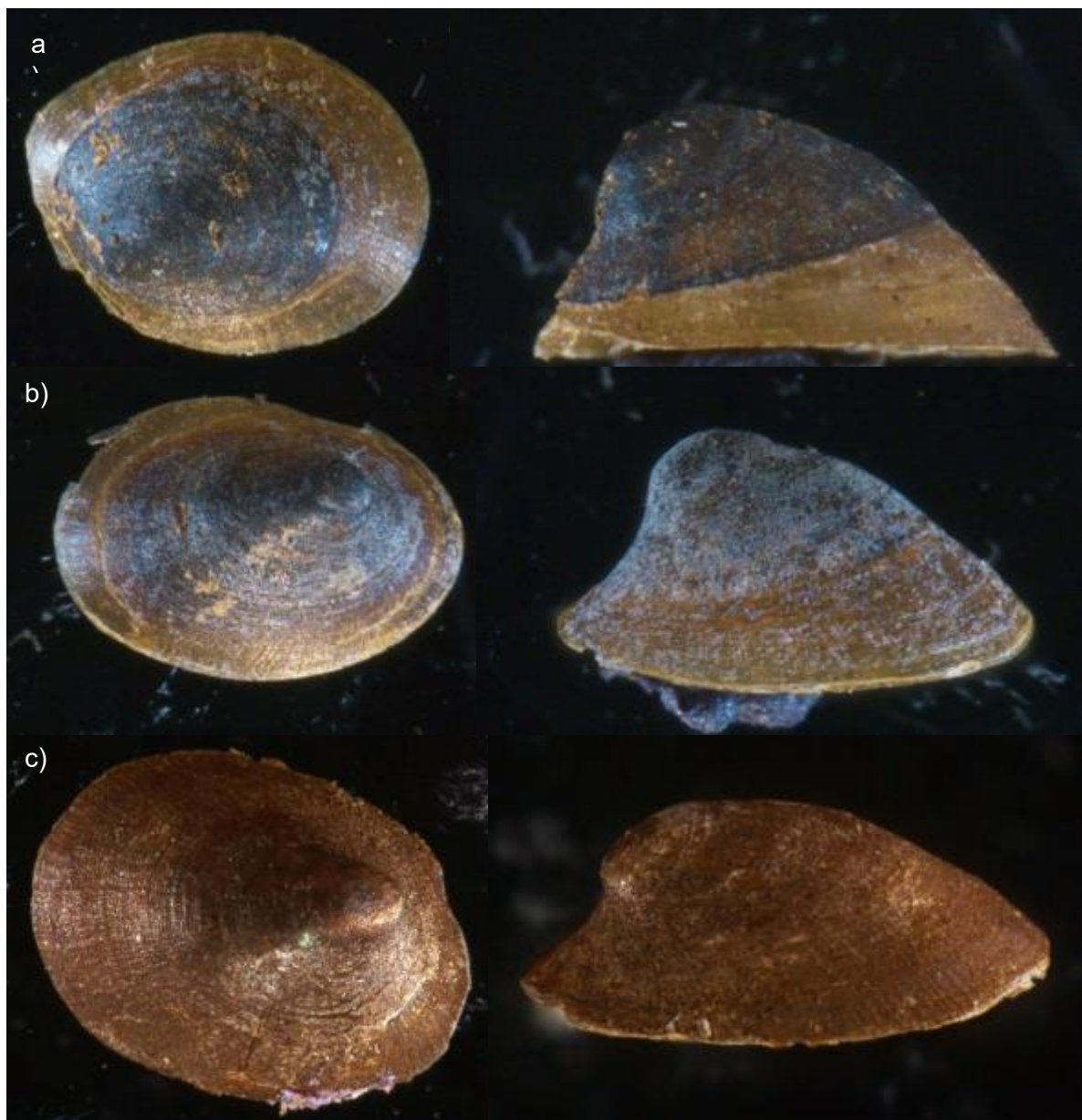


Figure 2.6: Photos of the *Burnupia caffra* (Krauss, 1848) specimens collected from the Mac-Mac River, b) Sabie River, and c) Suid-Kaap River.

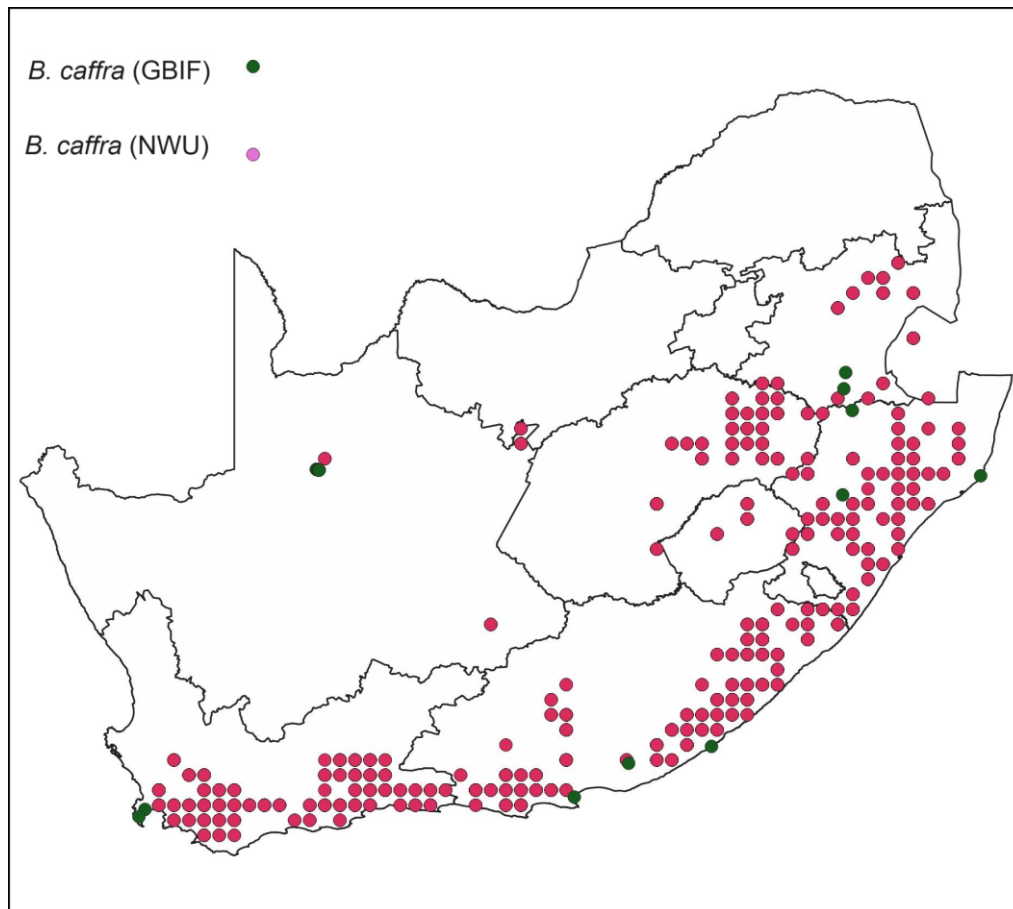


Figure 2.7: Distribution of *Burnupia caffra* (Kraus, 1848) where the green dots indicate information found on GBIF and pink dots indicate information found on the NWU snail database.

2.3.1.3 *Burnupia capensis* (Walker, 1912)

Type locality: South Africa – Cape Peninsula, Lakeside (Brown, 1994).

Burnupia capensis was first described by Walker in 1912 as *Ancylus (Burnupia) caffer* var. *capensis*. It has prominent radial ridges, and the apex is not very sharp (Brown 1994; Figure 2.8a). The species collected from the Vaal River also had these growth lines (indicated by the blue arrow in Figure 2.8b). The shell of *B. capensis* is oval in shape and can vary in width (Connolly, 1939), is thin and semi-transparent, and brownish coloured, with a darker area at the apex. The surface has extremely fine, close, somewhat uneven radial striae and fine growth-lines. The apex is noticeable, pointed, firmly turned to the right when viewed from above. The right gradient is convex; the left slope is somewhat concave; the rear slope is concave; and the anterior slope is convex and considerably flattened towards the peak (Walker, 1912).

Specimens that were collected in this study and that conform to this description were found in the Vaal River. *Burnupia capensis* has a wide distribution in South Africa, almost as extensive as *B. caffra* (Figure 2.9). The species can be found in almost every province in South Africa except for Gauteng and Limpopo (Figure 2.9).

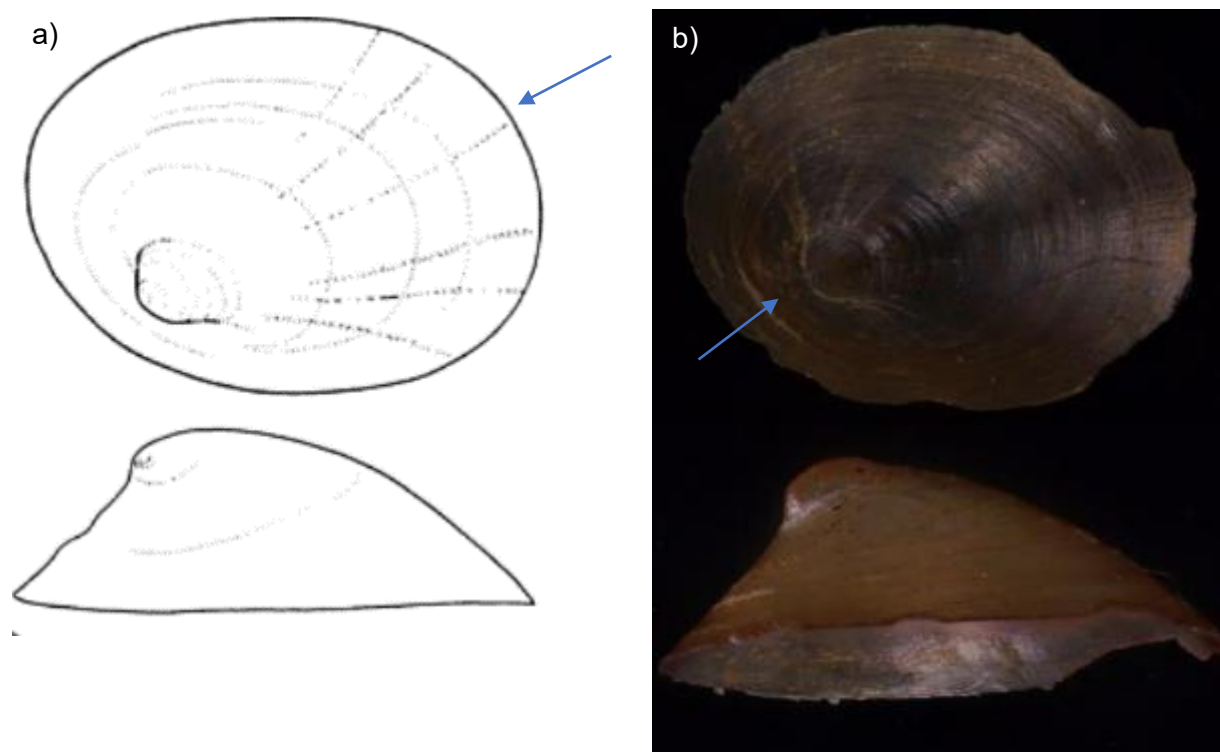


Figure 2.8: a) Line drawing of *Burnupia capensis* (Walker, 1912) from Brown (1994), and b) limpet from the Vaal River - V1A2.

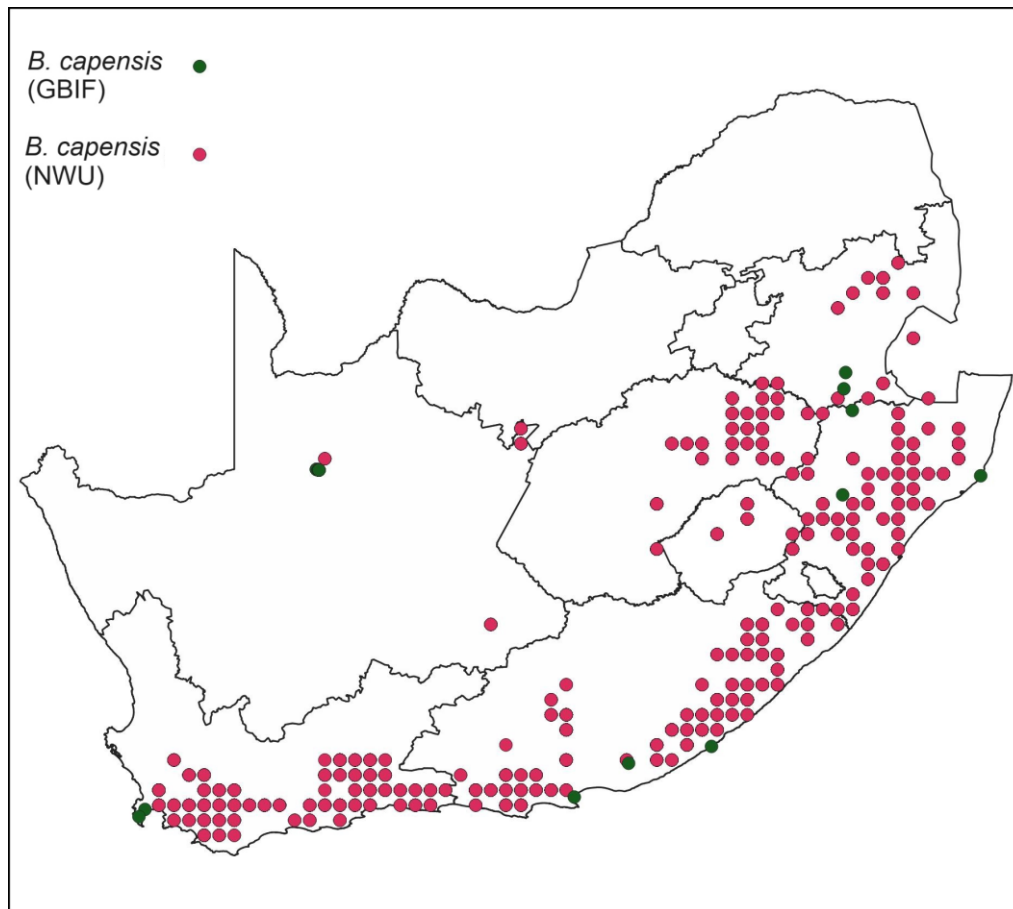


Figure 2.9: Distribution of *Burnupia capensis* (Walker, 1912) where green dots indicate information found on GBIF and pink dots indicate information found on the NWU snail database.

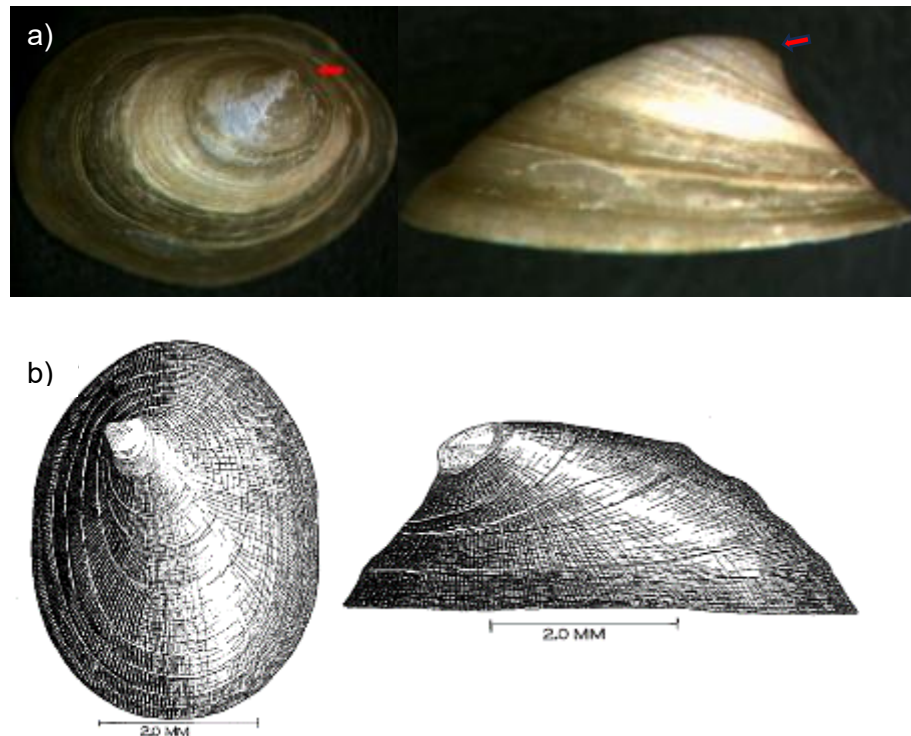
2.3.1.4 *Burnupia mooiensis* (Walker, 1912).

Type locality: South Africa – Mooi River, Potchefstroom (Walker, 1912).

Burnupia mooiensis was first described by Walker in 1912 as *Ancylus (Burnupia) mooiensis*. Walker (1912) characterised this species as having a somewhat oval shell shape with the widest point being anterior to the centre. The shell is thin, semi-transparent and is light brown in colour. The surface displays very fine growth lines, along with delicate wavy, radial striae. The apex is positioned halfway between the posterior and centre margin, being not very sharp, a bit turned to the right, and hardly noticeable. The right slope is slightly convex, but the left is almost straight. The anterior part is convex but broadens above and becomes nearly straight as it nearer the anterior border. The posterior slope is not severely concave (Figure 2.10).

Connolly (1939) described *B. mooiensis* as having a large, elevated shell and a somewhat oval shape. The ends of the shell are almost round, and the left border is more rounded than the right. The left and front slopes are nearly convex while the posterior end is long and not deeply concave. The right slope is somewhat concave halfway down. The apex is subacute and is turned to the right. The apex exhibits strong growth lines with strong wavy radials (Figure 2.10).

Burnupia caffra have a steep and short posterior region while *B. mooiensis* has a long and not very steep posterior end. The specimens collected in this study that fit the description of *B. mooiensis* were found in the Mooi River, Magalies River, and Crocodile River. *Burnupia mooiensis* has mostly been found in the North West, Gauteng, and Mpumalanga, have been reported a few times in the Free State, and only reported once in KwaZulu Natal (GBIF record) (Figure 2.11).



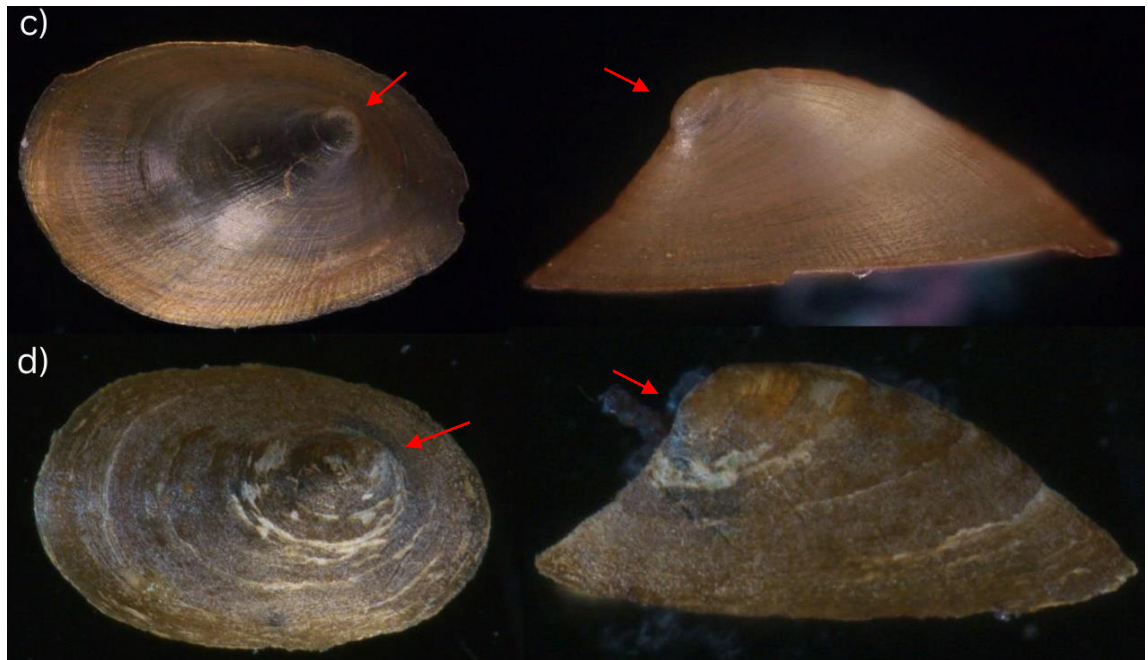


Figure 2.10: Comparison of *Burnupia mooiensis* (Walker, 1912) from the a) NWU snail collection, and b) a drawing from Eeden & Oberholzer (1963) c) the Mooi River (sample MR1A4), d) the Bronkhorstspuit (sample BSA1). The red arrow indicates the apex of the species.

