Morphological plasticity among polystomatid flatworms (Monogenea: Polystomatidae)

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THIS DISSERTATION IS DEDICATED TO

My grandmother, Gerda de Villiers, who continually supported me throughout my studies and sadly passed away in July 2016.

My parents, Kobus and Gerda Coetzer, for giving me the opportunity and support for all of my studies.

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All the glory be to my primary Helper, Jesus, through Whom I am able to do all things!

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Abstract

Polystomes (Monogenea: Polystomatidae) are parasitic platyhelminths infecting a wide variety of hosts, including anurans, freshwater chelonians, caecilians, the Australian lungfish, salamanders, and the hippopotamus. Although polystome genera are collectively distributed across all zoological realms, most individual genera are restricted to one realm or even island.

Although molecular tools are now commonly used for the systematics and classification of polystomes, traditional morphological studies and morphometrics are still important. Yet there has been surprisingly little study of the morphological plasticity they display. Since it has been suggested that a high degree of morphological plasticity may have profound effects of the current classification of polystomes, this study aimed to evaluate the degree of plasticity displayed by some polystomatid genera occurring in both amphibians and chelonians.

Several morphological features of polystomes were assessed in this study, with the primary focus being on the sclerotized hooks, since classification of these soft-bodied parasites rests mainly thereupon. The effect of different chemical fixatives on the marginal hooklets was evaluated and found to be minimal. However, it seemed as though the age and life-stage of the parasite might have some influence on the sizes of these hooks in some species.

The validity of the genus *Metapolystoma* was evaluated based on morphology and molecular tools. The molecular analyses yielded similar results to those of previous studies suggesting that the genus is a junior synonym of *Polystoma*. The definite morphological differences between the two genera may be attributed to a high degree of plasticity very dependent on the ecology of the host.

Finally, the morphology of chelonian polystomes was also studied in an attempt to partially resolve the generic paraphyly displayed by previous molecular studies. Several morphological features have proven valuable for separation of species occurring in one of the three microhabitats inhabited by these polystomes. The most important features included the respective shapes of the eggs and testis, and the number and sizes of the genital spines, hamuli, and marginal hooklets respectively.

This study conclusively suggests that polystomes display a higher degree of morphological plasticity than previously suspected. However, the full extent still needs to be discovered.

Keywords

Polystomes; Monogenea; Polystomatidae; morphology; morphologie; plasticity; plasticité; morphological plasticity; plasticité morphologique; classification; *Metapolystoma*; *Polystoma*; *Protopolystoma*; *Polystomoides*; *Uropolystomoides*; *Neopolystoma*; marginal hooklets; crochets marginaux

Résumé

Les polystomes (Monogenea: Polystomatidae) sont des plathelminthes parasites qui infectent une grande variété d'hôtes, à savoir les amphibiens, les chéloniens d'eau douce, les caeciliens, le dipneuste australien et l'hippopotame. Bien que les genres de polystomes se retrouvent dans tous les domaines zoologiques, la plupart des genres individuels sont limités à un biotope ou même à une île.

Bien que les outils moléculaires soient maintenant couramment utilisés pour la systématique et la classification des polystomes, les études morphologiques traditionnelles et morphométriques restent toujours importantes. Pourtant, on a étonnamment peu étudié la plasticité morphologique qu'ils présentent. Comme il a été suggéré qu'un degré élevé de plasticité morphologique pouvait avoir des effets profonds sur la classification actuelle des polystomes, cette étude a visé à évaluer le degré de plasticité affiché par certains genres de ces parasites à la fois chez les amphibiens et les chéloniens.

Plusieurs caractéristiques morphologiques des polystomes ont été évaluées dans cette étude, l'accent étant mis principalement sur les crochets sclérotisés, puisque la classification de ces parasites à corps mou repose principalement sur ceux-ci. L'effet de différents fixateurs chimiques sur les crochets marginaux a été évalué et jugé minime. Cependant, il semblait que l'âge et le stade de vie du parasite puissent avoir une certaine influence sur la taille de ces crochets chez certaines espèces.