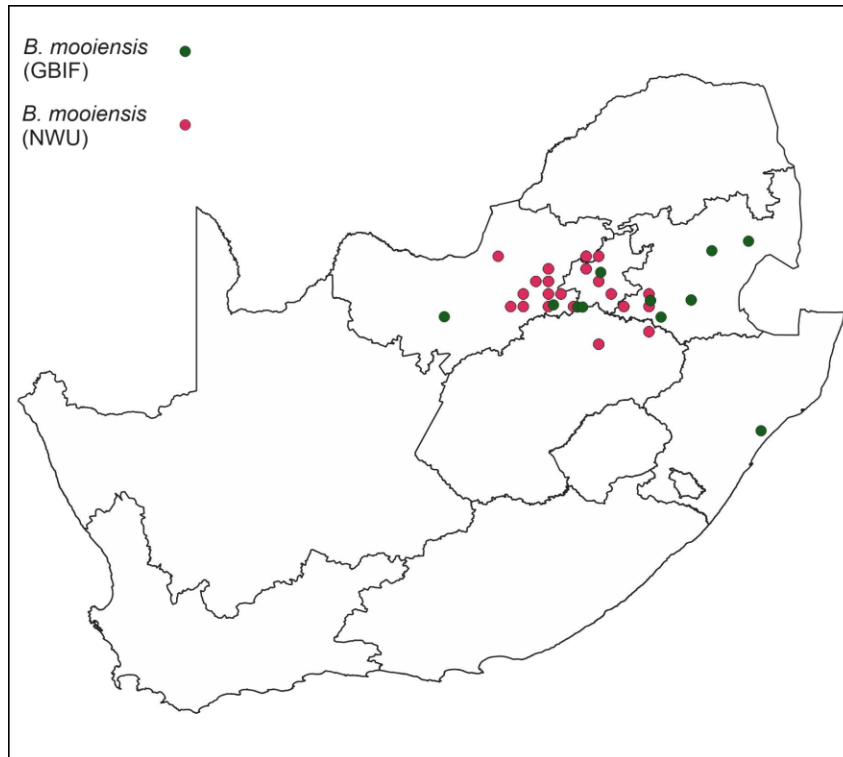


Figure 2.11: Distribution of *Burnupia mooiensis* (Walker, 1912) where green dots indicate information found on GBIF and pink dots indicate information found on the NWU database.

2.3.1.5 *Burnupia stenochorias* (Melvill & Ponsonby 1903)

Type locality: Eastern Cape (Melvill & Ponsonby 1903).

Burnupia stenochorias was first described by Melvill & Ponsonby in 1903 as *Ancylus (Ferrissia) stenochorias*. Brown (1994) and Connolly (1939) mentioned that the shell is very elongated and oval (see Figure 2.12), and this can mean that *B. stenochorias* lives on a slender plant stem. The left and anterior slopes are convex, the posterior region is concave, the apex is very prominent, and radial lines are strong (Connolly, 1939).

Compared to the above species (*B. caffra*, *B. capensis* and *B. mooiensis*), *B. stenochorias* does not have any growth lines present. The shell of *B. stenochorias* is elongated while the other species mentioned here are mostly oval.

It has mostly been found in the Eastern Cape, but there are a few occurrences close to KwaZulu-Natal and the Free State (see Figure 2.13).

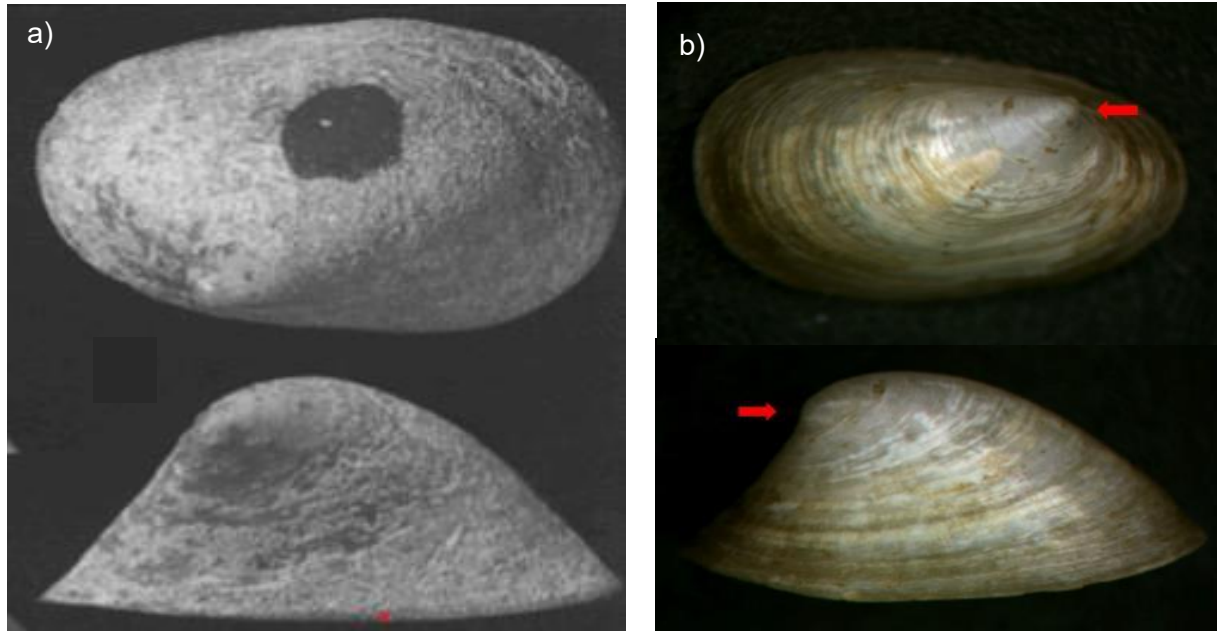


Figure 2.12: a) Photo of *Burnupia stenochorias* (Melvill & Ponsonby 1903) from Brown (1994), b) photos from the NWU snail collection.

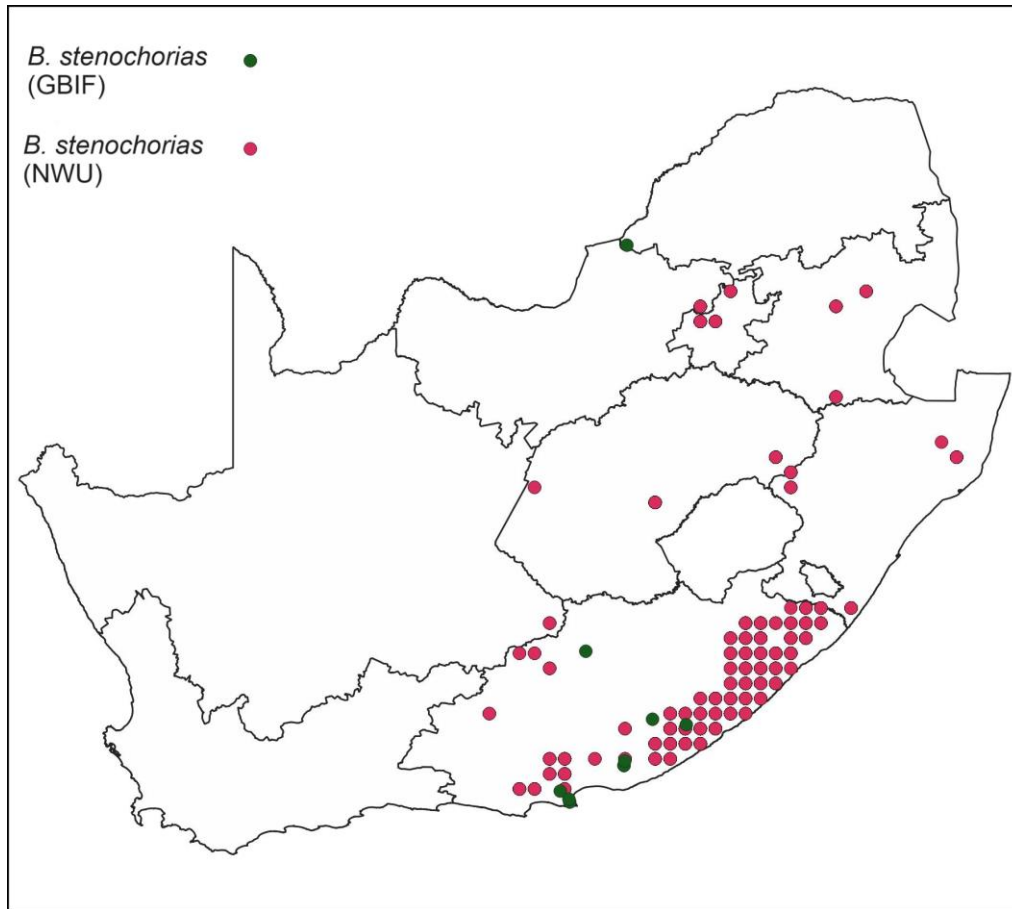


Figure 2.13: Distribution of *Burnupia stenochorias* (Melvill & Ponsonby 1903) where green dots indicate information found on GBIF and pink dots indicate information found on the NWU database.

2.3.1.6 *Burnupia transvaalensis* (Craven, 1881)

Type locality: South Africa – Mooi River, Transvaal (Craven, 1881).

Burnupia transvaalensis was first described by Craven in 1881 as *Ancylus transvaalensis*. Connolly (1939) described *B. transvaalensis* as having a large and somewhat oval shape. The apex of *B. transvaalensis* is not very sharp and is in the centre of the shell, making it a moderately high shell (Brown, 1994) (Figure 2.14). The lateral slopes are almost evenly convex, the anterior region is convex, and the posterior slope is straight. The striae are rough and low on the shell, and they are unevenly wrinkled.

The specimens that conform to this description were collected in the Vaal River, Elands River, and the Klein Olifants River. This species predominately occurs in the Northern Cape, North West, Gauteng, Mpumalanga, with a few occurrences in the Free State and Limpopo (GBIF record) (Figure 2.15).

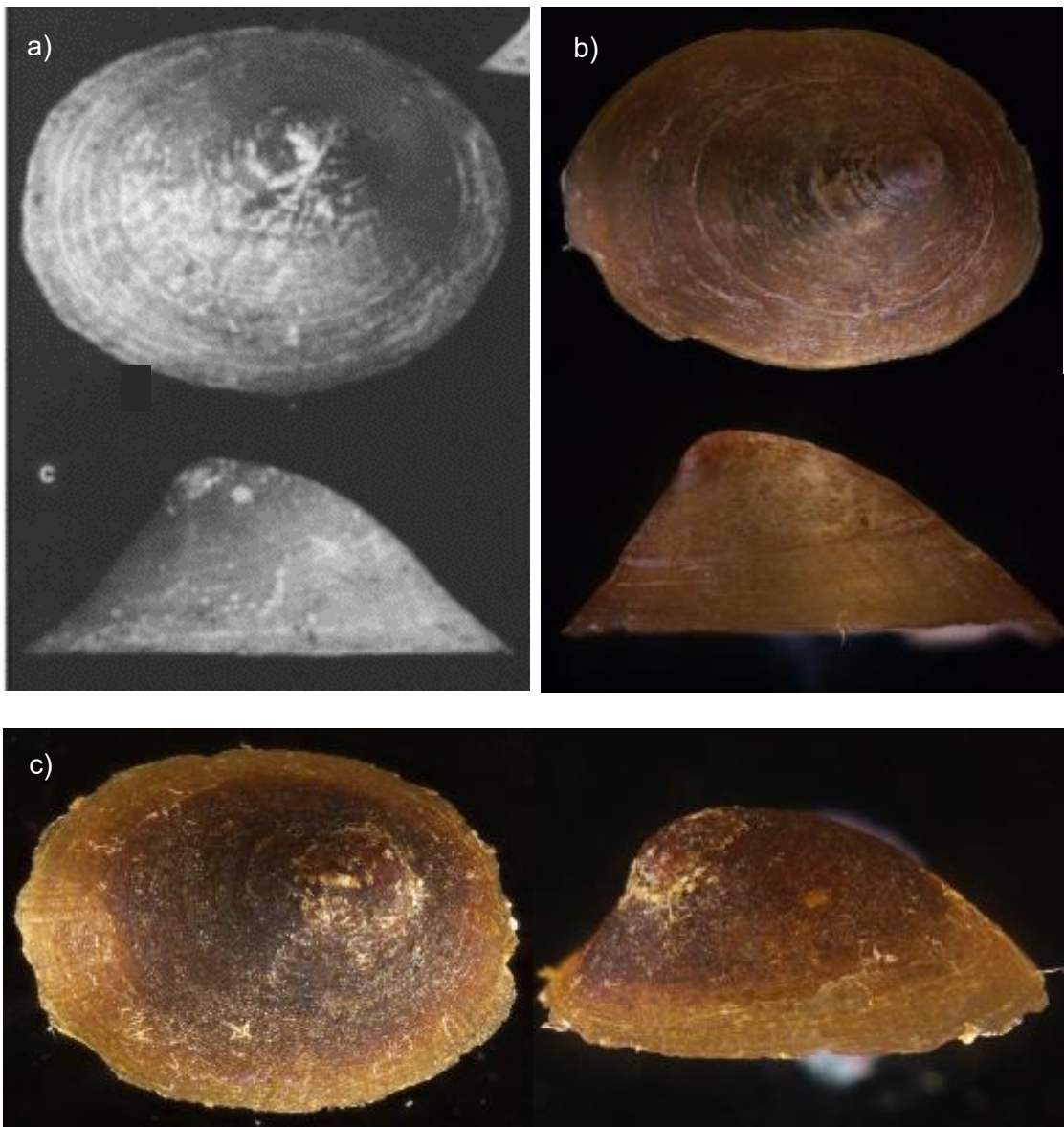


Figure 2.14: a) Photo of *Burnupia transvaalensis* (Craven, 1881) from Brown (1994), b) the sample collected from the Vaal River – VR1A3, and c) sample collected from the Elands River (sample ER1A8).

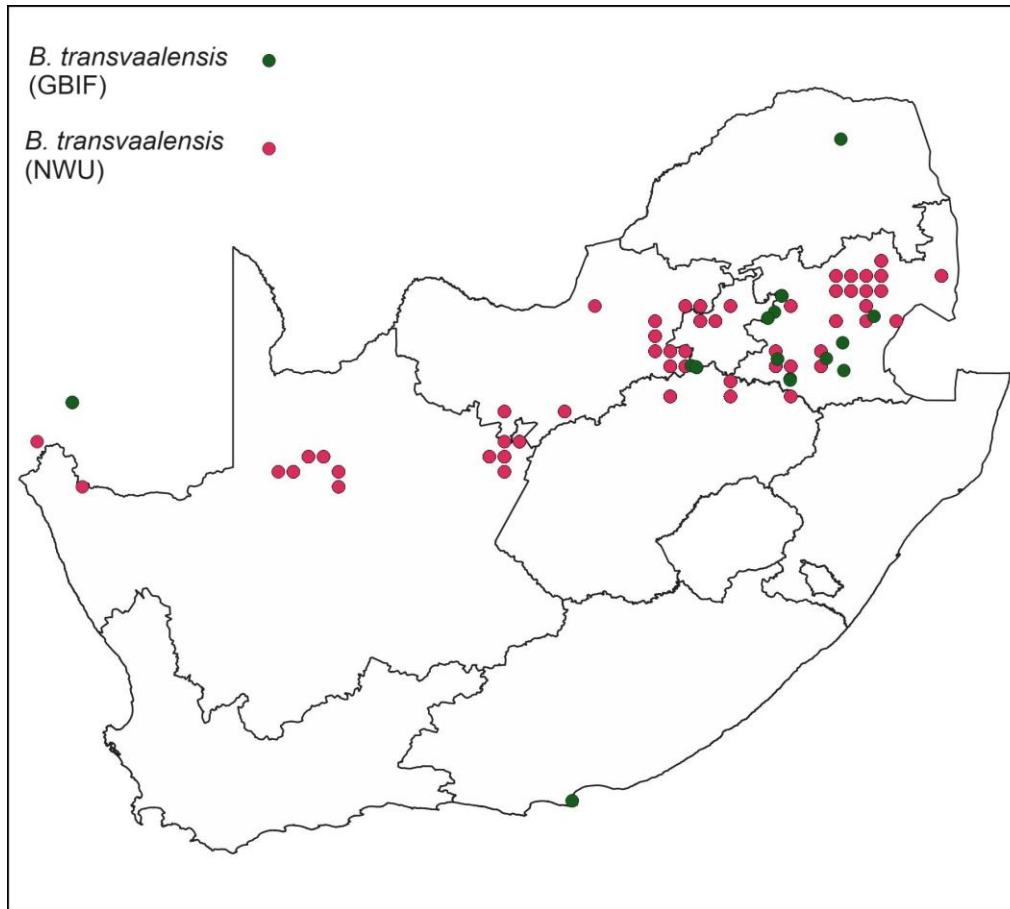


Figure 2.15: Distribution of *Burnupia transvaalensis* (Craven, 1881) where green dots indicate information found on GBIF and pink dots indicate information found on the NWU database.

2.3.1.7 *Burnupia vulcanus* Walker, 1924.

Type locality: South Africa – Mooi River, Potchefstroom (Walker, 1924).

Burnupia vulcanus was first described by Walker in 1924 and is the most recently described species of this genus from the Mooi River. This species has not been mentioned much since the original description. This species can also be found in the Vaal River catchment (Sands *et al.*, 2022). The shell of *B. vulcanus* is small and can be cone-shaped but it is usually oval (Connolly, 1939). The lateral regions are evenly bent, and the anterior slope is almost straight. The apex is prominent, and the tip is not very sharp (Connolly, 1939). The radial lines are close to each other, and slightly uneven. According to Connolly (1939), there are only two specimens known.

According to Connolly (1939), there are only two specimens known, one occurrence is in the Free State (Figure 2.16) and one in the Mooi River (type locality). No other records have been noted for this species.

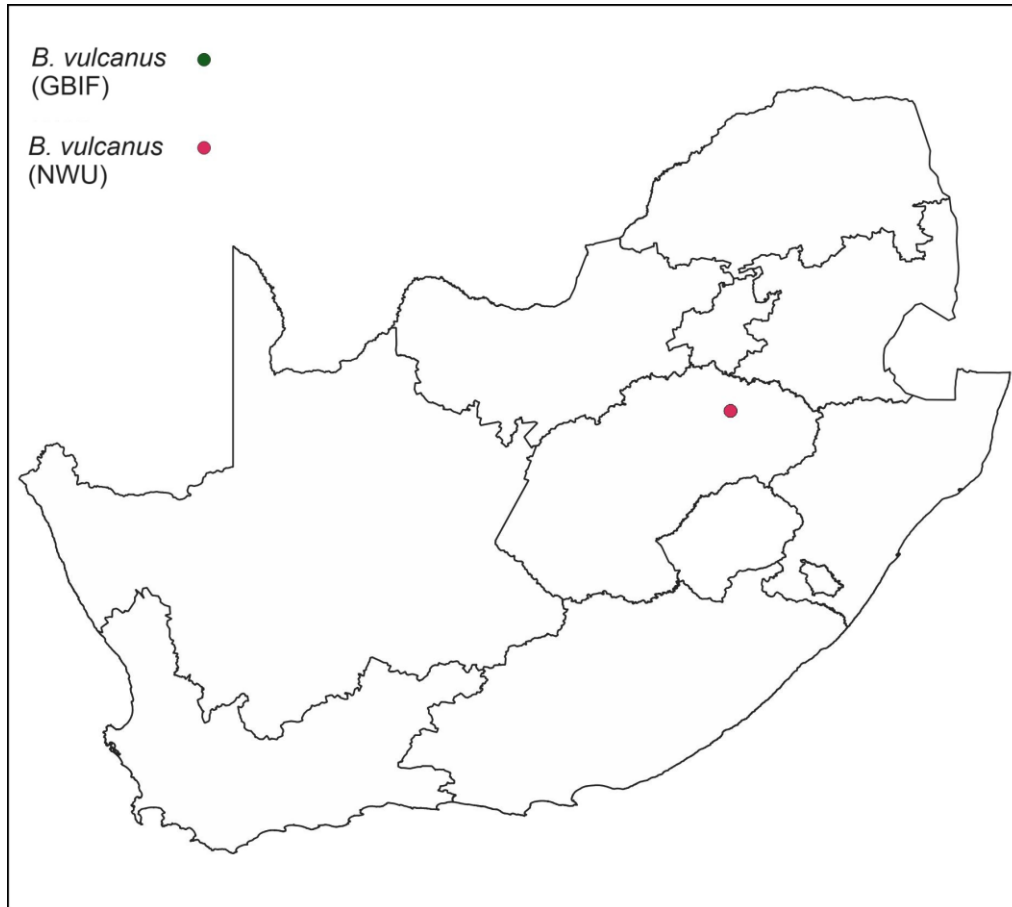


Figure 2.16: Distribution of *Burnupia vulcanus* (Walker, 1924) pink dots indicate information found on the NWU database.

2.3.1.8. Morphometrics

Another characteristic that can be used for the identification of limpets is the size and dimensions of the shell. Measurements of the collected limpets were taken according to Figure 2.2 and are provided in Table 2.5. Ratios based on these measurements are provided in Table 2.6.

Table 2.5: Morphometrical analysis (measured in mm) of the *Burnupia* shells collected in the present study.

Species	Sample code	Total length (T)	Anterior width (W1)	Posterior width (W2)	Apex height (H)
<i>B. transvaalensis</i>	V1A1	6,7	4,9	4,3	2,8
<i>B. capensis</i>	V1A2	5,3	4	3,5	1,6
<i>B. transvaalensis</i>	V1A3	7,2	4,9	4,4	2,8
<i>B. transvaalensis</i>	ER1A1	4,7	3,6	3,1	3,4
<i>B. transvaalensis</i>	ER1A3	5	3,6	3,3	3,6
<i>B. transvaalensis</i>	ER1A4	5,1	3,4	2,9	4
<i>B. transvaalensis</i>	ER1A5	6,2	4,6	4,5	1,9
<i>B. transvaalensis</i>	ER1A7	6,9	5,5	4,5	3,4
<i>B. transvaalensis</i>	ER1A10	6,4	4,4	3,5	3,1
<i>B. transvaalensis</i>	KOA1	4,7	3,2	3,4	2
<i>B. mooiensis</i>	MR1A1	5	3	2,3	2
<i>B. mooiensis</i>	MR1A2	6,4	3	2,5	2,2
<i>B. mooiensis</i>	MR1A4	5,5	3,5	3,1	2
<i>B. mooiensis</i>	MR2A1	6,6	4,7	4,3	2,4
<i>B. mooiensis</i>	MR2A5	6,4	4,4	3,8	2,2
<i>B. mooiensis</i>	MR2A6	5,6	4,1	3,3	2,4
<i>B. mooiensis</i>	MR2A8	5,1	3,7	2,8	1,6
<i>B. mooiensis</i>	MR3A1	6,4	4,5	3,7	2,3
<i>B. mooiensis</i>	MR3A5	6,2	4,3	3,4	2
<i>B. mooiensis</i>	NWA2	6,2	4,3	3,5	1,9
<i>B. mooiensis</i>	NWA3	4,4	3	2,2	2
<i>B. mooiensis</i>	CDA2	5,9	4,2	3,8	2,5
<i>B. mooiensis</i>	BSA1	5,7	3,5	3,2	2,4
<i>B. mooiensis</i>	BSA2	6,5	4	3,6	2,4
<i>B. caffra</i>	MMA2	4,5	3,3	2,5	1,9
<i>B. caffra</i>	SRA2	4	2,8	2,4	2,2
<i>B. caffra</i>	SRA3	4,4	3,1	2,6	2,3
<i>B. caffra</i>	SK1A4	5,9	4,2	3,3	2,5
<i>B. caffra</i>	SK1A6	5,7	3,8	3,4	2

Table 2.6: Morphometric ratios used based on the morphometrics in Table 2.5. Abbreviations: L – total length, W1 – anterior width, W2 – posterior width, H – apex height.

Species	Sample code	W1/L	W2/L	H/L	H/W1	W2/W1
<i>B. transvaalensis</i>	V1A1	0,731343	0,641791	0,41791	0,571429	0,877551
<i>B. capensis</i>	V1A2	0,754717	0,660377	0,301887	0,4	0,875
<i>B. transvaalensis</i>	V1A3	0,680556	0,611111	0,388889	0,571429	0,897959
<i>B. transvaalensis</i>	ER1A1	0,765957	0,659574	0,723404	0,944444	0,861111
<i>B. transvaalensis</i>	ER1A3	0,72	0,66	0,72	1	0,916667
<i>B. transvaalensis</i>	ER1A4	0,666667	0,568627	0,784314	1,176471	0,852941
<i>B. transvaalensis</i>	ER1A5	0,741935	0,725806	0,306452	0,413043	0,978261
<i>B. transvaalensis</i>	ER1A7	0,797101	0,652174	0,492754	0,618182	0,818182
<i>B. transvaalensis</i>	ER1A10	0,6875	0,546875	0,484375	0,704545	0,795455
<i>B. transvaalensis</i>	KOA1	0,680851	0,723404	0,425532	0,625	1,0625
<i>B. mooiensis</i>	MR1A1	0,6	0,46	0,4	0,666667	0,766667
<i>B. mooiensis</i>	MR1A2	0,46875	0,390625	0,34375	0,733333	0,833333
<i>B. mooiensis</i>	MR1A4	0,636364	0,563636	0,363636	0,571429	0,885714
<i>B. mooiensis</i>	MR2A1	0,712121	0,651515	0,363636	0,510638	0,914894
<i>B. mooiensis</i>	MR2A5	0,6875	0,59375	0,34375	0,5	0,863636
<i>B. mooiensis</i>	MR2A6	0,732143	0,589286	0,428571	0,585366	0,804878
<i>B. mooiensis</i>	MR2A8	0,72549	0,54902	0,313725	0,432432	0,756757
<i>B. mooiensis</i>	MR3A1	0,703125	0,578125	0,359375	0,511111	0,822222
<i>B. mooiensis</i>	MR3A5	0,693548	0,548387	0,322581	0,465116	0,790698
<i>B. mooiensis</i>	NWA2	0,693548	0,564516	0,306452	0,44186	0,813953
<i>B. mooiensis</i>	NWA3	0,681818	0,5	0,454545	0,666667	0,733333
<i>B. mooiensis</i>	CDA2	0,711864	0,644068	0,423729	0,595238	0,904762
<i>B. mooiensis</i>	BSA1	0,614035	0,561404	0,421053	0,685714	0,914286
<i>B. mooiensis</i>	BSA2	0,615385	0,553846	0,369231	0,6	0,9
<i>B. caffra</i>	MMA2	0,733333	0,555556	0,422222	0,575758	0,757576
<i>B. caffra</i>	SRA2	0,7	0,6	0,55	0,785714	0,857143
<i>B. caffra</i>	SRA3	0,704545	0,590909	0,522727	0,741935	0,83871
<i>B. caffra</i>	SK1A4	0,711864	0,559322	0,423729	0,595238	0,785714
<i>B. caffra</i>	SK1A6	0,666667	0,596491	0,350877	0,526316	0,894737

The morphometric data in Tables 2.5 and 2.6 were classified according to the river it was collected from as well as the identified species based on morphology and molecular data. The river classified data (Figure 2.17) indicated that there were significant differences between the H, H/L and W2/L values between the Elands and Mooi Rivers.

For the identified species plotted in Table 2.6, the values for *B. transvaalensis* seemed to indicate higher values especially for L, W1, W2 and H measurements (Figure 2.18). However, the ratios (Table 2.6) showed that all the species had similar ratios. There was only a significant difference between *B. transvaalensis* and *B. mooiensis* for the H/W1 ratio.

2.3.2 Phylogenetics and Species Diversity

In this study, 25 new partial COI *Burnupia* spp. sequences were obtained. The lengths of the sequences were between 680 and 780 base pairs. The base pair differences and the percentage similarity between different specimens from different sites and the GenBank sequences (details in Table 2.4) are shown in Table 2.6. Due to space constraints, only the representatives with the highest and lowest number of differences from each variant were included in the table.

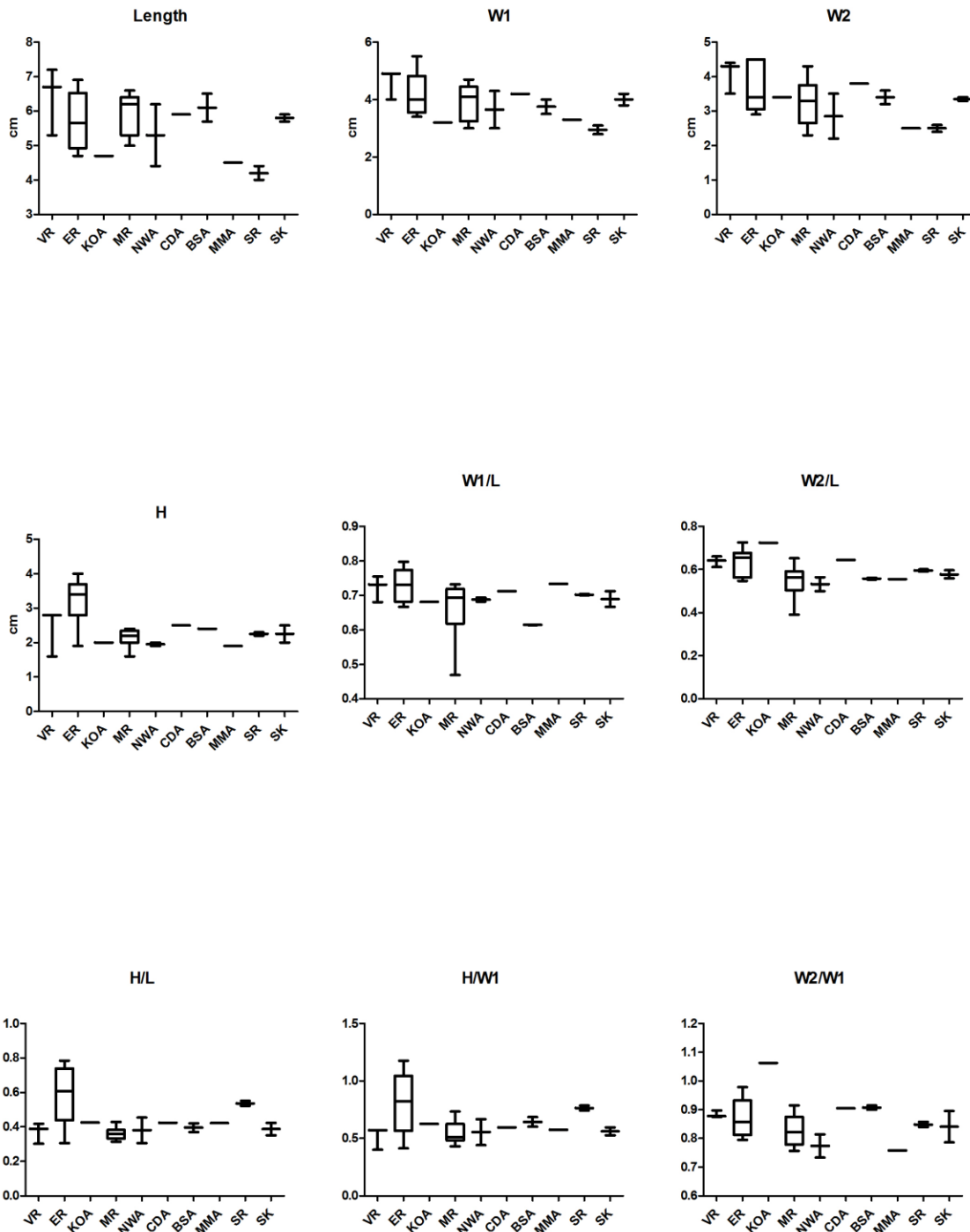


Figure 2.17:Box and whisker plot for morphometric analysis of *Burnupia* Walker, 1912 based on the rivers that were sampled. Abbreviations: VR – Vaal River, ER – Elands River, KOA, Klein Olifants River, MR – Mooi River, NWA – Magalies River, CDA – Crocodile River, BSA – Bronkhorstspuit, MMA – Mac Mac River, SR – Sabie River, SK – Suid-Kaap River, L – total length, W1 – anterior width, W2 – posterior width, H – apex height.

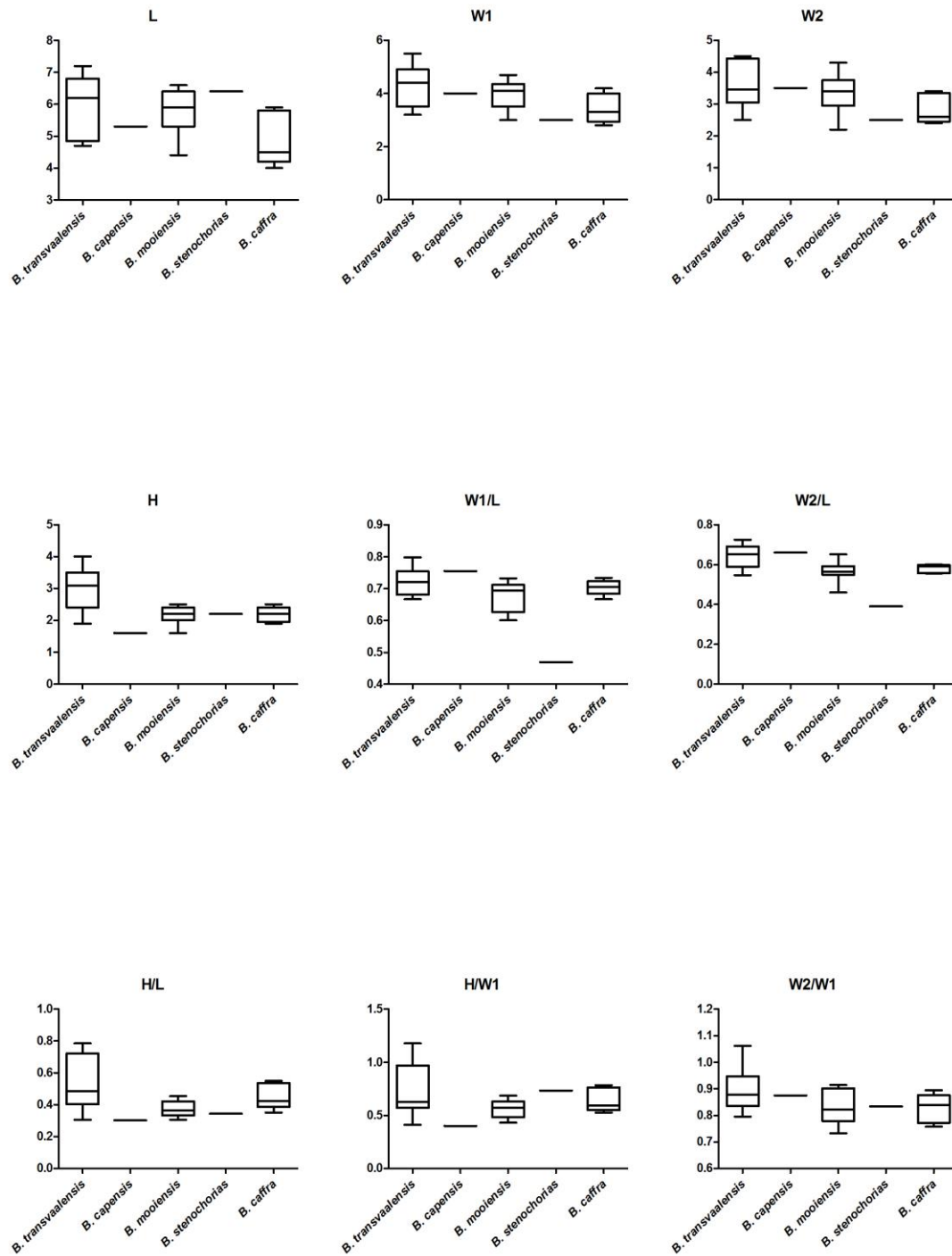


Figure 2.18: Box and whisker plot for morphometric analysis of *Burnupia* Walker 1912, based on the different species collected. Abbreviations: L – total length, W1 – anterior width, W2 – posterior width, H – apex height.

Table 2.7: Genetic divergence of aligned COI (LCO1490 and COR722b) sequences used in the *Burnupia* Walker, 1912 alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). The species obtained in this study are in bold and blue shade. AY82586 *Ferrissia rivularis* was used as the outgroup (green shade). Only the highest and lowest values from the same site are shown. Abbreviations: BS – Bronkhorstspruit, CD – Crocodile River, MR – Mooi River, NW – Magalies River, ER- Elands River, V1 – Vaal River, KO – Klein Olifants River, MM – Mac Mac River, SR – Sabie River, SK – Suid-Kaap 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	31
1	BSA1		4	13	9	14	18	19	21	22	21	42	43	42	42	43	19	22	23	24	20	18	23	22	22	45	38	46	43	78	39	109
2	CDA1	99.3		13	9	12	18	19	21	22	21	44	47	46	44	45	19	22	23	24	22	18	23	24	26	47	40	48	43	78	42	110
3	MR2A1	97.9	97.9		11	5	19	20	19	19	22	41	44	44	40	40	18	21	22	23	21	15	20	22	26	46	38	45	41	75	37	112
4	MR2A5	98.5	98.5	98.2		14	20	21	20	24	21	46	47	46	46	47	21	24	25	23	20	20	25	26	26	51	44	50	45	77	44	110
5	NWA2	97.8	98.1	99.2	97.8		23	24	21	19	25	45	48	47	44	45	20	23	24	24	24	17	23	25	30	43	40	50	44	77	42	109
6	ER1A4	97.1	97.1	97	96.8	96.4		1	12	13	11	42	45	45	45	45	10	13	14	17	15	11	18	18	24	53	39	45	43	76	40	108
7	ER1A10	97	97	96.8	96.7	96.2	99.8		13	14	10	43	46	46	46	46	11	14	15	18	16	12	19	19	25	54	40	46	44	77	40	108
8	V1A1	96.7	96.7	97	96.8	96.7	98.1	97.9		5	16	44	45	44	46	47	6	9	10	17	17	7	16	18	28	52	40	47	45	77	41	109
9	V1A3	96.5	96.5	97	96.2	97	97.9	97.8	99.2		17	44	47	46	45	46	6	10	11	18	17	6	17	19	29	49	38	48	46	80	40	112
10	KOA1	96.7	96.7	96.5	96.7	96	98.2	98.4	97.5	97.3		45	48	47	49	50	14	17	18	17	18	15	21	21	26	57	43	50	48	79	44	110
11	MMA2	93.4	93.1	93.5	92.8	92.9	93.4	93.2	93.1	93.1	92.9		3	3	14	14	42	41	40	46	47	39	42	41	41	57	13	14	26	79	12	103
12	SRA2	93.2	92.6	93	92.6	92.5	92.9	92.8	92.9	92.6	92.5	99.5		0	17	17	45	44	43	49	50	42	45	44	42	58	16	17	27	78	14	103
13	SRA3	93.3	92.7	93	92.7	92.6	92.9	92.7	93	92.7	92.6	99.5	100		17	17	44	43	42	48	49	41	44	43	42	58	16	17	27	78	14	103
14	SK1A4	93.3	93	93.6	92.7	93	92.9	92.7	92.7	92.9	92.2	97.7	97.3	97.3		0	42	43	44	48	48	39	44	43	42	55	4	10	30	77	7	111
15	SK1A6	93.2	92.9	93.7	92.6	92.9	92.9	92.8	92.6	92.8	92.1	97.8	97.3	97.3	100		43	44	45	49	49	40	45	44	42	55	4	10	30	77	7	111
16	ON256521 <i>Burnupia</i> sp. 1	97	97	97.1	96.7	96.8	98.4	98.2	99	99	97.8	93.4	92.9	93	93.3	93.2		7	8	16	17	5	14	16	26	48	34	45	43	78	37	111
17	ON256527 <i>Burnupia</i> sp. 2	96.5	96.5	96.6	96.2	96.4	97.9	97.8	98.5	98.4	97.3	93.5	93.1	93.2	93.2	93.1	98.9		1	19	20	8	17	19	27	49	37	44	42	79	37	109
18	ON256543 <i>Burnupia</i> sp. 2	96.4	96.4	96.5	96	96.2	97.8	97.6	98.4	98.2	97.1	93.7	93.2	93.3	93	92.9	98.7	99.8		19	20	9	18	20	28	50	38	45	41	79	38	108
19	ON256520 <i>Burnupia</i> sp. 3	96.2	96.2	96.3	96.4	96.2	97.3	97.1	97.3	97.1	97.3	92.8	92.3	92.4	92.4	92.3	97.5	97	97		12	14	22	25	30	48	43	50	48	82	44	111
20	ON256517 <i>Burnupia</i> sp. 3	96.8	96.5	96.6	96.8	96.2	97.6	97.5	97.3	97.3	97.1	92.6	92.1	92.2	92.4	92.3	97.3	96.8	96.8	98.1		16	23	22	27	51	43	51	49	80	44	112
21	ON256550 <i>Burnupia</i> sp. 4	97.1	97.1	97.6	96.8	97.3	98.2	98.1	98.9	99	97.6	93.9	93.4	93.5	93.8	93.7	99.2	98.7	98.5	97.8	97.5		13	17	23	47	35	42	40	78	34	109
22	ON256547 <i>Burnupia</i> sp. 5	96.4	96.4	96.8	96	96.4	97.1	97	97.5	97.3	96.7	93.4	92.9	93	93	92.9	97.8	97.3	97.1	96.5	96.4	97.9		10	24	54	40	45	44	82	38	111
23	ON256515 <i>Burnupia</i> sp. 5	96.5	96.2	96.5	95.9	96	97.1	97	97.1	97	96.7	93.5	93.1	93.2	93.2	93.1	97.5	97	96.8	96	96.5	97.3	98.4		26	55	38	44	47	78	37	107
24	ON256546 <i>Burnupia</i> sp. 6	96.5	95.9	95.9	95.9	95.3	96.2	96	95.6	95.4	95.9	93.5	93.4	93.3	93.3	93.4	95.9	95.7	95.6	95.3	95.7	96.4	96.2	95.9		46	39	41	38	78	36	108
25	ON256548 <i>Burnupia</i> sp. 7	92.9	92.6	92.7	92	93.2	91.7	91.5	91.8	92.3	91	91	90.9	90.8	91.3	91.4	92.5	92.3	92.1	92.5	92	92.6	91.5	91.4	92.8		52	60	56	83	50	102
26	ON256429 <i>Burnupia</i> sp. 8	93.6	93.2	93.6	92.6	93.2	93.4	93.2	93.2	93.6	92.7	97.8	97.3	97.3	99.3	99.3	94.2	93.7	93.6	92.7	92.7	94.1	93.2	93.6	93.4	91.2		10	27	72	6	103
27	ON256542 <i>Burnupia</i> sp. 8	92.8	92.5	92.9	92.1	92.1	92.9	92.8	92.6	92.5	92.1	97.8	97.3	97.3	98.4	98.4	92.9	93.1	92.9	92.1	92	93.4	92.9	93.1	93.5	90.6	98.3		26	79	9	112
28	ON256555 <i>Burnupia</i> sp. 9	93.2	93.2	93.5	92.9	93.1	93.2	93.1	92.9	92.8	92.5	95.9	95.7	95.7	95.2	95.3	93.2	93.4	93.5	92.5	92.3	93.7	93.1	92.6	94	91.2	95.4	95.9		81	25	105
29	ON256545 <i>Burnupia</i> sp. 10	87.8	87.8	88.2	87.9	87.9	88.1	87.9	87.9	87.5	87.6	87.6	87.8	87.7	87.8	87.9	87.8	87.6	87.6	87.1	87.5	87.8	87.1	87.8	87.8	87	87.9	87.6	87.3		70	113
30	AY282584 <i>Burnupia caffra</i>	93.5	93	93.8	92.7	93	93.4	93.4	93.2	93.4	92.7	98	97.6	97.6	98.8	98.8	93.9	93.9	93.7	92.7	92.7	94.4	93.7	93.9	94	91.7	98.9	98.5	95.8	88.4		109
31	AY282586 <i>Ferrissia rivularis</i>	82	81.9	81.4	81.9	82	82.2	82.2	82	81.5	81.9	83	83	82.9	81.5	81.7	81.7	82	82.2	81.7	81.5	82	81.7	82.4	82.2	83.2	81.7	81.5	82.7	81.4	82	

Between the BI and ML trees constructed in PhyML, MrBayes, and CIPRES, there were 19 supported nodes between the associations of 52 *Burnupia* specimens (Figure 2.19). There were 25 sequences obtained in the current study. The Vaal River specimens grouped together with 99% similarity (see Table 2.7) and 3 to 5 base pair differences between these three samples (Table 2.7). The Elands River species clustered together with 99% similarity and only differed with 1 base pair (Table 2.7). Most of the species from the Mooi River clustered together with the species from the Magalies River (NWA2 and NWA3), NWA2 and MR2A1 only differed by 5 base pairs and are 99% similar (Table 2.7). One species from the Mooi River (MR2A5) clustered with the species from the Crocodile River and Bronkhorstspuit. The species from the Crocodile River (CDA1 and CDA2) and the samples from Bronkhorstspuit (BSA1 and BSA2) are 99% identical and had a base pair difference of 4 and these species are 98% similar to MR2A5 (Table 2.7). The samples from the Suid-Kaap River are a sister clade to the species from the Sabie River and the Mac Mac River that grouped together. The Mac Mac River species and the Sabie River species are 99% similar (Table 2.7) with 3 base pair differences (Table 2.7). The species from Mac Mac and Sabie rivers differed by 14 base pairs from the Suid-Kaap River sequences (Table 2.7) and they are 97% similar (Table 2.7).