La validité du genre Metapolystoma a été évaluée en fonction de la morphologie et des outils moléculaires. Les analyses moléculaires ont donné des résultats similaires à ceux d'études antérieures suggérant que le genre est un synonyme du genre Polystoma. Les différences morphologiques définies entre les deux genres peuvent être attribuées à un haut degré de plasticité très dépendante de l'écologie de l'hôte.

Enfin, la morphologie des polystomes de chéloniens d'eau douce a également été étudiée dans le but de résoudre partiellement la paraphylie présentée par des études moléculaires antérieures. Plusieurs des caractéristiques morphologiques se sont avérées précieuses pour la séparation des espèces se retrouvant dans l'un des trois microhabitats fréquentés par ces parasites. Les caractéristiques les plus importantes comprenaient les formes respectives de l'œuf et du testicule, et le nombre et les tailles des épines génitales, des hamuli et des crochets marginaux, respectivement.

En conclusion, cette étude suggère que les polystomes présentent un degré de plasticité morphologique plus élevé que ce qui était suspecté auparavant. Cependant, il reste encore beaucoup à découvrir.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

Introduction and literature review

1.1. Importance of taxonomy and systematics

"The real voyage of discovery consists not in seeking new landscapes, but in having new eyes."

— Marcel Proust

Even after 250 years of scientists documenting thousands of newly-discovered plants and animals, the rate at which new species are described remains relatively stable. It is estimated that between 15,000 and 18,000 new species or taxonomic changes are documented each year (Nosowitz 2015). And yet, after centuries of discovering millions of new species, we still have little idea as to the extent of the unexplored biodiversity still to be discovered. Not only do we discover new species, but scientists are also continually re-describing, re-evaluating and reclassifying organisms, in addition to discovering all the intricacies of each known species. This is the essence of taxonomy and systematics (Mayr 1942; Oberholzer & Viljoen 1989).

The decision on whether organisms should be classified as one or several species is based upon the determination of similarities between organisms, and the origin of these similarities. For example, two species may morphologically be similar, but this similarity may be due to similar habitats, or to a close phylogenetic relationship. It is up to the taxonomist to study these species and make an informed decision about the validity of the species (Mayr 1942; Prudhoe & Bray 1982). Therefore, taxonomists are constantly searching for new informative characters or combinations thereof to assist in the process of taxonomy. Taxonomists always seek to identify morphometric descriptors of taxonomic value and with a high classification potential. To identify these characteristics can be a daunting task, since soft-bodied parasites often display large intraspecies variation and limited interspecies variation (Anton & Duthie 1981).

1.2. Morphological vs. molecular approaches to taxonomy and systematics of monogeneans

Taxonomists often apply several methods, including comparative morphology, genetics, physiology, biometrics, zoogeography, ethology, cytology, ecology, palaeontology, and biochemistry for the classification of parasitic helminths (Mayr 1942; Prudhoe & Bray 1982). In experienced, as opposed to theoretical helminthology, taxonomists seem to favour comparative morphology, genetics, and the patterns of life-history in a population for classification. Although systematists are often in disagreement as to which method is

more important, several authors suggest that using a combined approach is the most informative and reliable (Hillis 1987; Mayr 1942; Oberholzer & Viljoen 1989; Prudhoe & Bray 1982). Here we will compare the most important arguments for morphologists and geneticists, respectively.

Since organisms are usually centred, to a varying degree, around a specific morphological pattern (Prudhoe & Bray 1982), these traits and their measurements (morphometrics) have been used solely for classification of animals since the time of Linnaeus (Oberholzer & Viljoen 1989). Despite the current molecular tools available, morphology has remained very important for initial identification of species, as well as during the description of new species (Badets *et al.* 2013; Du Preez & Maritz 2006; Justine 1998; Verneau *et al.* 2009b). Mayr (1942) claimed that the most practical diagnostic characters were often the most clearly visible structures which have low variability. This may include characters that are not necessarily of particular importance for the species or individuals, but it may serve as a marker for the taxonomist. Yet, Prudhoe and Bray (1982) advised that morphological variation should be kept in mind during the classification process. More recently, Perkins *et al.* (2009) stated that morphological analyses are important, since they allow for synapomorphies to be identified, thus leading to the development of a strong set of characters which can be used to describe taxa.