The three sample sites that grouped together with the *B. caffra* sequences are 97–98% similar to it (Table 2.7) and the base pair differences range between 7 and 14 (Table 2.6). The species collected from the Vaal River (V1A1 and V1A3) are 99% similar and they only differed 5 base pairs (Table 2.7). The samples obtained from the Elans River (ER1A4 and ER1A10) are 99% similar and differed with 1 base pair. These species are 98% similar to samples from the Vaal River (V1A1) and the samples from the Klein Oifants River (KOA1) (see Table 2.6).

The *Burnupia stenochorias* (AY282585) sequence, as well as the *Burnupia* sp. 5 sequences from GenBank were between 92 and 96% similar to the sequences obtained in this study and it differed between 15 and 40 base pairs with the obtained sequences.



Figure 2.19: Phylogenetic tree based on the partial COI (LCO1490 and COR722b) sequences for selected *Burnupia* species. Nodal support is given as BI/ML (Bayesian Inference/Maximum Likelihood). Support values lower than 0.70 (BI) and 70 (ML) are not displayed. Sequences obtained from this study are in bold. The outgroup was *Ferrissia rivularis* (Say, 1817).

For the second set of primers used (ASMIT 1 and ASMIT 2) the lengths of the sequences were between 400 and 500 base pairs. The percentage similarity between the specimens from different sites, the base pair differences and the GenBank sequences are shown in Table 2.8.

Table 2.8: Genetic divergence of aligned COI (ASMIT 1 and ASMIT 2) sequences used in the *Burnupia* Walker, 1912 alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). The species obtained in this study are in bold. AY577509 *Ferrissia c.f clessiniana* Jickeli, 1882 was used as the outgroup (green shade). Abbreviations: BS – Bronkhorstspuit, CD – Crocodile River, MR – Mooi River, NW – Magalies River, ER – Elands River, V1 – Vaal River, KO – Klein Olifants River, MM – Mac Mac River, SR – Sabie River, SK – Suid-Kaap 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
1	V1A1		2	9	13	9	11	9	9	8	9	8	8	10	10	10	11	9	17	20	17	19	19	19	25	20	17	60
2	V1A3	99.4		11	15	11	13	11	11	8	9	10	10	12	12	12	13	9	17	20	17	19	19	19	25	20	17	62
3	MR1A1	97.6	97		7	5	6	5	5	14	13	4	4	7	7	7	8	16	22	25	22	26	26	26	30	23	22	65
4	MR1A4	96.5	96	98.1		9	11	9	9	18	16	8	8	12	12	12	13	16	20	23	20	28	28	28	32	25	22	67
5	MR2A1	97.4	96.9	98.6	97.4		10	0	0	14	13	1	1	9	9	9	10	16	22	25	22	26	26	26	30	23	23	65
6	MR2A5	97.1	96.5	98.4	97	97.1		10	10	13	12	8	9	3	3	3	4	17	22	24	22	27	27	27	30	25	22	63
7	MR2A6	97.6	97.1	98.6	97.6	100	97.3		0	14	13	1	1	9	9	9	10	16	22	25	22	26	26	26	30	23	23	65
8	MR3A1	97.6	97.1	98.6	97.6	100	97.3	100		14	13	1	1	9	9	9	10	16	22	25	22	26	26	26	30	23	23	65
9	ER1A4	97.7	97.7	96.1	94.9	95.8	96.3	96.1	96.1		3	13	13	14	14	14	15	10	19	21	19	18	18	18	25	19	14	57
10	ER1A10	97.5	97.5	96.2	95.2	95.9	96.5	96.2	96.2	99.5		12	12	13	13	13	13	11	17	19	17	16	16	16	24	19	12	48
11	NWA2	97.8	97.3	98.9	97.8	99.7	97.8	99.7	99.7	96.3	96.5		0	7	7	7	8	15	20	23	20	24	24	24	29	21	21	64
12	NWA3	97.9	97.3	98.9	97.8	99.7	97.6	99.7	99.7	96.3	96.5	100		8	8	8	9	15	21	24	21	25	25	25	29	22	22	64
13	CDA1	97.3	96.8	98.1	96.8	97.4	99.2	97.6	97.6	96.1	96.2	98.1	97.9		0	0	1	17	21	25	22	26	26	26	29	24	22	63
14	CDA2	97.3	96.8	98.1	96.8	97.4	99.2	97.6	97.6	96.1	96.2	98.1	97.9	100		0	1	17	21	25	22	26	26	26	29	24	22	63
15	BSA1	97.3	96.8	98.1	96.8	97.4	99.2	97.6	97.6	96.1	96.2	98.1	97.9	100	100		1	17	21	25	22	26	26	26	29	24	22	63
16	BSA2	97.1	96.5	97.8	96.5	97.1	98.9	97.3	97.3	95.8	96.2	97.8	97.6	99.7	99.7	99.7		18	22	26	23	27	27	27	30	25	23	62
17	KOA1	97.3	97.3	95.2	95.2	95.2	94.9	95.2	95.2	96.9	96.4	95.4	95.5	94.9	94.9	94.9	94.6		15	17	15	20	20	20	24	19	18	59
18	SRA2	94.8	94.8	93.3	93.9	93.3	93.3	93.3	93.3	93.9	94	93.7	93.6	93.6	93.6	93.6	93.3	95.4		0	0	10	10	10	19	15	9	65
19	SRA3	94.7	94.7	93.3	93.9	92.9	93.6	93.4	93.4	94.1	94.2	93.8	93.7	93.4	93.4	93.4	93.1	94.9	100		0	12	12	12	22	18	9	66
20	MMA2	94.8	94.8	93.3	93.9	93.3	93.3	93.3	93.3	93.9	94	93.8	93.6	93.3	93.3	93.3	93	95.4	100	100		10	10	10	19	15	9	65
21	SK1A2	94.9	94.9	93	92.5	92.6	92.8	93.1	93.1	94.9	95.2	93.5	93.3	93.1	93.1	93.1	92.8	94.1	96.9	96.8	96.9		0	0	14	13	1	64
22	SK1A4	94.9	94.9	93	92.5	92.6	92.7	93	93	94.9	95.2	93.4	93.3	93	93	93	92.7	94.1	96.9	96.7	96.9	100		0	14	13	1	64
23	SK1A6	94.9	94.9	93	92.5	92.6	92.8	93	93	94.9	95.2	93.4	93.3	93	93	93	92.8	94.1	96.9	96.8	96.9	100	100		14	13	1	64
24	AY577508 <i>Burnupia kemp</i> i	93.4	93.4	91.9	91.4	91.5	92	92	92	93	92.5	92.2	92.3	92.3	92.3	92.3	92	92.8	94.2	94.1	94.2	96.2	96.2	96.2		10	9	62
25	AY55750 <i>Burnupia sthulmani</i>	94.7	94.7	93.8	93.3	93.5	93.4	93.9	93.9	94.7	94.2	94.3	94.2	93.7	93.7	93.7	93.4	94.3	95.4	95.2	95.4	96.5	96.5	96.5	97.3		11	65
26	AY282584 <i>Burnupia caffra</i>	94.1	94.1	92.4	92.4	92	92.4	92	92	94.8	95.2	92.5	92.4	92.4	92.4	92.4	92	93.7	96.8	96.8	96.8	99.6	99.6	99.6	96.8	96.2		56
27	AY577509 <i>Ferrissia c.f clessiniana</i>	82.6	82	81.1	80.5	81.1	81.7	81.1	81.1	82.6	83	81	81.4	81.7	81.7	81.7	82	82.5	80.3	80.8	80.3	81.4	81.4	81.4	81.9	81.1	80.6	

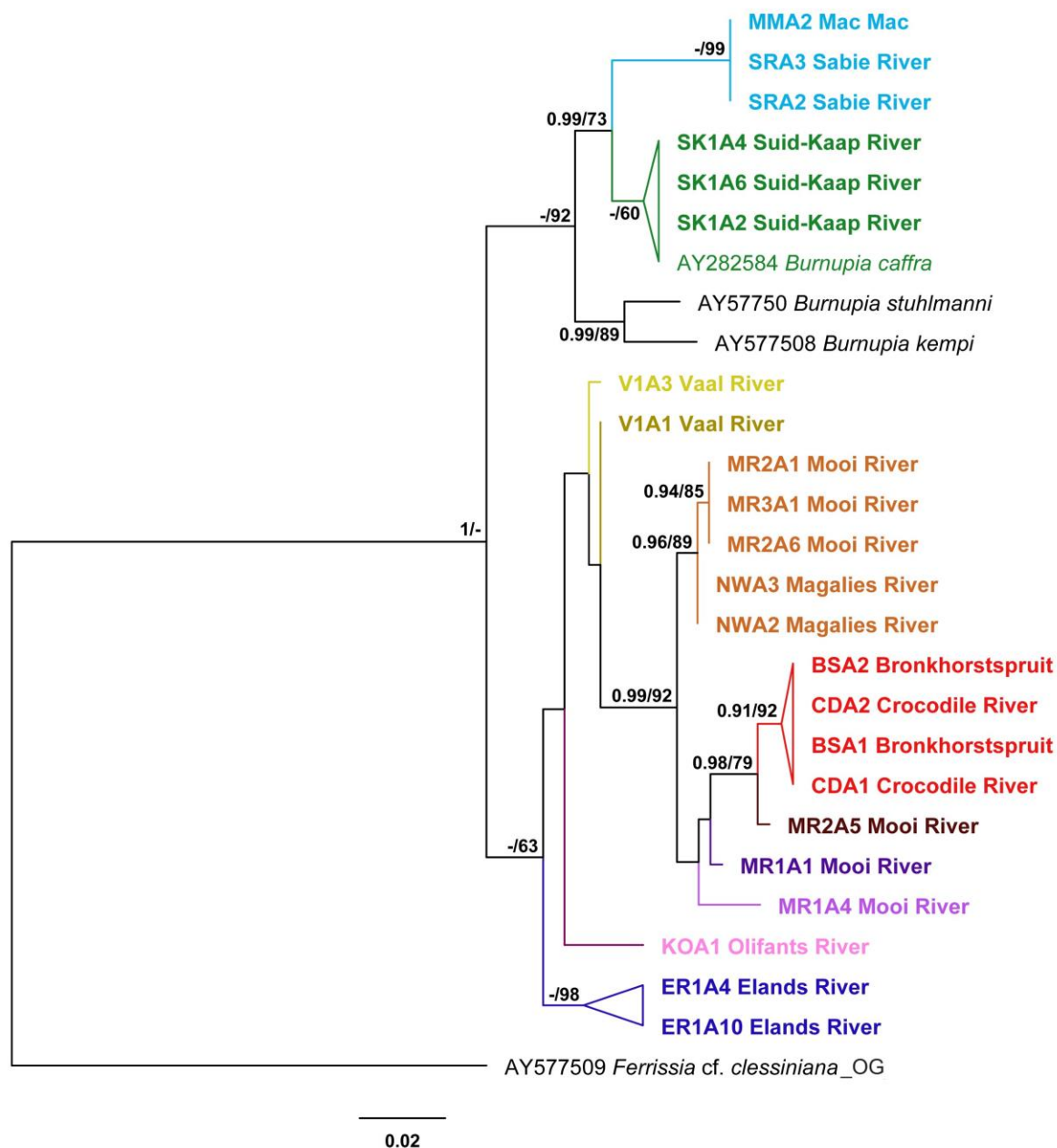


Figure 2.20: Phylogenetic tree based on the partial COI sequences (using primers ASMIT1 and ASMIT2) for selected *Burnupia* species. Nodal support is given as BI/ML (Bayesian Inference /Maximum Likelihood). Support values lower than 0.60(BI) and 60 (ML) are not displayed. Sequences used from GenkBank are in black. The outgroup was *Ferrissia clessiniana* (Jickeli, 1882).

Between the BI and ML trees constructed in PhyML, MrBayes, and CIPRES, there were 13 supported nodes between the associations of 26 *Burnupia* specimens (Figure 2.20). The Mac Mac River and the Sabie River grouped together and according to Table 2.8 they are 100% identical. The Suid-Kaap River sequences clustered together with an existing sequence on GenBank, *B. caffra*. All these samples differed with 1 base pair (Table 2.8). The specimens from the Vaal River grouped together and they are 99% identical and they only differed by 2 base pairs (Table 2.8). Mooi River (MR2A1; MR3A1; MR2A6) and Magalies River (NWA2; NWA3) grouped together and matched 99% with each other. Bronkhorstspuit and Crocodile River grouped together, and they are 100% identical (Table 2.8). The Elands River species grouped together, and they are 99% similar and they differed by 3 base pairs (Table 2.8).

2.4. Discussion

The simple way that Brown (1994) and Walker (1912) described shells makes it extremely difficult and occasionally impossible to distinguish between various species (Oberholzer & van Eden, 1963). Morphology-based identification includes the use of shell size and form, which might vary due to environmental conditions (Brown, 1994), because shapes and patterns can be altered by external variables. For instance, certain molluscs that have been attached to a reed conform exactly to the reed's contours, entirely departing from the typical shell shape of an animal that has been living on the surface of a solid rock. Therefore, when using the shell shape to differentiate between species, caution should be used as they could be somewhat misleading. Furthermore, morphology-based taxonomy can be limited and time-consuming, requires in-depth familiarity with a group, and may result in inaccurate species identification (Layton *et al.*, 2014; Feng *et al.*, 2011; Weigand *et al.*, 2011; Nantararat *et al.*, 2019; Raphalo *et al.*, 2021). Consequently, species cannot be identified on morphology alone and molecular studies are also needed to help eliminate misidentification (Brown, 1994; Appleton *et al.*, 2002).

Relatively simple changes in molluscs' surroundings, such as pollution, current velocity, temperature, the elimination of riparian vegetation and predator abundance, have been shown to cause shell morphology changes and can cause acceleration of morphological variations within a species too (Lacerda *et al.*, 2011; Kistner & Dybdahl, 2013; Sands *et al.*, 2022). The same conclusion is reached for *Burnupia*, where reviewing type material will be crucial in updating the genus' systematics and giving the newly discovered phylogenetic species their proper names (Sands *et al.*, 2022).

2.4.1 Taxonomy

The only way to identify South African species is to refer to descriptions and drawings of the shell as well as the region where the specimens were found. However, the degree of variety displayed by these structures severely restricts their usefulness in identifying different species (Oberholzer & van Eden, 1963). Assigning names to phylogenetic species also remains a difficult task. *Burnupia's* taxonomic complexities and conflicts have been anticipated (Sands *et al.*, 2021).

Connolly (1939) stated that *B. caffra* has a length of 5,4 mm and the height of the apex is 2,5 mm. The specimens obtained in this study that were identified as *B. caffra* (Suid-Kaap river) had a length between 5,7 and 5,9 and the apex is 2 mm (see Table 2.6). These measurements are relatively close to each other. In 2022, Sands *et al.* completed a study on limpet species from the Suid-Kaap, Mac Mac and Sabie rivers. These limpets were also identified as *B. caffra*. From the phylogenetic tree (Figure 2.19), it can be observed that the samples from the Sabie River, Suid – Kaap River and the Mac Mac River grouped together with the previously sequenced *B. caffra* sample. Figure 2.6 shows the species that were collected in the current study, but there are no other photos or drawings from existing literature to compare these samples to. This identification was based on the locality where these samples were collected, molecular results (Figures 2.19 and 2.20), and their morphological characteristics. The species obtained in this study conforms to the description of *B. caffra*, because the limpets had a noticeable apex, not very sharp and turned far to the right, and the posterior part is short and steep. Since *B. caffra* has a wide distribution range and Brown (1994) stated that *B. verreauxi* can be considered as a refined variant of *B. caffra*, both species need to be studied further by generating sequence data and revising using type material.

Connolly (1939) gave two different measurements for *B. capensis*. The one specimen had a length of 7,5 mm and an apex height of 2,5 mm and the other was 5,7 mm in length and the apex was 2,1 mm high. The specimen collected in this study (V1A2) had a length of 5,3 mm and a height of 1,6 mm (Table 2.6). These measurements are close to the second samples measurements in Connolly (1939). In the study completed by Sands *et al.* (2022), they collected specimens from the Moremoholo River in Lesotho. The Moremoholo River flows into the Orange River and then it links up with the Vaal River. They identified this limpet as *B. capensis striatissima* and it is indicated on Figure 2.17 as *Burnupia* sp. 1. Pilsbry (1932) stated that *B. capensis striatissima* is a subspecies for *B. capensis*. With the relatively close relationship between the Vaal River and Moremoholo River, similar size range, and the genetic similarity (Figure 2.19), it is possible that the Vaal River species (V1A2) is similar to *Burnupia* sp. 1. (aka *B. capensis*

striatissima). From the comparison of morphological characteristics between *B. capensis* and VR1A2 (shown in Figure 2.8), the newly collected limpet (V1A2) has the same characteristics that were given by Brown (1994), such as the oval shape, an apex that is noticeable and nearly pointed, and prominent radial ridges. This species collected from the Vaal River (V1A2) thus appears to be *B. capensis*.

Burnupia mooiensis described in Connolly (1939) had a length of 7 mm and an apex height of 3 mm. The specimen from this study, collected from the Mooi River had a length between 5 and 6,6 mm and an apex height of 2,3 – 4.3 mm (Table 2.6). The Magalies River (NW) species had a length between 4,4 and 6,2 mm and an apex height of 2 mm (Table 2.6). The limpets collected from the Crocodile River had a length of 5,9 mm and an apex height of 2,5 mm and the Bronkhorstspuit species had a minimum length of 5,7 mm and a maximum length of 6,5 and the apex height of 2 mm. These measurements differ from the measurements given in Connolly (1939) but the phylogenetic analysis (Figures 2.19 and 2.20) indicated that the samples from the Mooi River, Magalies River and the Crocodile River grouped together. The samples collected from the Mooi River (i.e. MR1A1, MR2A1, MR2A5, MR2A6, MR3A1, MR3A5), Magalies River (NWA2, NWA3), Crocodile River (CDA1, CDA2), and from Bronkhorstspuit (BSA1; BSA2) morphologically correspond to *B. mooiensis* since the limpets collected have a large, oval, elevated shell and are wide at the posterior end, the apex is not very noticeable and the posterior slope is long and not steep. *Burnupia mooiensis* usually occurs in the Gauteng and North West Provinces.

According to Connolly (1939), *Burnupia stenochorias* has a shell length of 8,5 mm and an apex height of 3,5 mm. The phylogenetic analysis (Figure 2.19) indicates that no species obtained in this study grouped together with *B. stenochorias*. This was corroborated by the morphology as the limpets collected during the course of this study did not conform to the description given for *B. stenochorias*. Furthermore, this species mostly occurs in the Eastern Cape and this study's sampling areas did not overlap with these species' distribution. However, the three sequences from the Albany Museum, collected in three sites in the Eastern Cape, did group together with *B. stenochorias*.

For *Burnupia transvaalensis*, two shells were measured by Connolly (1939). One of the shells lengths was 7,5 mm and the apex was 3,2 mm high, and the second shell was 8 mm in length and the apex was 3,5 mm in height. The specimen V1A3, collected in this study from the Vaal River, had a length of 7,2 mm and an apex height of 2,8 mm (Table 2.6). The Elands River's limpets had a length between 4,7 and 6,9 mm and the apex height can be from 1,9 to 4 mm (Table

2.6). The limpets from the Klein Olifants River had a length of 4,7 mm and an apex height of 2 mm. This is relatively close to the first shell that Connolly (1939) measured. The phylogenetic analysis (Figures 2.19 and 2.20) indicates that the Elands River and the Klein Olifants River grouped together. The type locality for *Burnupia transvaalensis* is in the Mooi River, a tributary of the Vaal River. The Elands River flows into the Wilge River, and this forms part of the Vaal River basin. The Elands River and the Wilge River is a tributary of the Klein Olifants River. The species collected from the Vaal River (V1A1), the Elands River (i.e. ER1A1, ER1A3, ER1A4, ER1A7, ER1A10), and the Klein Olifants River (KOA1) conforms to *B. transvaalensis* description as they are large and oval in shape. This species has a high shell due to the fact that the apex is in the centre of the shell. *Burnupia transvaalensis* can be found in the North West, Gauteng, Mpumalanga, Free State, and Northern Cape.

Burnupia vulcanus can possibly be a synonym for *B. mooiensis* (Brown, 1994) because these two species have localities extremely close to each other, even overlapping. Due to the fact that *B. vulcanus* is similar to (and possibly a synonym for) *B. mooiensis*, it is difficult to know whether *B. vulcanus* was collected during the course of this study. In Figure 2.16, it can be observed that the information on the NWU snail database indicates that this was found in the Free State, but this species type locality is in the Mooi River. There are no sequences of this species available, thus phylogenetic analysis could not be done. The description given for *B. vulcanus* which are usually small and coned shaped, with a prominent apex that is not very sharp and a large apical depression (Connolly, 1939), does not conform to any of the species found in this study. In a study conducted by Wolmarans *et al.* (2015), the molluscan diversity of the Mooi River was compared to surveys that were conducted in 1963. The results of this paper indicated that *B. vulcanus* were not found in in the Mooi River. Thus, *B. vulcanus* has not been collected in the Mooi River in over 50 years, meaning that the species may have either been damaged from the environment (and thus unrecognizable as *B. vulcanus*) or has since gone extinct. This highlights the need for further studies to be done on *B. vulcanus* and *B. mooiensis*.

In Figures 2.17 and 2.18, it was indicated that there were some significant differences between the morphometrics of the species. *Burnupia transvaalensis* values indicate that it has a larger shell size and the ratios (Table 2.6) show there were a significant difference with *B. mooiensis*. The height of the apex and the length of the shell was compared to the literature in Connolly (1939). The observed morphometric resembles between the different *Burnupia* species can possibly be because of their close geographical proximity (Pfenninger *et al.*, 2003). Species identification with shell morphometry alone are still challenging because there was not a

significant difference in the morphometric aspect of this species. Drawings were made of the species found or species that were used to compare the limpets found (Figure 2.21).

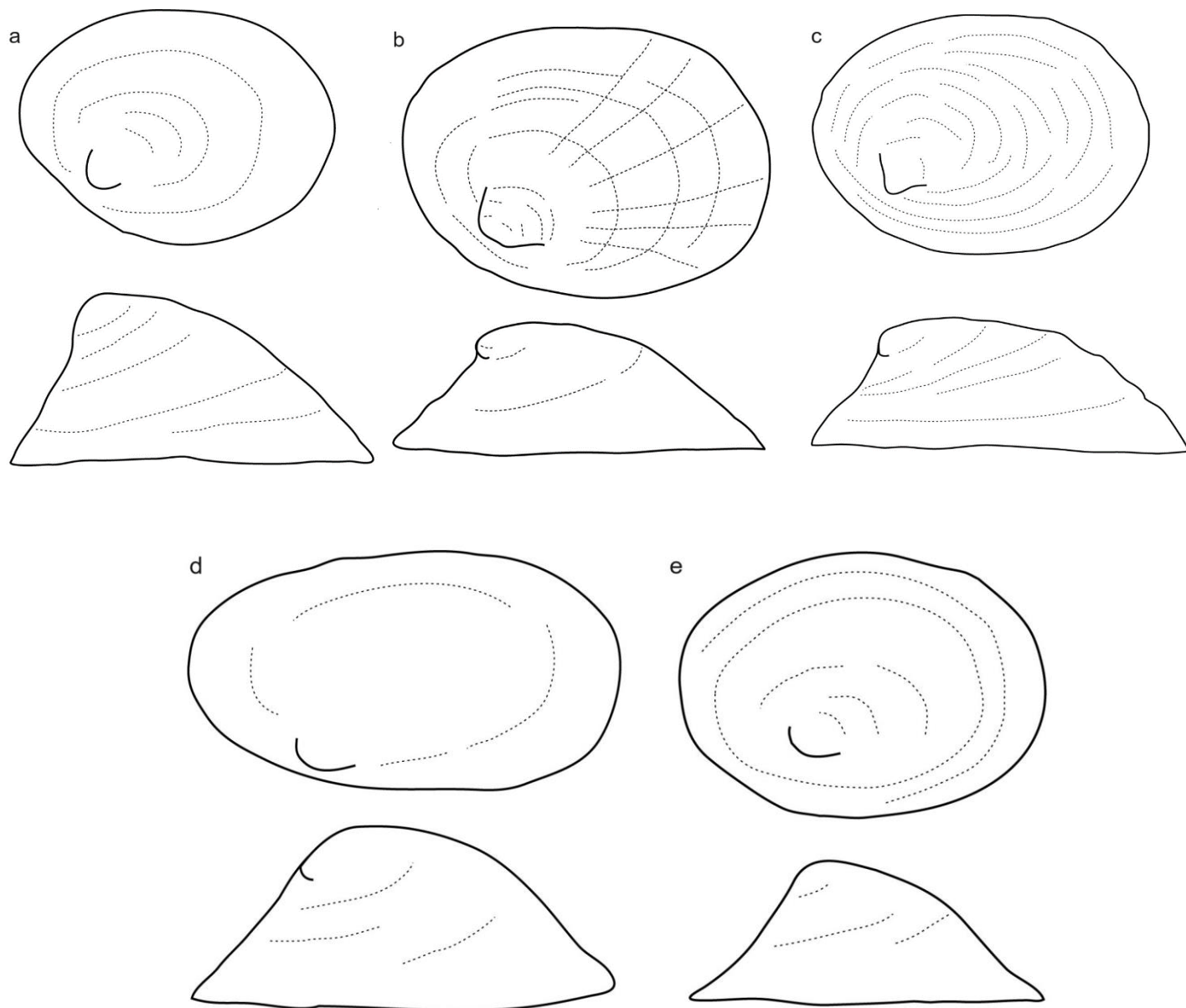


Figure 2.21: Drawings made of *Burnupia* Walker, 1912 based on the microphotographs that were taken of the limpets collected in this study. a) *Burnupia caffra* (Krauss, 1848), b) *Burnupia capensis* (Walker, 1912), c) *Burnupia mooiensis* (Walker, 1912), d) *Burnupia stenochorias* (Melvill & Ponsonby, 1903) (not collected in the study), e) *Burnupia transvaalensis* (Craven, 1881).

The utilisation of distinct primers and the undertaking of two distinct COI phylogenetic analyses were required because of the incompatibility of certain sequences available on GenBank with the LCO, HCO, COR, ASMIT 1, and ASMIT 2 primers. The divergence in phylogenetic tree construction was an approach used to incorporate all accessible sequences from GenBank, recognising the limited quantity therein, with the aim of enhancing the precision of the analytical outcomes.

2.4.2 Conservation

De Kock and Wolmarans (2017) stated that molluscs have suffered the most documented extinctions of any taxon. In South Africa, the conservation of freshwater limpets is important because, as already mentioned, they are very sensitive to anthropogenic pressures and global warming. In the coming century, the impact of climate change is expected to escalate substantially, emphasising how urgent it is to address these issues (Sands *et al.*, 2022). There has been a noticeable decrease in both species diversity and gastropod numbers attributed to climatic warming (De Kock & Wolmarans, 2017). The understanding of the conservation status of freshwater molluscs in South Africa is deficient (De Kock & Wolmarans, 2017).

According to the IUCN Red List category, most of the species in the Burnupiidae family have conservation statuses that are data deficient. This is because there is insufficient data available to accurately estimate the species' risk of extinction based on its distribution and current population status (IUCNN, 2022). All species in this genus have taxonomic inconsistencies, and as specimens in this genus are so small (2–4 mm), they frequently go undetected in sampling surveys, suggesting that the actual distribution may be significantly larger (Van Damme & Lange, 2016; ICUN, 2022).

Burnupia caffra's conservation status is of least concern (Van Damme & Lange, 2016). This species has a wide distribution range, and currently, there are no risks to this species. Only two species, *Burnupia crassistriata* and *Burnupia stuhlmanni*, have near threatened conservation statuses (Lange, 2010; Van Damme & Lange, 2017). Threats to these species, including their heightened susceptibility to pollution, the effects of global warming, and the risk of habitat loss (De Kock & Wolmarans, 2017) means that these species have the potential to face rapid extinction (Van Damme & Lange, 2017). There is no information regarding conservation available for *Burnupia ingae* and *Burnupia verreauxi*.

Given that members of this genus are susceptible to desiccation and are predominantly located in streams and rivers, the increase in global warming poses a potential threat to their survival (De Kock & Wolmarans, 2017).

2.5. Conclusion

Overall, 25 *Burnupia* samples were sequenced throughout the course of this study. The main aims of this study were to use COI data to revise the taxonomy of *Burnupia* sp. in South Africa, to fill the knowledge gaps and answer some questions that are there regarding freshwater limpets' taxonomy. With the 25 sequences obtained only four species could be identified with molecular and morphological analyses. These four species were *Burnupia caffra*, *B. capensis*, *B. mooiensis*, and *B. transvaalensis*. Our sampling regime did not particularly target the type localities of the *Burnupia* species specifically. Morphological identification of limpets is a difficult task, and the characteristics are limited. Shell morphometry coupled with molecular analysis and morphological descriptions may result in more accurate identification of freshwater limpets. This chapter did reveal that there is limited information available on freshwater limpets in terms of taxonomy and morphology. Therefore, this study contributed to the knowledge gaps, but there are still a lot of work to be done before this species can be fully understood. More studies will lead to a refined taxonomy and a better understanding of the diversity of limpets. It is hoped that the outcome and the results of this study will update the gaps in literature regarding freshwater limpets and encourage more research in freshwater environments.

Chapter 3: Host-parasite interactions within *Burnupia* spp. from South Africa.

3.1 Introduction

Molluscs and parasitic helminths interact in a variety of ways (Outa & Avenant-Oldewage, 2023). Helminths resemble worm-like organisms and are clinically classified based on which organ in the host they reside in as well as overall external morphology (Castro, 2011). They are divided into Platyhelminthes (or flatworms as it has a flattened body shape) and nematodes (which are roundworms, with nemato meaning “thread”. They encompass both bisexual and hermaphroditic species, with definitive classification relying on internal and exterior morphological features of the eggs, larval and adult stages. Subdivisions within these groups are based on the specific host organ they inhabit, such as intestinal roundworms, lung flukes and extraintestinal tapeworms (Castro, 2011).

Nematodes have an elongated, slender, cylindrical body shape (Scholz *et al.*, 2018) while platyhelminths, in comparison, have a flat body type. Members of Nematoda have a cuticle on the surface of their body (Scholz *et al.*, 2018), and in some cases, the cuticle can have ridges that are longitudinal and known as alae (Castro, 2011). They consist of a mouth and an anus, and it has lips with sensory papillae around the mouth. They usually have a body cavity and an oesophagus and they possess a fully developed digestive system (Castro, 2011; Scholz *et al.*, 2018). The females are generally longer than the males.

Trematodes can occur in the digestive tract as well as in other organs, and all trematodes are endoparasites (Scholz *et al.*, 2018). Characteristics that distinguish trematodes and platyhelminths from other parasites include a distinct anterior end, bilateral symmetry, and a dorsoventrally flattened body. Trematodes have an oral and ventral sucker present (Castro, 2011; Scholz *et al.*, 2018) and their outer body layer is covered with a tegument and, in some cases, spines can be present (Scholz *et al.*, 2018). The digestive system is fully developed. These parasites require an intermediate host in their life cycle and, generally, it is a gastropod (Castro, 2011; Scholz *et al.*, 2018).

3.1.1 Nematoda

Nematodes can interact with their molluscan hosts in four different ways: a) some parasite juveniles grow inside their hosts; b) adult parasites are free-living or are parasites of vertebrate organisms; c) a molluscan host is necessary for the whole life cycle; and d) a molluscan

organism is necessary for nematodes to complete their life cycle, finally killing the host (Zimmerman *et al.*, 2011a). Nematodes of molluscs are less researched than some of the more commonly associated relationships, such as trematodes (see section 3.1.2; Morley, 2010).

Molluscs are the primary intermediate hosts for 61 species of Rhabditida with vertebrates acting as the final hosts. On the other hand, molluscs serve as the primary hosts for 47 species of Rhabditida where their entire life cycle is concluded within the mollusc (Grewal *et al.*, 2003).

In-depth research on nematodes that use molluscs as hosts have typically focused on livestock- and human-infecting species (Outa & Avenant-Oldewage, 2023). However, research on nematodes that parasitise aquatic molluscs is lacking when compared to that of their terrestrial counterparts (Outa & Avenant-Oldewage, 2023). According to global studies (Grewal *et al.*, 2003; Zimmermann *et al.*, 2011a, 2011b), a minimum of 11 rhabditid species finish their life cycles in freshwater gastropods. Depending on the individual, nematode infections can cause minor diseases or result in the host's death (Anderson and Bartlett, 1993; Grewal *et al.*, 2003).

Species of the family Daubayliidae are known to cause death in their hosts (Chitwood & Chitwood, 1934). The infected adult female daubayliids abandon their dying hosts to infest new ones after swiftly reproducing in them and inflicting fatal illness (Poinar & Richards, 1979; Anderson and Bartlett, 1993; Zimmermann *et al.*, 2011a; Zimmermann *et al.*, 2013; Outa & Avenant-Oldewage, 2023). *Daubaylia* Chitwood & Chitwood, 1934 is the only genus in Daubayliidae and contains slow-moving species that are thin parasites of freshwater leeches and snails (Poinar, 2016). All the species currently recognised in *Daubaylia*, along with their host and country where they are found, are indicated in Table 3.1.

Table 3.1: Table of the known *Daubaylia* species Chitwood & Chitwood, 1934, the hosts they parasitise, where they are found and the corresponding references.

Species	Host	Country	Reference
<i>Daubaylia burnupiae</i> Outa & Avenant-Oldewage, 2023	<i>Burnupia stenochorias</i> (Melvill & Ponsonby, 1903)	South Africa	Outa & Avenant-Oldewage (2023)
<i>Daubaylia dewiti</i> Schuurmans-Stekhoven, 1956	<i>Bahyomphalus contortus</i> (Linnaeus, 1758)	Holland	Schuurmans-Stekhoven (1956)
<i>Daubaylia elegans</i> Honer & Jansen, 1961	<i>Planorbis carinatus</i> Müller, 1774	Netherlands	Honer & Jansen (1961)

<i>Daubaylia helicophilus</i> Poinar & Richards, 1979	<i>Gyraulus spirullus</i> (Gould, 1859)	Taiwan	Poinar & Richards, (1979)
<i>Daubaylia malayanum</i> Sullivan & Palmieri, 1978	<i>Gyraulus convexiusculu</i> (Hutton, 1849)	Malaysia	Poinar & Richards, (1979)
<i>Daubaylia pearsoni</i> Anderson & Bartlett, 1993	<i>Glyptophysa gibbose</i> (Gould, 1847)	Australia	Anderson & Bartlett (1993)
<i>Daubaylia potomaca</i> Chitwood & Chitwood,1934	- <i>Planorbella trivolvis</i> (Say, 1817) - <i>Biomphalaria glabrata</i> (Say, 1818) - <i>Helisoma anceps</i> (Menke, 1830) - <i>Bulinus globosus</i> (Morelet, 1886) - <i>Radix natalensis</i> (Krauss, 1848)	Virginia USA, Nigeria, Kenya.	- Chernin <i>et al.</i> (1962) - Outa & Avenant- Oldewage (2023)
<i>Daubaylia seistanensis</i> Baylis & Daubney, 1922	- <i>Gyraulus</i> <i>convexiusculus</i> (Hutton, 1849)	India	Baylis & Daubney (1922)

Daubaylia species are of notable interest due to Chernin's (1962) observation that *Daubaylia potomaca* Chitwood & Chitwood,1934 exclusively parasitises pulmonate snails such as *Biomphalaria glabrata* (Say, 1818). Chernin *et al.* (1962) tested *D. potomaca*'s infections and observed that this species infects planorbids (ram's horn snail) including *Helisoma* Swainson, 1840, *Planorbarius* Duméril, 1805 *Australorbis* Pilsbry, 1934 and *Bulinus* Müller, 1781.

According to Zimmermann *et al.* (2011a), *D. potomaca* have a very uncommon life cycle. The infective phase is only present in the adult female nematode, and this is the only phase that is shed from the limpet or host (Zimmermann *et al.*, 2011a). The completion of the life cycle happens in approximately ten days (Chernin, 1962). This species can detect if the limpet is about to die and then the nematode will exit their host. Another reason for the nematode to leave their host is when the nematode reaches maturity and if there is minimum resources and space available within the host (Zimmerman *et al.*, 2013; Outa & Avenant-Oldewage, 2023). *Daubaylia potomaca* usually decreases the lifetime of the host (Poinar & Richards, 1979; Zimmermann *et al.* 2011a; Outa & Avenant-Oldewage, 2023). Zimmermann *et al.* (2011b) stated that because there are no parasites in the intestinal tract of the hosts the transmission of *D. potomaca* might take place via direct perforation of the snail foot.

The life cycle of *D. malayanum* Sullivan & Palmieri, 1978 is similar to the life cycle of *D. potomaca* (see Chernin, 1962; Sullivan & Palmieri, 1978). The adult female is also the nematode that parasitises the limpet. The time that is needed for the nematode to complete their life cycle is approximately 11 to 27 days (Sullivan & Palmieri, 1978). *Daubaylia helicophilus* Poinar & Richards, 1979- also decreases the lifespan of the host and Poinar and Richards (1979) stated that adult females are transmitted through ingestion. The female parasites proceed through the intestine and go into the haemocoel, and this is where reproduction takes place. It is still uncertain whether daubayliids are transmitted passively or actively (Outa & Avenant-Oldewage, 2023).

The producing and the development of eggs and larvae can be different in the *Daubaylia* spp. (Outa & Avenant-Oldewage, 2023). Some of the species only have one egg that is not developed, such as *D. potomaca* and *D. seistanensis* Baylis & Daubney, 1922. The egg development only occurs when the egg is deposited into the host tissue (Baylis & Daubney, 1922; Chernin *et al.*, 1960; Outa & Avenant-Oldewage, 2023). For *D. potomaca*, the time that is necessary for the egg to develop before the egg hatches is approximately five days (Chernin, 1962; Outa & Avenant-Oldewage, 2023). The uterus of species such as *D. pearsoni* and *D. malayanum* have eggs that are well developed, and these eggs contain first- stage larvae (Outa & Avenant-Oldewage, 2023). *Daubaylia malayanum*'s eggs hatch in about twelve hours, after exiting the uterus (Sullivan & Palmieri, 1978). Eggs were infrequently seen in the uterus in other species, like *D. elegans* Honer & Jansen, 1961 and *D. olsoni* Poinar, 1984, and this potentially indicates fast oviposition (Poinar, 1984).

Due to the fact that larvated eggs are present in the uterus of *Daubaylia burnupiae* Outa & Avenant-Oldewage, 2023, this species reproductive behaviour might be similar to the reproductive behaviour of *D. pearsoni* and *D. malayanum* (Outa & Avenant-Oldewage, 2023).

3.1.2 Trematoda

Molluscs and digenean trematodes are believed to have survived a lengthy evolutionary history. Some researchers, such as Llewellyn (1965), stated that before vertebrate organisms appeared, adult trematodes first infected molluscs. Whether true or false, both communities likely have a lengthy history of interaction (Esch & Fernandez, 1994). Ewald (1980) stated that the parasite may develop a strategy to control the host by changing the host's behaviour to improve the chance that the parasite will be successfully transmitted to the next or final host. These changes will increase the susceptibility of the host to predation, for example, changes in the host's colour (Esch & Fernandez, 1994).

Numerous interactions between trematodes and molluscs can cause the host to become sterile. The rediae may occasionally consume the gonad directly, leading to sterility. In other cases, sterilisation occurs indirectly and is probably related to hormonal changes brought on by parasites (Esch & Fernandez, 1994). Based on the characteristics of their life cycles and the specific hosts they inhabit, digenean trematodes in marine and freshwater environments can be divided into two groups. One category consists of autogenic organisms, which live their entire lives inside the boundaries of a freshwater or marine ecosystem, with both intermediate and final hosts living in that environment permanently. A second category consists of allogenic organisms, which complete their life cycles in transient or ephemeral hosts that inhabit the ecosystem (Esch & Fernandez, 1994).