Some advantages of the use of morphological methods include (1) the availability of a vast amount of museum-archived specimens (little, if anything, is known about the genetics, behaviour, or ecology of the vast majority of these specimens); (2) the applicability to fossil species, especially attempting to find relationships with extinct species; (3) the use of ontogenetic (development of an organism/the organism's lifespan) information, since structures can change over the course of an organism's life; and (4) the cost, which may be somewhat lower than expensive experiments and molecular analyses (Hillis 1987).

However, there are also some problems with using morphological traits and morphometrics for classification. To use morphological characteristics for species identification and classification requires a very sound knowledge of the taxonomic groups, which can become somewhat of a problem once a specialist retires (Berthier *et al.* 2014). Using only morphology to describe species can also be a daunting task, especially where simple organisms like parasites are concerned, as these small organisms often harbour few reliable morphological characteristics and high interspecies variation (Poisot *et al.* 2011). The extent of morphological variation is such that Prudhoe and Bray (1982) even suggested that a number of morphologically-based described species exist that may be regarded as artificial, especially among soft-bodied organisms. This may be because the

specimens used for the description of the species might have been unnaturally flattened or distorted. This problem is regularly encountered among parasites, especially monogeneans. Therefore, scientists working on these parasites rely heavily on sclerotized skeletal structures (Du Preez & Maritz 2006).

Mayr (1942) was also of the opinion that simple morphological features often have a simple genetic basis, therefore allowing for the derivation of certain underlying genetic factors if these characters can be adequately traced. Perkins *et al.* (2009), however, warned against the use of morphology to derive phylogenetic relationships among species. They claimed that parasites tend to have simplified and conserved body plans compared to their free-living relatives and suggested that using more than six morphological traits may provide some phylogenetic insights. Inter-individual changes within a species may also demand the use of several individuals during the morphological study and description of a species. The number of specimens used should be reflective of a population (Perkins *et al.* 2009; Prudhoe & Bray 1982). It is also important to note that the host and environment may have an effect on the morphology and development of parasite species (Olstad *et al.* 2009; Prudhoe & Bray 1982).

Morphological traits used for species separation have become trivial, especially in cryptic taxa, and there is no clear distinction between species harbouring extensive intraspecific variation. Due to this, several authors have suggested that the morphological approaches alone may be inadequate for the description of taxa (Bentz, *et al.* 2001; Du Preez *et al.* 2007; Kok & Van Wyk 1986; Tinsley & Jackson 1998b). They now recommend the use of physiological, biochemical, and genetic tools (Aisien & Du Preez 2009; Prudhoe & Bray 1982), although numerical approaches (Du Preez & Kok 1993; Du Preez & Martiz 2006), and host identity in host specific species (Du Preez 1994; Du Preez *et al.* 2003; Du Preez & Kok 1997) should also be used. The latter can, however, only be used if the genera are proven to be host-specific. Among polystomatids, some authors have suggested, and implemented, a large scale verification of all polystomatid species using molecular techniques (Aisien & Du Preez 2009; Bentz, *et al.* 2001; Tinsley 1974).

Molecular techniques have some advantages to morphology. These include that (1) larger data sets can we analysed and compared during the same timeframe; (2) there is little to no phylogenetic limits, as the data contain a phylogenetic record from very recent times; and (3) molecular data is confounded less by environmental influences, therefore showing a smaller extent of non-heritable variation (Hillis 1987).

Establishing a threshold value for molecular divergence is, however, very important, as the lack thereof may lead to closely related species being classified as one. For polystomes, Bentz *et al.* (2001) suggested that 1% divergence of the ITS1 gene as the threshold under which individuals can be considered conspecific. For the amphibian genus *Polystoma*, Du Preez *et al.* (2007) suggested that a 2% threshold for COI and 0.07% uncorrected pairwise divergence for 28S is usually indicative of a well-differentiated species. The former is similar to the proposed threshold for chelonian polystomes of 1.5 - 2% suggested by Verneau *et al.* (2011).