It is vital to know how a digenean trematode's life cycle work to comprehend the dynamics of parasite communities in molluscan hosts. The life cycle (see Chapter 1, Section 1.5.1, Figure 1.2) starts with the production of eggs by the mature adults, the eggs are excreted and ingested by a suitable host. The eggs can hatch in the water and free-swimming miracidia will be released (Esch & Fernandez, 1994). These miracidia will find and infiltrate the intermediate host (Esch & Fernandez, 1994). When the parasite is in the host, the larvae usually move to the hepatopancreas, the gonads or other organs and then the miracidium changes into a sporocyst. These sporocysts are the first intramolluscan generation (Scholz *et al.*, 2018). The sporocyst reproduces and produces redia or daughter sporocysts—known as the second intramolluscan generation (Scholz *et al.*, 2018). When the redia and sporocysts reproduce they give rise to the larvae which are known as the cercaria. The cercaria arise in sporocysts or redia through metagenesis which is the alternation of asexual and sexual generation (Scholz *et al.*, 2018; Faltýnková *et al.*, 2016). Typically, cercariae emerges and seek out the second intermediate host, utilising various species of snails, frogs, tadpoles and fish. Once inside the second intermediate host, the cercariae undergo encystment, and transform into metacercariae (Toledo & Esteban, 2016). The infection of the final host occurs through ingestion of the second intermediate host that contains the encysted metacercariae. After ingestion, the metacercariae undergo excystation in the duodenum and the juvenile parasites move to the small intestine. There they use their ventral sucker to attach to the mucosa and undergo maturation to reach the adult stage (Toledo & Esteban, 2016).

Human infections can occur when a person consumes raw or undercooked freshwater fish, amphibians, crustaceans, and molluscs (Toledo & Esteban, 2016). These infections are more common in regions where traditional cultural practices promotes the consumption of these animals. In the case of schistosomes, when they encounter a human's skin they adhere to it, shed their tails, and then penetrate, thereby fulfilling their life cycle (Deep *et al.*, 2023).

Numerous zoonotic diseases with significant public health implications are caused by parasites that utilise snails as intermediate hosts. Freshwater snails play a crucial role as transmission vectors and intermediate hosts for parasites, which results in serious diseases including clonorchiasis, fascioliasis, opisthorchiasis and schistosomiasis (Shamsi *et al.*, 2023). The infection of freshwater snails with these parasites not only pose a threat to public health but also present a threat to food security and aquaculture. Given the potential that snail populations must thrive in aquaculture ponds, there is an increased likelihood of parasitic infection among stocked fish (Shamsi *et al.*, 2023).

Echinostomiasis is a zoonotic parasitic disease transmitted through the consumption of contaminated food, and it is caused by digenean trematodes. Echinostome adults are hermaphroditic digeneans, residing in the bile ducts and intestine of a wide range of vertebrate hosts. They are particularly prevalent in aquatic or semi-aquatic birds and mammals which include humans (Toledo & Esteban, 2016). The majority of these species parasitize poultry, as well as other bird species and various mammals excluding humans, at least 20 species have the potential to cause disease in humans (Saijuntha *et al.*, 2011)

The family Echinostomatidae comprises over 60 species that have a widespread distribution (Saijuntha *et al.*, 2011). Adult members of Echinostomatidae exhibit a distinctive characteristic in the form of a head collar adorned with collar spines encircling the oral sucker (Toledo & Esteban, 2016). The spines of the cephalic collar can be presented either in one or two circles. There is a big variation in the size of echinostomes, which can range from 5mm to over 10mm in length. The tegument, covering both the ventral and dorsal surfaces. The oral and ventral sucker are closely situated to each other (Toledo & Esteban, 2016).

Trematodes in South Africa exhibit a higher frequency of development in freshwater snails with shell lengths ranging from 15 to 20 mm, however, even species with shells measuring from 4 to 6 mm in diameter can act as sporadic carriers of parasites. Since trematodes were found for the first time in several species of *Burnupia* and *Ferrissia* Walker, 1903, it can not be assumed that molluscs which have large shell sizes are the only ones that trematodes will parasitise (Cawston, 1924).

Hypoderaeum Dietz, 1909 consists of species with underdeveloped head collars that can be distinguished by their body proportions and sucker ratio (Esteban *et al.*, 2000). Members in this genus are frequently encountered widespread parasites occurring all over the world (Esteban *et al.*, 2000; Azizi *et al.*, 2015). This genus is present in the posterior region of the small intestine and the cercaria can utilise the same host of another host as an intermediate host (Azizi *et al.*, 2015). The parasite infects the final host when it consumes the infected

mollusc. This species has been observed and reported from around the world (Azizi *et al.*, 2015).

Cawston (1924; 1925) detected cercariae in *Burnupia trapezoidea*. The tails of the cercariae were divided and he mentioned that these monostomes (indicates a flatworm with a single oral sucker) are not yet described. These samples were found in the Free State, at Schutte's Draai. In *Burnupia capensis natalensis* almost identical monostomes were found in Natal (Cawston, 1924). Cawston (1924) mentioned that rediae were present in two different *Burnupia* species found in Natal. *Burnupia caffra*, *B. capensis natalensis* and *B. stenochorias* are known species from this area (Cawston, 1924). Cawston (1923) also lists all the different species of *Burnupia* that was infected with different stages of trematode larvae and the locations where these specimens were found in South Africa.

The current study aimed to investigate the presence and distribution of parasites in freshwater *Burnupia* limpets from the Mooi and Vaal River. This was completed through morphological analysis and genetic characterization of the limpet infecting taxa.

3.2. Materials and methods

3.2.1 Collection of limpets

Limpets were collected from the Mooi River in North-West (-26.6863, 27.1023) and the Vaal River in the Free State (-26,9057, 27,4364). The sampling was completed on the 20/04/2023 and 03/05/2023. A total of 42 limpet specimens were collected from the Mooi River and 35 from the Vaal River. Sample sites are shown in the map (Figure 3.1) below. The limpets were found attached to rocks, stones, and vegetation in shallow, fast-flowing water and collected using a hand-picking method. Specimens were placed into plastic buckets filled with water from the river. The samples were kept alive, and oxygen was added to the buckets.

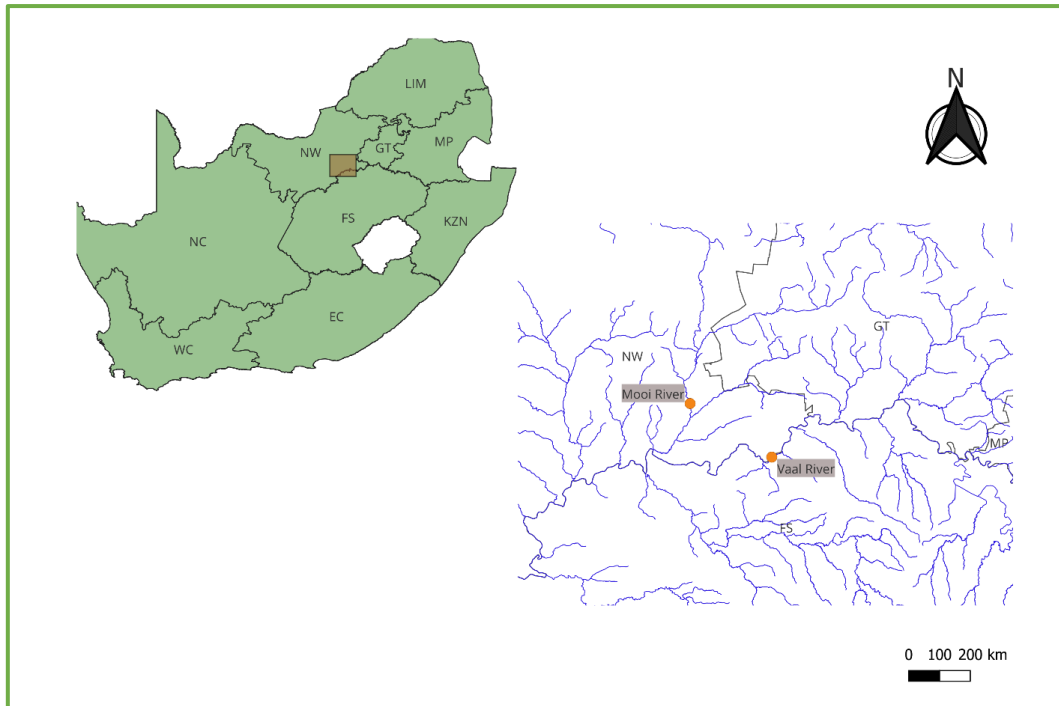


Figure 3.1: Map with locations or rivers which were sampled in 2023 in the Free State and North West.

3.2.2 Collection of parasites

To extract the parasites from the limpets, each limpet was placed in a small plastic cup, approximately 25 ml in size, filled with water obtained from the collection site. The cups were then placed under a light source to induce shedding of parasites (Born-Torrijos *et al.*, 2014). This procedure was conducted with the aim of triggering a stress response in the host molluscan species, artificially prompting the shedding of cercariae. After the third day under the light source (see Figure 3.2), each limpet was screened with a dissection microscope (Zeiss, AxioCam ERc 5s) for parasites. A total of two screenings was completed two days apart. A second screening was conducted to ensure that the pre-patent infections that may have matured in the meantime were detected. After the second screening, the limpets were crushed by placing the limpet into the bottom part of a clean glass petri dish, the top part of the petri dish was positioned over the limpet and then pressure was applied by using the top part of the petri dish, crushing the limpet against the bottom part. Afterwards, the soft anatomy of the limpet was evaluated under a dissection microscope for any parasite movement (Figure 3.3). Any parasites present were removed from the host with fine needles. The nematodes that were obtained were placed into boiled tap water placed into labelled tubes in molecular-grade ethanol (Scholz *et al.*, 2018) and stored in a freezer for further analysis.

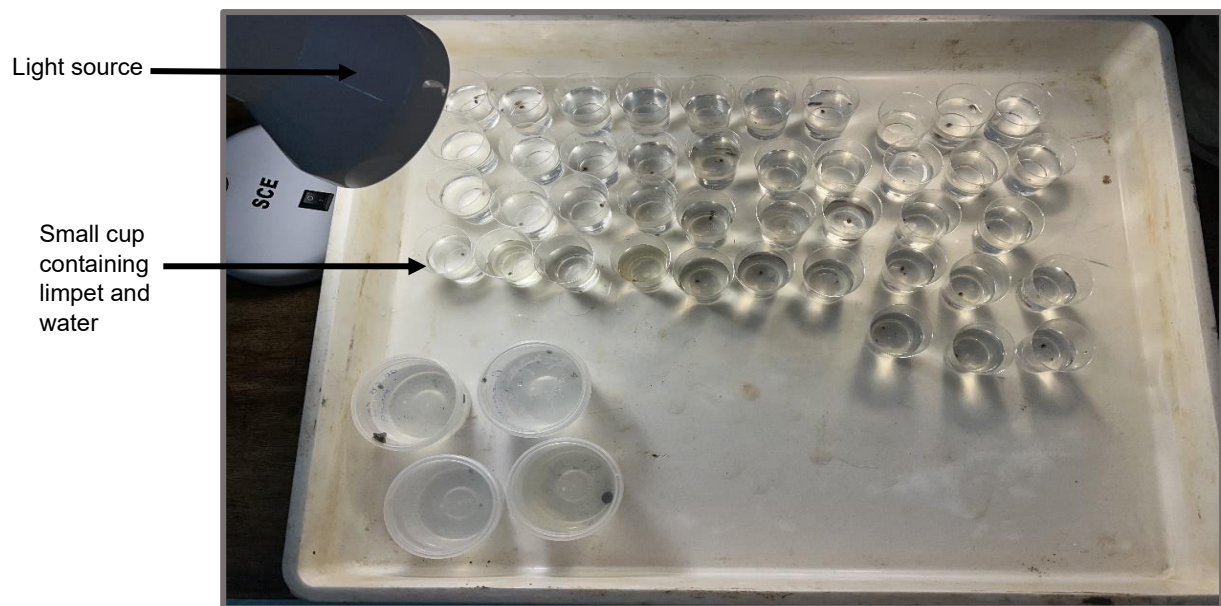


Figure 3.2: Photo indicating the shedding experiments that were used for the limpets collected.

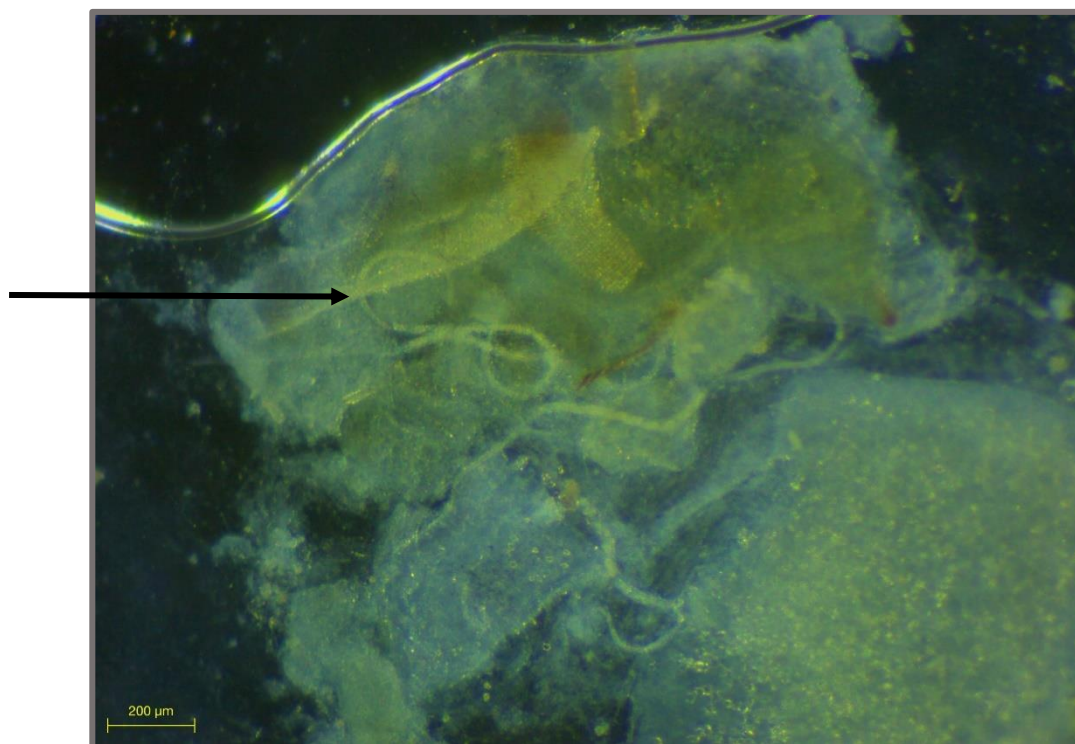


Figure 3.3: Soft tissue of limpet after crushing experiment was done. Nematodes can be observed in the soft tissue, indicated by the black arrow.

3.2.3 Morphological analysis

In the NWU laboratory, the nematodes were identified to family level using the information provided on morphological characteristics via photos, drawings and published descriptions (such as Outa & Avenant-Oldewage, 2023). Individuals were selected from each site for morphological analysis and molecular analysis. For morphology, one individual was placed in

lactophenol to make the specimen more transparent and make it easier to see certain morphological characteristics. Three individuals were placed in 70% ethanol to preserve the specimen (Scholz *et al.*, 2018). Photomicrographs were taken with a Zeiss, Axiocam ERc 5s, and with a Nikon Eclipse light microscope. The microphotographs were used as a digital voucher and for further morphological analysis.

For scanning electron microscopy, two parasite specimens (one parasite from each site) were used. The specimens underwent dehydration through a graded series of ethanol concentrations (80%, 90%, 95% and twice at 100%) where the specimens were left for 10 minutes in each concentration. This was followed by a graded series of hexamethyldisilazane (Merck) at concentrations of 30%, 70% and twice at 100%, for 5 minutes each to remove the ethanol. Afterwards, the dried specimens were mounted and placed in a SPI-MODULE sputter coater (SPI supplies) to be coated with gold (Outa & Avenant-Oldewage, 2023). After coating, the specimens were examined under a scanning electron microscope (Desktop Phenom Pro) at 10 kV.

3.2.4 Infestation rates:

The infection rates were shown as mean abundance and prevalence. The mean abundance is the mean number of parasites per host specimen (Scholz *et al.*, 2018). This refers to the average of parasites counted in the host, divided by the total host specimens (both uninfected and infected hosts). This calculation provides a comprehensive measure by considering the presence or absence of parasites in each host specimen (Scholz *et al.*, 2018). The prevalence represents the percentage or fraction of hosts that show the evidence of infection among the overall host population sampled (Scholz *et al.*, 2018).

3.2.5 Molecular analysis

For molecular analysis of the nematodes, three parasites in total were used. A small piece of the posterior extremity of the parasite was carefully cut out using a dissection scalpel and placed in molecular-grade ethanol (Scholz *et al.*, 2018) in a separate tube and used for molecular analysis (Vermaak, 2021). This was completed carefully to ensure that no damage was done to any other important structures that can affect the morphological analysis of the parasite. The anterior and posterior body parts were fixed in separate tubes for additional morphological examination if needed.

The mitochondrial DNA was extracted using the PCR BIO Rapid Extraction PCR Kit (PCR biosystems; Analytical Solutions Primers were based on existing literature (Olson *et al.*, 2003; Svitin *et al.*, 2019; Pearson, 2022; Outa & Avenant-Oldewage, 2023). The primers that were used to amplify a fragment of the 28S gene were LSU5 (5' –TAG GTC GAC CCG CTG AAY

TTA AGC A – 3') and 1500R (5' – GCT ATC CTG AGG GAA ACT TCG – 3') as reported from Olson *et al.*, (2003) and Outa & Avenant-Oldewage (2023). The polymerase chain reaction (PCR) conditions that were used by Olson *et al.* (2003) were modified by Outa & Avenant-Oldewage (2023). The modified PCR conditions that were used are provided in Figure 3.4. The PCR amplification was completed in volumes of 25 µl, which contained 8 µl deionised water, 1.25 µl each of forward and reverse primers, 2 µl DNA, and 12.5 µl master mix (KAPA HotStart & Dream Taq).

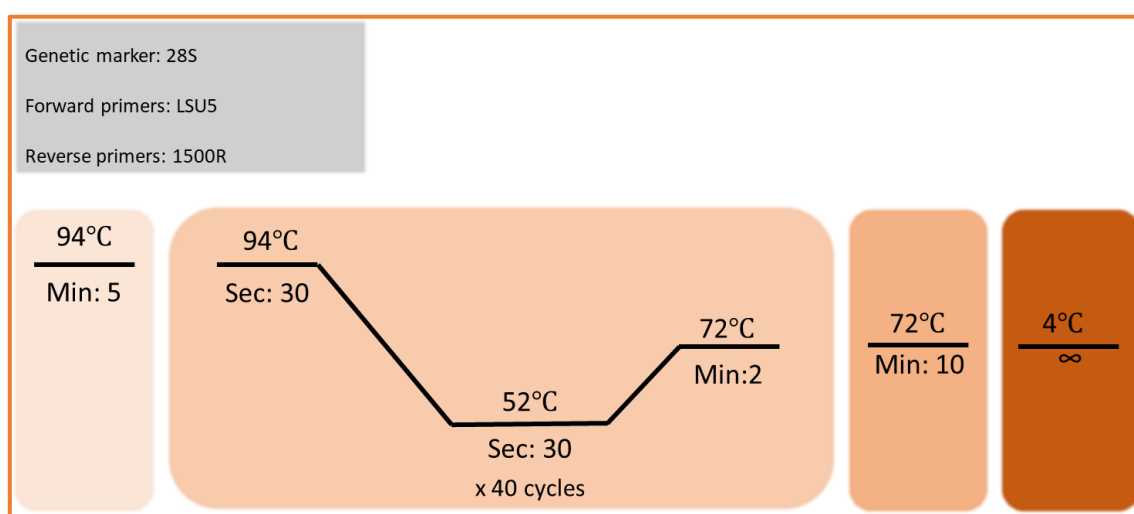


Figure 3.4: Polymerase chain reaction (PCR) conditions for primers LSU5 and 1500R.

For molecular analysis of the limpet- hosts (for identification purposes), the soft tissue of the limpet was extracted from the shell and placed in molecular-grade ethanol. DNA was extracted with the PCR BIO Rapid Extraction PCR Kit. The genetic marker that was targeted was COI. The primers used were ASMIT 1 (5'-TTT TTG GGC ATC CTG AGG TTT AT-3') and ASMIT 2 (5' -TAA AGA AAG AAC ATA ATG AAA ATG-3') based on previous literature (Jørgensen *et al.*, 2004). The PCR amplifications were completed in volumes of 25 µl, which included 8 µl deionised water, 1.25 µl of each primer, 2 µl DNA, and 12.5 µl mastermix (KAPA HotStart & Dream Taq). The PCR conditions followed Jørgensen *et al.* (2004) and can be seen in Figure 3.5.