The use of some gene sequences, such as ITS1 and COI, has been shown to support the morphological and morphometrical descriptions of polystomatid flatworms (Berthier *et al.* 2014). Therefore, more and more systematists suggest using a combination of these two methods, including SEM, to maximise phylogenetic data (Berthier *et al.* 2014; De Leon *et al.* 2016; Du Preez *et al.* 2007; Hillis 1987; Kok & Van Wyk 1986). However, they specify that the material should be collected and preserved correctly and with care. When used optimally, molecular systematics are able to address questions and problems not addressed by morphological systematics, and vice versa (Hillis 1987).

1.3. Monogenea and the Polystomatidae

1.3.1. Classification

The most recent (Héritier *et al.* 2015) classification for polystomes is as follows:

Phylum: Platyhelminthes (Gegenbaur, 1859)

Class: Monogenea (Van Beneden, 1858)

Order: Polystomatidea (Lebedev, 1988)

Family: Polystomatidae (Gamble, 1896)

Platyhelminthes, better known as flatworms, constitute a diverse phylum of aquatic and terrestrial invertebrates. They can be either parasitic Neodermata, or free-living turbellarians. Prudhoe and Bray (1982) hypothesized that parasitic platyhelminths had evolved from rhabdocoelid-like turbellarians, which had facultative commensialistic associations with several aquatic invertebrates. It is suspected that these associations later became an obligatory relationship and may be why there is a low degree of pathogenicity among polystomatid flatworms to their hosts, although little is currently known about this (Verneau *et al.* 2011).

The Neodermata are a monophyletic clade consisting of three classes, namely the Monogenea, Trematoda and Cestoda. Monogeneans are parasitic mainly on fish, but also occasionally on amphibians and chelonians, crustaceans and molluscs (Llewellyn 1957).

The Monogenea are separated into two general sub-groups namely the Polyopisthocotylea Odhner, 1912, and Monopisthocotylea Odhner, 1912, based on the morphology of the adults' attachment organs as well as their feeding habits (Olson & Tkach 2005; Sinnappah *et al.* 2001). Some authors prefer the additional terms for these two groups, namely Polyonchoinea and Oligonchoinea (Bychowsky 1937). Although the two terminologies (Monopisthocotylea and Polyopisthocotylea versus the Polyonchoinea and Oligonchoinea) do not exactly overlap (Justine 1998), it depends on the preference of the author to decide which nomenclature should be used.

There are still lengthy on-going debates on the monophyly and the validity of the Monogenea (Euzet & Combes 2003; Justine 1998; Mollaret *et al.* 1997; Olson & Tkach 2005) with most favouring the two monophyletic lineages representing the Monopisthocotylea and the Polyopisthocotylea, that have been shown to have evolved separately. The weight of available evidence, including molecular work on 18S rRNA genes, strongly favours the paraphyly of the Monogenea, but according to Olson and Tkach (2005) the '*burden of proof is on the side of supporting monophyly*'. The Polyopisthocotylea are regarded as the sister clade of the Digenea–Cestoda clade (Badets *et al.* 2013). Morphologically there is still divided evidence as well as opinions supporting monophyly and paraphyly respectively. According to morphological studies using synapomorphies from the eyes and ciliated bands on the larvae, the monogeneans are monophyletic. However, cladistic studies on sperm ultrastructure do not support synapomorphies (Mollaret *et al.* 1997).

Despite this phylogenetic uncertainty, several authors have commented on the Monogenea being an ideal group for all kinds of studies. The traits making them ideal include the available knowledge of their morphological and numerical diversity, the fact that they are generally host-specific, and their phylogeny, to family level, which is pretty well resolved (Kaci-Chaouch *et al.* 2008; Poulin 2002)

The family Polystomatidae consists of more than 200 species in 26 genera inhabiting a variety of hosts, including the Australian lungfish, anurans, caecilians, salamanders, freshwater chelonians, and even a mammal, the African hippopotamus (Badets *et al.* 2009; Badets *et al.* 2011; Badets & Verneau 2009; Héritier *et al.* 2015; Prudhoe & Bray 1982; Verneau *et al.* 2009a). This diversity makes them the most varied family among all monogeneans. Two of the genera are almost globally distributed, namely *Polystoma* (sub-

family Polystomatinae) occurring in anurans and *Polystomoides* (sub-family Polystomoidinae) occurring in freshwater turtles (Morrison & Du Preez 2001). African amphibians are host to four genera, namely *Polystoma*, *Eupolystoma*, *Protopolystoma*, and *Metapolystoma*. Other genera infecting anurans include *Madapolystoma*, *Diplorchis, Kankana*, *Mesopolystoma*, *Neodiplorchis*, *Parapolystoma*, *Parapseudopolystoma*, *Pseudodiplorchis*, *Sundapolystoma*, and *Wetapolystoma*, to name a few. Furthermore, *Polystomoides*, *Uropolystomoides, Neopolystoma,* and *Polystomoidella*, are found to infect freshwater turtles, *Concinnocotyla* infects the Australian lungfish, *Oculotrema* the hippopotamus, and *Nanopolystoma* infects caecilians.

1.3.2. General morphology

The main morphological characteristic of the family Polystomatidae is the well-developed opisthaptor with distinctly visible three pairs of cup-like suckers in all genera (Prudhoe & Bray 1982), except *Sphyranura*, which only has two (McAllister *et al.* 1991; Sinnappah *et al.* 2001).

Polystomes are flatworms in the true sense of the word, being dorso-ventrally flattened, and lanceolate, elliptical or discoid in outline. Their outer covering is a cytoplasmic, syncytical tegument (Prudhoe & Bray 1982). More often than not, the anterior part is not as broad as the posterior part, consisting of the haptor (Figure 1.1). However, some genera, for example *Protopolystoma*, differ in this regard, with the haptor being small compared to the rest of the discoid body (Prudhoe & Bray 1982).

The larvae, called oncomiracidia, are ciliated and free-swimming, at least for the first couple of hours after hatching (Tinsley & Owen 1975; Theunissen *et al.* 2014). They generally have two pairs of anterior eye-spots with lenses and eight pairs of marginal hooks on their haptor which may indicate some ancestral traits (Llewellyn 1957).

Polystomes depend on several structures for attachment. While oncomiracidia depend solely on the sixteen marginal hooklets, they no longer serve as attachment organs in adults, even though they are retained in the latter (Du Preez & Martiz 2006; Williams 1995). The adults depend to differing degrees on six large suckers and large anchors, or hamuli, also situated on the opisthaptor (Figure 1.2). The absence or presence, shape and number of hooks when present, and ratios with other structures of the hamuli are important as taxonomical characters (Tinsley & Tinsley 2016).

Figure 1.1: Example of a *Polystomoides* sp. Photo: L.H. du Preez. Labels: Mo - mouth surrounded by an oral sucker, Ph - pharynx, Gb - genital bulb, Ov - ovary and oviduct, Va - vaginae, Te - testis, Su - haptoral suckers, Ha – opisthaptor.

The mouth usually has a false oral sucker surrounding it, and is followed by a pharynx, oesophagus, and ultimately the intestinal caeca. Intestinal caeca can often extend into the opisthaptor and may be diverticulated. Diverticulae from both caecae may join to form anastomoses or even a reticulated network. The presence and number of anastomoses differ between species (Prudhoe & Bray 1982; Tinsley 1974).

Polystomes are hermaphrodites, but cross-fertilisation, rather than self-fertilisation, is the norm. Male and female reproductive systems open into a genital atrium with a common aperture, or through separate pores to the outside (Prudhoe & Bray 1982). In the female, the uterus may be absent, especially in neotenic forms like *Protopolystoma*, which display no need for egg storage (Tinsley & Jackson 1998b). Vitelline follicles are well-developed and may be dispersed throughout the body, depending on the genus (Enabulele *et al.* 2012).