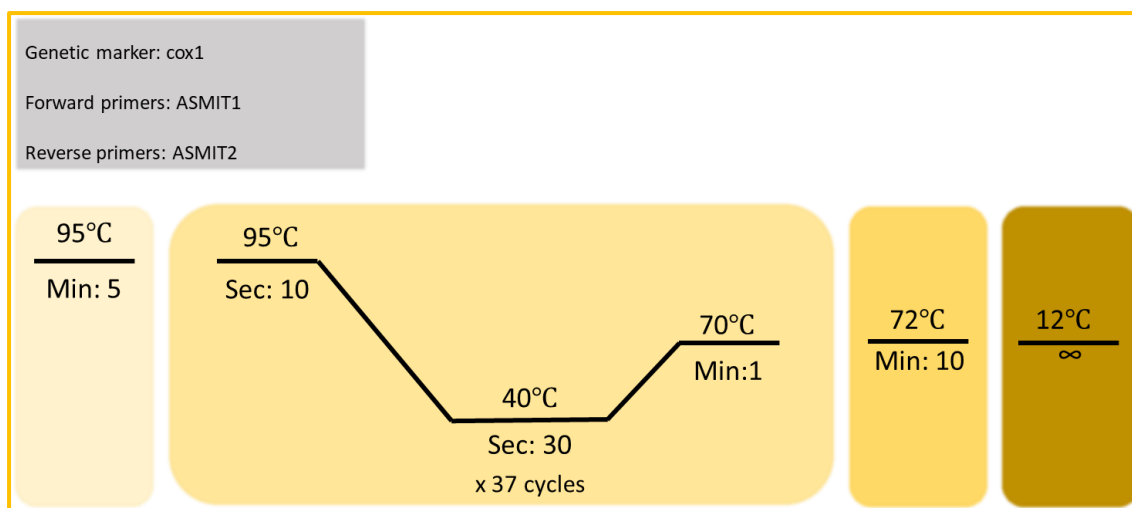


Figure 3.5: Polymerase chain reaction (PCR) conditions for primer ASMIT 1 and ASMIT 2.

For both the parasites and the molluscs, a 1% agarose gel electrophoresis, run for 30 min at 90V, was used to determine the success of the DNA amplification. Afterwards, the amplicons were examined under an ultraviolet light. Inqaba Biotechnical Industries Pty. Ltd. in Pretoria, South Africa received the successful amplicons, and then purified and sequenced these samples. Geneious Prime bioinformatics software v 2.1 was used to trim, align, and assemble the sequences. The sequences obtained in this study were compared to other sequences present on GenBank. For this, the Basic Local Alignment Search Tool (BLAST) was used.

The optimal nucleotide models for the alignment were determined using jModelTest2.1 based on the Akaike information criterion. Phylogenetic analysis involved both Bayesian inference (BI) and maximum likelihood (ML) analyses. The ML analysis employed PhyML v. 3.0 with 1000 repetitions (accessible at <http://www.atgc-montpellier.fr/phyml/>), while BI was conducted using MrBayes v3.2.6 software through the CIPRES science Gateway v.3.3. (accessible at <https://www.phylo.org/>). The resulting tree was visualized using FigTree v. 1.4.3, and MEGA v. 7 was utilized for calculating p-distance. Differences in the numbers of base pairs were generated using Geneious Prime bioinformatics software v 2.1. The sequences utilised in the phylogenetic analysis for *Daubaylia* Chitwood & Chitwood, 1934 are detailed in Table 3.2 and for Trematodes in Table 3.3.

Table 3.2: Sequences from GenBank, species name, accession number, sequences length and corresponding reference are available for *Daubaylia* Chitwood & Chitwood, 1934. Sequences obtained during the study are in bold.

Species name	Accession number (GenBank) or sample code	Sequence length	Reference
<i>Daubaylia</i> sp.	VRB2301	1137	Present study
<i>Daubaylia burnuipae</i>	OQ269618	1041	Outa & Avenant-Oldewage (2023)
<i>Daubaylia potomaca</i>	KU180680	3087	Holovachov <i>et al.</i> (2015)
<i>Daubaylia</i> sp.	KY319365	1103	GenBank (unpublished)

Table 3.3: Sequences used from GenBank for phylogenetic analysis. Species name, accession number, sequences length and corresponding reference are available. New sequences obtained in this study are in bold, and the out group are shaded in green.

Species name	Accession number (GenBank) or sample code	Sequence length	Reference
<i>Trematode</i> sp.	MR1A2a	421	Present study
<i>Echinoparyphium aconiatum</i>	NC_067751	14865	Gacad <i>et al.</i> (2023)
<i>Echinostoma cinetorchis</i>	MT577587	432	Izrailskaia <i>et al.</i> (2021)
<i>Echinostoma mekongi</i>	MZ822066	444	Chomchoei <i>et al.</i> (2022)
<i>Echinostoma revolutum</i>	NC_046395	15714	Ran <i>et al.</i> (2020)
<i>Fasciola gigantica</i>	MN913873	1533	Chougar <i>et al.</i> (2020)
<i>Fasciola hepatica</i>	OQ392481	448	GenBank (unpublished)
<i>Hypoderaeum conoideum</i>	MT159512	1041	Tantrawatpan & Saijunth (2020)
<i>Hypoderaeum conoideum</i>	MT159523	1041	Tantrawatpan & Saijunth, (2020)
<i>Patagifer</i> sp. 1	OQ606512.1	337	GenBank (unpublished)
<i>Notocotylus atlanticus</i>_OG	OL629687	445	Gonchar & Galaktionov (2022)

3.3. Results

3.3.1 Morphological analysis

The parasitic nematodes obtained from the study have different characteristics that were used to identify it. On the latero-dorsal portion of the cephalic end, amphids are subterminal (Figure. 3.6A). A distinctive corpus joins with a small isthmus. The excretory pore and nerve ring (Figure. 3.6B) are close to the anterior extremity. The uterus includes six eggs, comprising larvated eggs (Figure 3.6C). There is a short post-vulvar sac in the uterus (Figure 3.6C) and the spiral and cone shaped shortened tail is shown in Figure 3.6D (Outa & Avenant-Oldewage, 2023). In Figure 3.7, the nerve ring, corpus, isthmus, excretory pore, glandular basal bulb, and anus can be observed.

The parasite species obtained from the current study conforms to the description given for *Daubaylia* by Chitwood and Chitwood (1934) and Outa and Avenant-Oldewage (2023). This identification was based on the following morphological characteristics: the presence of ant elongated oesophagus, presence of a distinct corpus, a nerve ring surrounding the isthmus, dorsolateral amphids, a reduced stoma, and a single ovary (Chitwood & Chitwood, 1934; Outa & Avenant-Oldewage, 2023). All the nematodes that were collected during this study were females. These females are usually longer than the males.

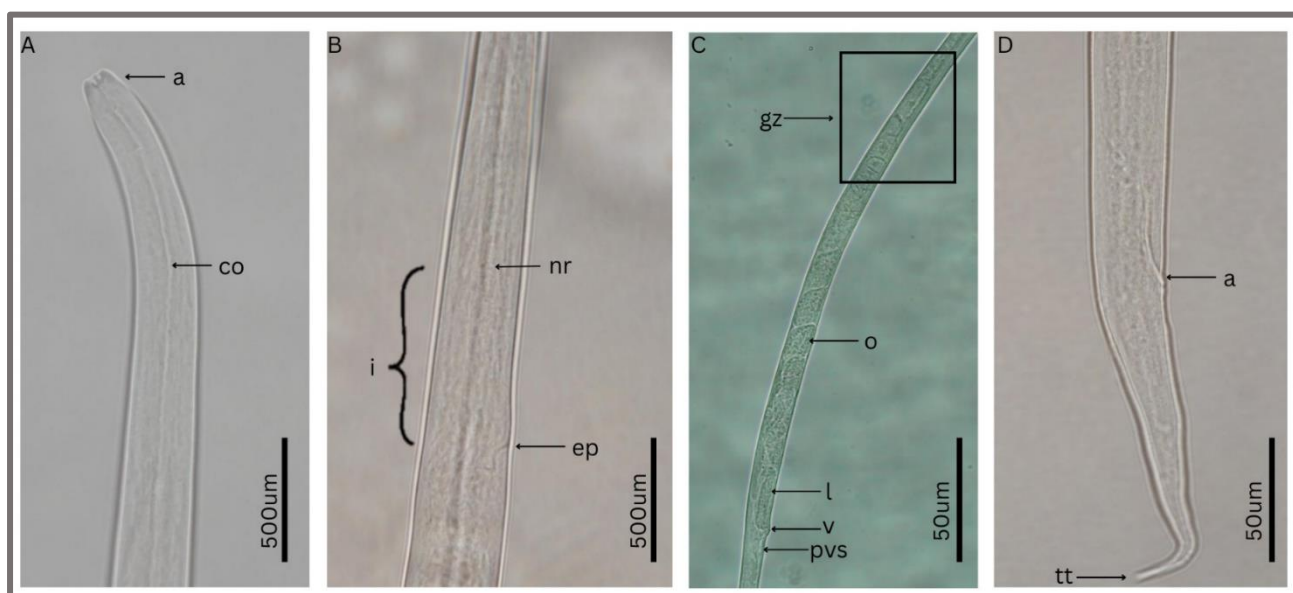


Figure 3.6: Light microscope photos of the *Daubaylia* Chitwood & Chitwood, 1934 found. A – anterior end, B – mid region, C – mid region, D-posterior end. Abbreviations: amphids (a), anus (a), corpus (co); excretory pore (ep), germinal zone (gz), isthmus (i); larva (l), nerve ring (nr), oocyte (o), post – vaivular sac (pvs), tt – tail tip, vulva (v).

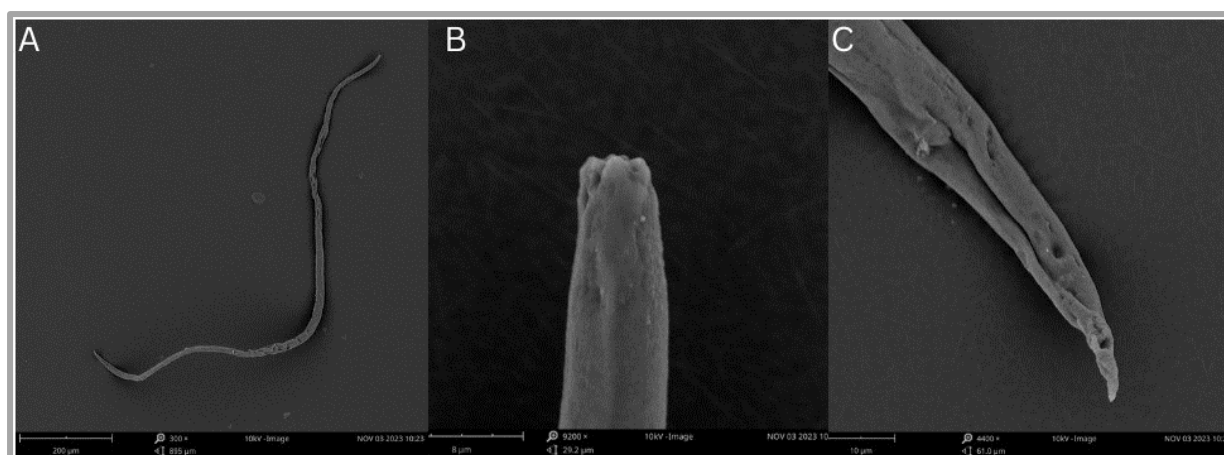


Figure 3.7: Scanning electron microscope photos of *Daubaylia* Chitwood & Chitwood, 1934 found in the present study. A – whole specimen, B – anterior part, C – posterior part.

3.3.2 Infestation rates

The nematodes infection intensity averaged four nematodes per sample site and these nematodes were found in the body cavities and in the internal tissue of the limpets. There were four nematodes collected from the Mooi River samples and four from the Vaal River. In total, eight nematodes were collected in this study. The overall infection of nematodes was very low (see Table 3.4). The overall infection rates of the two sites together were 10.3%.

Table 3.4: Number of limpets collected, number of parasite infection, sample locations, intensity of abundance and prevalence. The results from Outa & Avenant-Oldewage (2023) were also included in this study.

Sample sites	Number of limpets	Number of parasites collected	Intensity of abundance	Prevalence	Reference
Mooi River	42	4	0.09	9.5%	Present study
Vaal River	35	4	0.11	11.4%	Present study
Vaal Dam	442	Not stated	6.65	52.3%	Outa & Avenant-Oldewage (2023)
Vaal Barrage	304	Not stated	3.11	26.6%	Outa & Avenant-Oldewage (2023)

3.3.3 Molecular analysis

3.3.3.1 Nematoda

One sequence was obtained from this study with a length of 1137 base pairs. The sequence obtained in this study matched 99.6% with an existing sequence on GenBank, *Daubaylia burnupiae* (accession number: OQ269618) and only differed by seven base pairs (see Table 3.5). Therefore, it is confirmed that the species collected in this study conforms to *Daubaylia burnupiae*. The sequence obtained in this study differed by 4.5% from *Daubaylia potomaca* (accession number: KU1180680) and had 48 base pair differences (Table 3.5).

Table 3.5: Genetic divergence of aligned 28S sequences used in the *Daubaylia* Chitwood & Chitwood, 1934 alignment, expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). The species obtained in this study are in bold and blue shade

		1	2	3	4
1	VRB2301		48	61	7
2	KU180680 <i>Daubaylia potomaca</i> ,	95.648		10	43
3	KY319365 <i>Daubaylia</i> sp.	94.649	99.098		41
4	OQ269618 <i>Daubaylia burnupiae</i>	99.664	95.858	96.103	

3.3.3.2 Trematoda

During the shedding method that was used in this study, no shedding of trematodes from limpets occurred. However, a trematode sequence was obtained while doing molecular analysis for the COI gene of limpets. The sequence length obtained was 421 base pairs.

BLAST was used to compare this sequence to existing sequences on GenBank. In Table 3.6 the available sequences of different trematodes, the base pair differences and percentages similarities are shown. These sequences were used to show the relation between the trematode sequences obtained in this study and other available trematode sequences.

Table 3.6: Genetic divergence of aligned COI sequences alignment expressed as percentage identity (below diagonal) and differences in the number of nucleotides (above diagonal). The species obtained in this study are in bold and in blue shade. OL629687 *Notocotylus atlanticus* was used as the outgroup (green shading).

		1	2	3	4	5	6	7	8	9	10	11
1	MR1A2a		52	49	76	73	80	81	61	84	82	113
2	MT159512 <i>Hypoderaeum conoideum</i>	87.5		3	102	74	79	114	57	109	80	112
3	MT159523 <i>Hypoderaeum conoideum</i>	88.2	99.5		100	73	79	113	57	108	79	111
4	NC_067751 <i>Echinoparyphium aconiatum</i>	81.6	82.9	83.2		61	69	110	52	100	66	97
5	MT577587 <i>Echinostoma cinetorchis</i>	81.7	82.9	83.1	85.9		43	51	41	67	64	91
6	MZ822066 <i>Echinostoma mekongi</i>	80.6	82.2	82.2	84.5	90.0		61	47	62	60	93
7	NC_046395 <i>Echinostoma revolutum</i>	80.5	80.9	81.1	81.6	88.2	86.3		45	105	70	98
8	OQ606512.1 <i>Patagifer sp. 1</i>	82.0	83.1	83.1	84.6	87.7	86.1	86.6		52	51	73
9	MN913873 <i>Fasciola gigantica</i> ,	79.9	81.8	82.0	83.3	84.6	86.1	82.5	84.7		27	89
10	OQ392481 <i>Fasciola hepatica</i>	80.3	82.1	82.4	85.3	85.3	86.6	84.4	85.0	94.0		91
11	OL629687 <i>Notocotylus atlanticus</i>	72.5	74.9	75.2	78.3	79.1	79.2	78.1	78.5	80.2	79.8	

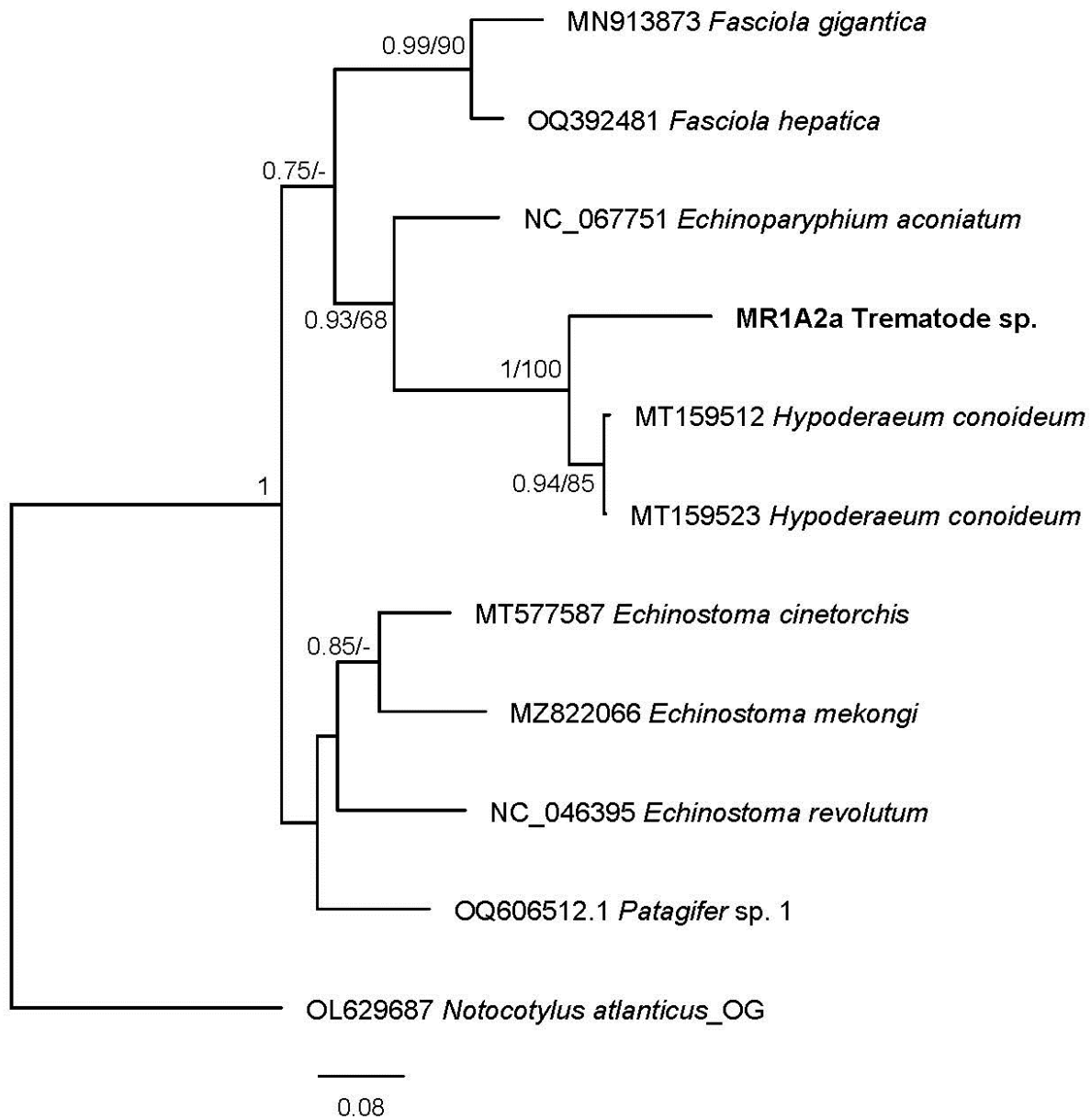


Figure 3.8: Phylogenetic tree based on the partial COI sequences for available sequences in the family. Nodal support is given as BI/ML (Bayesian Inference /Maximum Likelihood). Support values lower than 0.70 (BI) and 70 (ML) are not displayed. Sequences obtained from this study are in bold. The outgroup was *Notocotylus atlanticus* Stunkard, 1966.

Between the ML and BI trees constructed in PhyML, MrBayes, and CIPRES, there are seven supported nodes between the associations of 11 trematode specimens (Figure 3.8). The one sequence obtained from this study formed a sister clade with *Hypoderaeum conoideum*. Between these 3 sequences there was a 49 and 52 base pair difference, and they are 88% similar (Table

3.6). Due to this fact it can be stated that the trematode species found in this study is not *Hypoderaeum conoideum* but could potentially be part of the *Hypoderaeum* genus.

3.4. Discussion

3.4.1 Taxonomy

The nematodes obtained in this study conform to the description of *Daubaylia burnupiae* described by Outa and Avenant-Oldewage (2023). The relatively short isthmus in the oesophagus is a distinguishing trait of this species. The portion after the corpus of the oesophagus of other species of this genus is either longer or almost the same length compared to the corpus. The species collected in this study differ morphologically from other known species in a number of unique characteristics. *Daubaylia pearsoni* usually have more than one egg present in the uterus, and they are normally larvated (Anderson & Bartlett, 1993) and this is absent in the current species. With *D.olsoni*, both males and females have tails that are longer (Pionar, 1984) than the current species collected. Furthermore, *D.olsoni* usually do not have eggs in the uterus (Pionar, 1984), but the specimens in this study do.

Daubaylia elegans is similar in length to the existing species, according to Honer & Jansen (1961). However, both genders of *D. elegans* have a longer tail. Additionally, the uterus of *D. elegans* is roughly half as big as that of the current species and does not contain eggs that are embryonated, and the female of *D. elegans* is slimmer with the vulva located more closely to the anterior part of the nematode (Honer & Jansen, 1961). *Daubaylia bonaerensis* is easily distinctive from the current species since it is at minimum three times bigger than the existing species (Camino & Gonzalez, 2011). The present species matches *D. malayanum* due to the quantity and position of the larvated eggs in the uterus. Although *D. malayanum* is smaller than *D. burnupiae*, both sexes of the *D. burnupiae* have shorter tails and a shorter oesophagus (Sullivan & Palmieri, 1978). Female and male *D. potomaca* are almost the same size, in contrast to *D. burnupiae* whose females are bigger than males (Chitwood & Chitwood, 1934; Chernin *et al.*, 1960). Adult females of *D. potomaca* have a single egg developed in the uterus and pregnant females of *D. burnupiae* could carry a maximum of twelve eggs, many of which had developed larvae (Chitwood & Chitwood, 1934). In comparison to *D. seistanensis*, the adult females only consist of one egg (Baylis & Daubney, 1922), while *D. burnupiae* can contain a maximum of twelve eggs. The vulva of *D. burnupiae* is at the posterior part of the nematode, while *D. dewiti* has its vulva almost evenly spaced between the posterior and anterior parts of the body (Outa & Avenant-Oldewage, 2023).