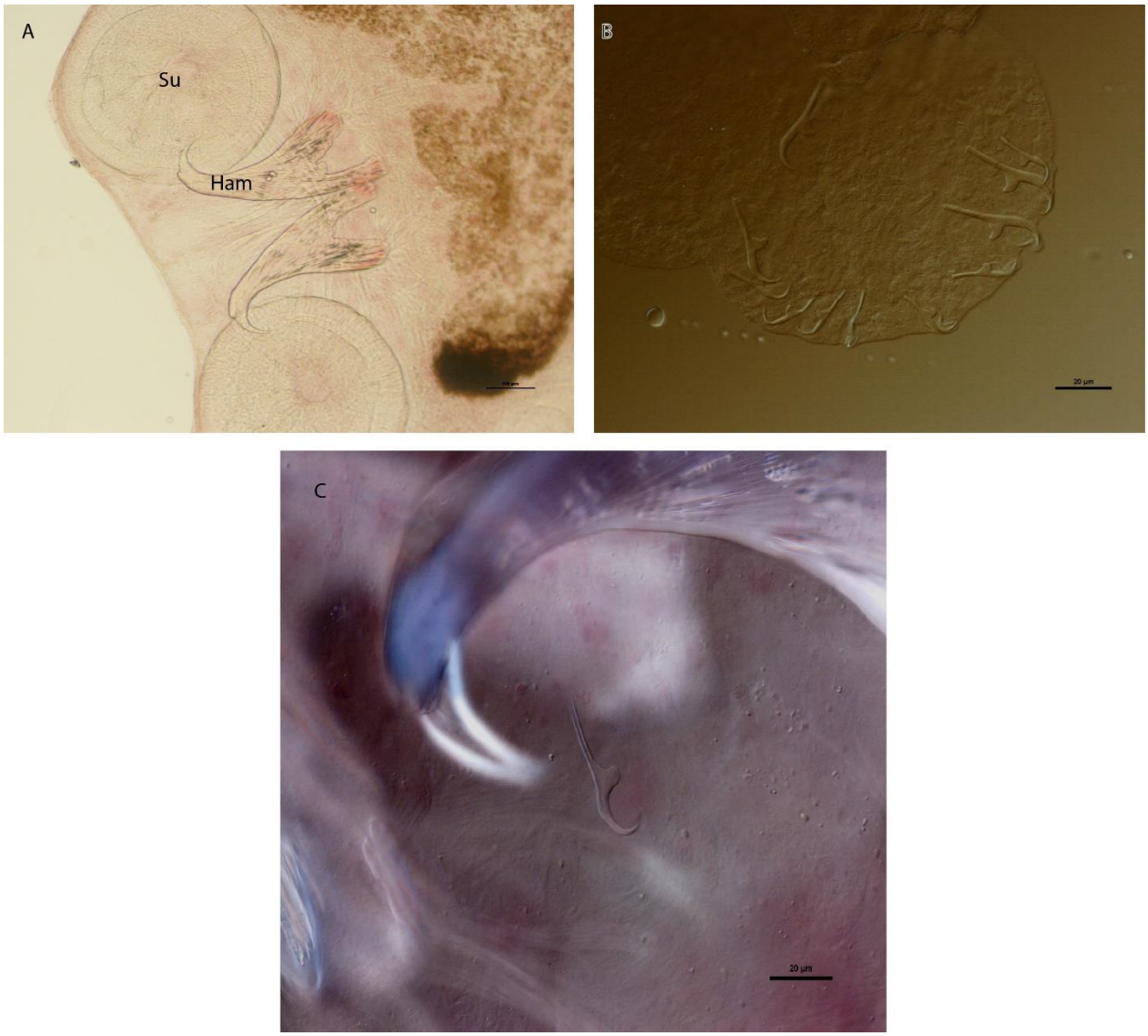

Figure 1.2: Attachment structures of polystomes. A - Hamuli and suckers of *Metapolystoma porosissimae*. (Scale 100µm). B - Marginal hooklets on the oncomiracidial haptor of *M. porosissimae*. (Scale: 20µm) C - Indication of the size difference between the hamulus and marginal hooklet on *Polystoma marmorati*. (Scale: 20µm). Labels: Su – Sucker; Ham – Hamulus.