This is the same case if the current species is compared to *D. helicophilus* where the vulva of *D. helicophilus* is positioned closer to the anterior part of the nematode (Poinar & Richards, 1979).

Daubaylia species are distinguished from one another mainly based on differences in their overall length and physical characteristics that can be seen under a light microscope (Outa & Avenant-Oldewage, 2023). However, it is very difficult to identify and distinguish species based only on body length and size as there are species whose lengths overlap with one another. For example, *D. burnupiae* is hard to differentiate from most of the species from this genus if this is only based on the length of the body. Thus, other characteristics are needed to distinguish between species and *D. burnupiae*, including eggs in the uterus and the development of the larvae. The most distinguishing characteristic for *D. burnupiae* is the presence of a short isthmus with an oesophagus (Outa & Avenant-Oldewage, 2023).

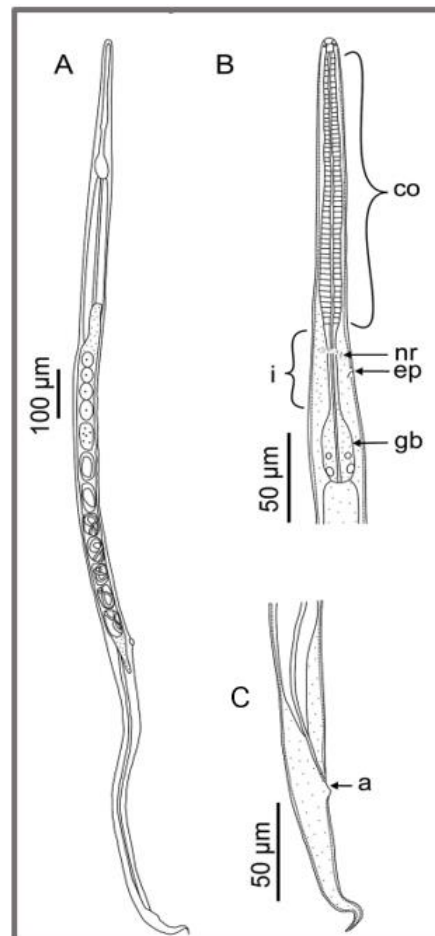


Figure 3.9: Line drawings of *Daubaylia burnupiae* from Outa & Avenant-Oldewage, 2023. A, female; B, anterior end female; C, posterior end female. Abbreviations: a – anus, co – corpus, ep – excretory pore, gb – glandular basal bulb, i – isthmus, nr – nerve ring.

3.4.2 Molecular analysis

On GenBank there are only three daubayliid sequences present. There is one sequence where the 18S gene were targeted and two sequences for 28S (Table 3.5). The sequences that were obtained in the current studies, matched with one of the existing sequences *Daubaylia burnupiae* and there was a significant difference between this species and the other species of daubayliid present. Thus, it can be stated that the DNA, and the morphological analysis, supports the idea that the current species is *Daubaylia burnupiae* and is unique from the other *Daubaylia* species. Due to the lack of sequences, a phylogenetic tree could not be constructed. A comprehensive examination on the evolutionary history of *Daubaylia* has not yet been done. Since, out of 10 *Daubaylia* sp. only four species have been sequenced. This underscores the imperative for a more thorough investigation into this group in the future.

During the molecular analysis, it was determined that one of the snails had a trematode present. This were discovered when the sequences from a *Burnupia* spp. were obtained, and the Basic Local Alignment Search Tool showed a match of 88% with a trematode species, *Hypoderaeum conoideum* Dietz 1909. The percentage is too low to assume that the species obtained in this study and *Hypoderaeum conoideum* are the same species. However, it was the highest percentage given by GenBank when BLAST was used. Based on phylogenetic tree, the current trematode species grouped with the *Hypoderaeum conoideum* and is most closely related to this genus.

For phylogenetic analysis (see Figure 3.8), the representatives were chosen as they were the only available COI sequences on GenBank for this family. *Fasicola* was also included as it was the next closely related species according to BLAST. Based on the tree, the obtained sequence is most closely related to *Hypoderaeum* and *Echinoparyphium* (Figure 3.8). Between the species in the genus of *Echinostoma* the species were 86% - 90% similar and they differed 43 – 61 base pairs (Table 3.6). Between the sequence obtained in this study and *Hypoderaeum conoideum*, it was 87% – 88% similar, with between 49 and 52 base pair differences (Table 3.6). Based on the differences within one genus and the differences between the obtained sequence and *Hypoderaeum conoideum*, it can be assumed that the sequences obtained in the study are part of the genus *Hypoderaeum*.

This tree does not show the family as monophyletic, but more sequences and different genetic markers are needed to determine if this is in fact the case. Also, the three species of *Echinostoma* group together but not as close as one would expect within in the same genus.

The trematode obtained in this study cannot be identified further because a notable level of genetic variation can be observed in *H. conoideum* populations across diverse geographical locations (Tantrawatpan & Saijuntha, 2020). Temporal factors such as seasonal and climatic changes might have played a role in influencing the genetic variation and sub-structuring of *H. conoideum* populations. Tantrawatpan and Saijuntha (2020) mentioned that previous evidence of genetic differentiation in *Echinostoma revolutum* Looss, 1899 populations linked to temporal factors, has also been reported.

3.4.3 Infection rate

In the results of this current study, it can be observed that the infection of parasites in the *Burnupia* spp. were low. If this infection rate is compared to Outa and Avenant-Oldewage (2023), it can be determined that this is a very low infection rate as Outa and Avenant-Oldewage (2023) indicated by a 41.6% infection rate, with the highest number of nematodes that were discovered in a limpet being 72. Both the diversity and abundance of parasites can fluctuate over time and across different locations (Scholz *et al.*, 2018). Biotic variables associated with hosts also display temporal and spatial variations; for example, seasonal alterations in water temperature can lead to changes in the host's immunity, subsequently influencing the level of parasite infection (Scholz *et al.*, 2018).

This was the case for the article written by Outa & Avenant-Oldewage (2023), these results indicate that the pollution of a watercourse or river can lead to the decrease of nematodes in *Burnupia* spp. This article results suggests that the environment in which the snail lived may have an impact on the spread of the infection. The pollution may weaken the hosts' immunological defences, allowing the parasite to infect them more frequently (Outa & Avenant-Oldewage, 2023). Thus, it may seem that the nematodes are more susceptible to pollution than their hosts are.

It is expected that the exposure of pollution to the snail may lead to a potential decrease in the survival of the adult parasite. This could be attributed to the fact that daubayliids undergo a brief free-living stage during the transmission of adult nematodes between different hosts, making them susceptible to adverse effects from environmental pollution (Sullivan & Palmieri, 1978; Outa & Avenant-Oldewage, 2023). Thus, nematodes can possibly be a bioindicator or aquatic contamination (Outa & Avenant-Oldewage, 2023).

An additional confounding factor to consider is the host body size, as parasite diversity (and abundance) tends to rise with an increase in host body size, driven by allometric relationships. Larger hosts provide a more extensive and stable habitat for parasite colonisation (Scholz *et al.*,

2018). Therefore, when comparing parasite communities of a specific host species across various sites, it is essential to choose hosts with similar body sizes to mitigate the potential impact of host size on the observed differences (Scholz *et al.*, 2018).

The samples obtained from the Vaal River are slightly bigger than the ones from the Mooi River (Chapter 2; section 2.3.1.8, Tables 2.5 and 2.6), however, it was concluded that there were no significant differences in the morphometrics of the different sites. As mentioned in section 3.3.2 (Figure 3.4), Outa & Avenant-Oldewage, (2023) found 72 nematodes in a *Burnupia* spp. With this it can be stated that the shell sizes of *Burnupia* sp. may not be a good validation for a high or low infection rate.

Another possible reason for the very low trematode prevalence obtained from this study is that the *Burnupia* spp. collected in the study from Outa and Avenant-Oldewage (2023) is ten times higher than the number of *Burnupia* sp. obtained in this current study.

3.4.4 Interactions between nematodes and trematodes

Chaetogaster limnaei limnaei has been extensively studied in connection to a wide variety of trematode parasites. This symbiont, *C. l. limnaei*, has the ability to completely avoid parasite infections and to decrease the emergence of trematode parasites (Fernandez *et al.*, 1991, Rodgers *et al.*, 2005).

In a study conducted by Zimmerman *et al.* (2011), the interactions between *Daubaylia potomaca* (nematodes), *Chaetogaster limnaei limnaei* (annelid) and *Helisoma anceps* (trematode) were conducted. The results stated that when the annelid is present the intensity of *D. potomaca* increases because *C. l. limnaei* negatively impacts the *Echinostoma* sp. The annelid restricts the infection of trematode parasites because they ingest the miracidia and cercariae. When this happens, the miracidia are not able to penetrate the host, subsequently rediae development cannot occur. If the rediae of *Echinostoma* sp. are present, this causes a reduction in *D. potomaca*'s intensity, since the rediae ingest the adult and juvenile nematode. *Chaetogaster limnaei limnaei* can prevent *Echinostoma* sp. from infecting the host. Due to this, *D. potomaca* can reproduce and the intensity of infection can increase since, the predation by the rediae and the competition is reduced (Zimmerman *et al.*, 2011).

3.4.4 Zoonotic trematode species

The trematode identified in this study probably belongs to the genus *Hypoderaeum*, which is of significant public health and veterinary importance. This genus includes parasites such as

Hypoderaeum conoideum and *Stellantchasmus inermis*. *Hypoderaeum conoideum* is recognised as a neglected trematode capable of infecting humans. It is among the 23 zoonotic species which represents eight genera within the family Echinostomatidae, with a distribution predominantly in Southeast Asia and Europe (Saijuntha *et al.*, 2016, Shamsi *et al.*, 2023). Transmission of these trematodes occurs through the consumption of undercooked or raw fish, molluscs and amphibians, leading to symptoms in humans such as abdominal pain, diarrhoea, weight loss and fatigue (Chai, 2019; Shamsi *et al.*, 2023).

Hypoderaeum conoideum is frequently observed in the small intestine of various bird species, including geese, ducks, swans, wild aquatic birds, and chickens across many regions worldwide. The life cycle of this trematode involves different species of freshwater snails acting as the first intermediate host, while bivalves, tadpoles and fish play a role as the second intermediate host (Azizi *et al.*, 2015). The completion of its life cycle involves transmission between these hosts, contributing to the distribution and prevalence of *Hypoderaeum conoideum* in avian populations.

Migratory birds play a crucial role in parasite transmission serving not only as final hosts but also as key carriers due to their migration across various geographic territories and areas. An example of this can be seen in an investigation conducted in Mazandaran and Gilan provinces in Iran, where eight species of wild ducks were studied. In the Guilan Province, the practice of breeding ducks and geese is widespread, and free-grazing ducks and geese are commonly moved among various rice paddy fields across different regions within the province (Eslahi *et al.*, 2023). The study found several helminths in *Anas crecca*, including *Apatemon gracilis*, *H. conoideum*, *Cotylurus cornutus*, *Echinostoma revolutum*, *Amidostomum anseris* and *Hymenolepis collaris*, (Youssefi *et al.*, 2014). *Anas crecca*, are widespread and usually occur in North Canada and Alaska. When it is winter these birds migrate to Africa (Atkinson *et al.*, 2006). Parasites infecting migratory birds are of significant importance because these birds may transport parasites over long distances. Additionally, some parasites have the capability to infect suitable hosts across extensive geographic areas, highlighting the potential impact of migratory bird populations on the spread of parasitic infections (Youssefi *et al.*, 2014).

3.5. Conclusion and future recommendations

In this study, there were two sites sampled and 77 *Burnupia* spp. were collected. All the *Burnupia* spp. were used for the shedding methods to induce the shedding of parasites. Overall, two parasite species were collected. Three of the parasites were used for molecular studies and the rest were studied with different microscopes to identify the parasites. All the nematodes that were

collected were identified as *D. burnupiae* and the morphological and molecular analysis supports this identification. One trematode was found with molecular analysis and a sequence for this species was obtained during this study. This trematode was identified to be a part of the genus *Hypoderaeum*. This was established with the assistance of a phylogenetic tree (BI and ML), a base pair difference and percentage identical table. *Hypoderaeum* is part of the Echinostomatidae and this family can cause Echinostomiasis which are a zoonotic parasitic disease. These trematodes can be transmitted over a wide geographical area because migratory birds can serve as carriers for these parasites. The aim of this study was to evaluate if *Burnupia* spp. are an intermediate host to parasites.

Chapter 4: Discussion and Future Recommendations

4. 1 Summative discussion and conclusion

Approximately 400 species of freshwater snails, including limpets, are present in Africa. Freshwater limpets are small organisms, with a cap-like shell and an apex. These limpets can usually be found in fast-flowing water of rivers attached to stones, rocks, and vegetation. Identification of limpets is difficult because their morphological characteristics are limited, and the characteristics can be affected and altered by environmental factors such as the habitat they inhabit. The existing literature on limpet taxonomy is sparse and only a few major publications are available (Walker, 1912; Connolly, 1939; Oberholzer & van Eeden, 1969; Raphalo *et al.*, 2021; Sands *et al.*, 2022). This reinforces the fact that information on freshwater limpets is limited and more studies on their taxonomy are needed.

Prior to the commencement of this study, there were only four available sequences that offered assistance in the identification of freshwater limpets. Morphological identification of limpets poses a challenge due to its limitations and difficulties due to the restricted number of characteristics that can be used for identification, which can be influenced by various environmental factors.

4.1.2 *Burnupia* taxonomy

The primary aim of this research was to enhance the taxonomy of freshwater limpets within South Africa with a particular focus on *Burnupia* spp. To achieve this goal, the study involved the collection of limpets from ten different sites. A total of 26 limpets were gathered, and a combination of morphological and genetic analyses were employed to aid in the identification of these limpet species. The study identified these species as *Burnupia caffra*, *B. capensis*, *B. mooiensis*, *B. transvaalensis*.

Species were identified based on the locations they were collected from, on morphological descriptions and on molecular analysis. The phylogenetic analysis indicated that the species found in the Sabie River, Mac Mac River and the Suid-Kaap River are *B. caffra*. The genetic variation of these samples and the species showed a similarity of 96–99% with a base pair difference of 1–9. Based on the morphological descriptions and the molecular results it can be confirmed that these sampled are *B. caffra*. The samples from the Mooi River grouped together

with the Crocodile and Magalies River. The genetic variations between these samples indicate that they are 99% similar. The morphological descriptions of these limpets are similar to the description given to *B. mooiensis* and linked with the distribution and type locality of *B. mooiensis*. It can be stated that the limpets collected from these sites are *B. mooiensis*. The samples from the Elands River, Vaal River and Klein Olifants River grouped together and are 98–99% similar and only have 1 base pair difference. Based on the sample locality and the distribution of *B. transvaalensis* and the morphological descriptions, these samples are *B. transvaalensis*. *Burnupia transvaalensis*'s type locality is in the Mooi River. One sample from the Vaal River was identified as *B. capensis*, this identification was made based on the morphology of the two shells and from the reason that *B. capensis* is very widely distributed.

This study highlighted the existing limitations in the available information concerning freshwater limpets, particularly in the realms of morphology and taxonomy. Furthermore, this research provided contributions, including photomicrographs and morphometrical data for the collected 29 limpets as well as new sequences for *B. caffra* (5), *B. capensis* (1), *B. mooiensis* (14) and *B. transvaalensis* (9). While this study added to knowledge gaps in the existing literature on these limpets, further research is necessary to gain a comprehensive understanding of these molluscs. Species were identified based on the locations they were collected from, on morphological descriptions and on molecular analysis. The phylogenetic analysis indicated that the species found in the Sabie River, Mac Mac River and the Suid-Kaap River are *B. caffra*. The genetic variation of this samples and the species showed a similarity of 96-99% with a base pair difference of 1–9.

4.1.3 Host-parasite interactions

This paper also delved into the role of limpets as intermediate hosts for different parasites. The aim of this study was to investigate the presence and distribution of parasites associated with freshwater limpets. The research revealed that freshwater molluscs have been relatively understudied when compared to their terrestrial counterparts. The study involved the collection of limpets from two different sites. Shedding was induced with a light source because phototactically active trematodes respond to the light source and therefore induces the emission of the cercariae. These methods resulted in the collection of eight parasites. All eight parasites collected within the tissue of the snails after dissection, were identified as nematodes, while a further parasite, a trematode species, was identified using molecular analyses. The nematodes were identified through a combination of molecular and morphological studies. Interestingly, a prior study had recently discovered the same nematode species in *Burnupia stenochorias* (Outa & Avenant-

Oldewage, 2023). They identified the nematode species as *Daubaylia burnupiae*. With the aid of morphological analysis and molecular analysis, the nematode species collected throughout this study conformed to the description of *Daubaylia burnupiae*. The identification of the limpet in which the parasite was found might be a misidentification. Since, *Burnupia stenochorias* only have documented occurrences in the Eastern Cape. In Outa & Avenant-Oldewage (2023) they collected *Burnupia* species from the Vaal River in this current study none of the Vaal River sequences grouped or matched with the existing *B. stenochorias* sequences on GenBank and none of the samples conformed to the morphological description given of this species.

This research adds valuable information since the parasites were found in a different *Burnupia* species from the species mentioned in the article from Outa & Avenant-Oldewage (2023). In addition, this parasite was found in a different location (Mooi River), Outa & Avenant-Oldewage (2023) sampled in the Vaal River.

In conclusion, this study made a significant contribution by enhancing our understanding of the molecular and morphological aspects of freshwater limpets. Additionally, it provided valuable insights into the role that freshwater limpets play as intermediate hosts for both nematodes and trematodes. This study also touched on the significance of zoonotic parasites and the migratory birds that spread this disease over a large geographical scale.

The identification of the species that were researched and the provision of molecular data in the current study are expected to enhance the accurate identification of these species in future research. Additionally, this study underscores the ongoing necessity for in-depth taxonomic and biodiversity investigations. Understanding the diversity of a given region is crucial for inclusive species incorporation into conservation efforts and future planning. Moreover, it's possible that some yet undiscovered species might hold biological, ecological, or economic significance within these ecosystems. Thus, having a comprehensive understanding of the organisms at play is advantageous. In the context of our ongoing global biodiversity loss, research endeavours like this one have become increasingly vital for assessing the overall spectrum of species that inhabit our world before they potentially disappear.

4.2. Future recommendations

Based on the results and findings of the current research there were gaps in knowledge apparent, underscoring the need for further research. To address these gaps and expand our understanding, future investigations could explore various aspects related to the studied topic. These investigations can include:

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- It is advisable to prioritise sampling from type localities in future limpet studies, especially of the *Burnupia* spp. that have not been collected much since the original description. This approach can yield valuable morphological data regarding the characteristics used for the identification of species.
 - It is suggested that environmental factors and the habitat the limpets inhabit can affect the shell morphology. There is a need for research to investigate whether this factor can induce shell alterations.
 - There are limpet samples available at the NWU snail database, these limpets can be used to compare the morphometrics between the information given in this study and the available samples. This is needed to obtain more information regarding the morphometrics and if there is a difference between species or the sample sites, they were collected from.
 - Explore the other limpet species such as *Pettancylus*, especially in terms of distribution, molecular, morphological analysis and conservation.
 - The data obtained from this study can be utilised to update the conservation status of *Burnupia* species since many of these species are classified as data deficient.
 - Explore the ecological dynamics of freshwater limpets, investigating the environmental factors that influence their distribution, abundance, and diversity. This could involve studying water quality, habitat preferences, and ecological interactions.
 - Investigate the specific host-parasite relationships between freshwater limpets, nematodes, and trematodes. This includes identifying the parasite species, understanding their life cycles, and determining the factors influencing parasitic infections.
 - Assess the potential effects of climate change on the distribution and ecology of freshwater limpets considering how changing environmental conditions may influence their populations and associated parasites.
 - In the context of the nematodes, it is highly beneficial to conduct more comprehensive research on the taxonomy of species within the *Daubaylia* genus. Much of the existing research in this field is outdated.
 - There's a need for further exploration to understand the transmission mechanisms of these parasites between hosts. It is also essential to examine whether pollutants can influence the reproductive behaviour of parasites and whether parasites have a measurable impact on the survival rate of their hosts.
 - It is valuable to consider molecular identification for upcoming cases of human echinostomiasis. This approach not only enhances our comprehension of the life cycle between host and parasite but also holds the potential to furnish crucial insights on

disrupting its maturation. This, in turn, could be instrumental in preventing future infestations among both intermediate and human hosts.

- To deepen the knowledge of the population structure and genetics of *H. conoideum*, it is recommended to collect samples from diverse intermediate and final hosts throughout different seasons in a particular locality. Conducting genetic analyses on these collected samples would significantly contribute to a more thorough comprehension of the genetic dynamics within *H. conoideum* populations.
- Additional helminthological investigations are essential to gather more information on the diversity of helminths in aquatic birds in Iran, especially across different seasons of the year. These investigations aim to delineate the taxonomic structure and seasonal diversity of parasites, providing insights into their potential role in the transmission of parasitic infections among aquatic birds.

By exploring these research avenues, forthcoming studies have the potential to provide valuable insights into the ecology, biology, and public health significance of South African freshwater molluscs, along with their associated parasites.

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