1.3.3. Phylogenetic history

Williams (1995) stated that polystomes might have evolved with their hosts as the terrestrial habitat was invaded by ancestral amphibians. This co-evolution has been tested numerous times, supporting the statement by Williams (1995). Verneau *et al.* (2002) proposed that the four major amphibian polystomes may have originated through the diversification of their hosts, about 250 Mya. This early divergence has been described as perhaps being simultaneous to the transition of organisms from an aquatic to a terrestrial lifestyle (Héritier *et al.* 2015). It is supported by some species of Polystomatidae being found in lungfish and tetrapods, as well as their proposed high degree of host-specificity,

and their global distribution (Verneau *et al.* 2002; Verneau *et al.* 2009a). They then dispersed to freshwater chelonians in the Upper Triassic era (Verneau *et al.* 2002; Verneau *et al.* 2011). The most recent phylogenetic study conducted by Héritier *et al.* (2015) suggested that amphibian polystomes originated in the middle-late Devonian era and co-evolved with their hosts though the Mesozoic and Cenozoic periods. He further suggests that chelonian polystomes diverged in the late Jurassic period, after modern turtles had split into pleurodires and cryptodires (Héritier *et al.* 2015). However, final conclusions could not be reached due to inadequate sampling of this group of polystomes.

With the anuran polystome genus *Polystoma* being so abundant and diverse in Africa (32 species in Africa alone), several taxonomists have speculated on its origin. In a relatively early study by Prudhoe and Bray (1982), it was suggested that *Polystoma* and *Eupolystoma* originated in the Ethiopian region, with *Polystoma* radiating from there into the Palearctic, Nearctic, Neotropic and Oriental regions, while *Eupolystoma* dispersed into the Indian sub-continent. They suggested that the genus *Pseudopolystoma*, found in Japan, probably became ecologically isolated and evolved from there on. Isolation following continental fragmentation and drifting is proposed as the major event in the subsequent evolution of *Polystoma*.

According to Bentz *et al*. (2006) molecular phylogenies suggest the origin of *Polystoma* to be in South America, subsequently colonising North America, Europe and then Africa. Their conclusion that Africa was the final continent to be invaded, was derived from the fact that all African *Polystoma* species were monophyletic, compared to the paraphyly of the American and European taxa. Even though only a couple of African representatives were included in her analysis, her findings were supported by Bentz *et al.* (2001), who suggested that this colonisation from Europe to Africa may have happened some 5 Mya. Bentz *et al.* (2006) concluded that *Polystoma* originated in South America, after the separation of the continent from Africa. They further proposed that *Polystoma* might have colonised North America in the Palaeocene, Eurasia by the mid-Cainozoic and Africa in the Messinian period.

The anuran species thought to have been the host for the transmission from South America may have been either *Hyla* or *Pelobates* species. The same species (*Polystoma gallieni*) infecting *Hyla* can be found across the Mediterranean Sea, which may indicate that less time has passed since the colonisation than the proposed 5 Mya. Bentz *et al.* (2006) suggested that the original hosts were hyloids, which was supported by Badets *et al.* (2011). The latter authors found several host-switching events from hyloids to ranoids. However, their findings suggested the origination of *Polystoma* species in ancestral ranoid

hosts on Gondwana, therefore challenging the Eurasian origin suggested by Bentz *et al.* (2001).

Support for the *Pelobates* species includes the molecular affinities among several widespread pelobatids and their associated polystomes. There is also a large similarity in the molecular time estimates, which all lead to taxonomists favouring this genus as the host responsible for the transportation of polystomes to Africa (Bentz *et al.* 2001). However, the latter hypothesis implies several host switching and host dispersal events, which has also been supported by several other authors (Bentz *et al.* 2006; Verneau *et al.* 2002; Verneau *et al.* 2009b).

On a morphological level, larval characteristics suggest that *Protopolystoma* may have been the first to infect anurans (Tinsley 1981; Llewellyn 1957; Williams 1995). This can be seen in the ciliated cell patterns being closely related to the chelonian genus *Polystomoides*, as well as *Oculotrema* found in the hippopotamus. This close relation may indicate that these three genera were basal in polystome evolution and that all other genera radiated from them. The antiquity of the relationship between these three genera, is, however, shown in their very different adult morphologies (Tinsley 1981). Tinsley and Tinsley (2016) suggested that especially chelonian polystomes can be considered living fossils, due to the high stability of their morphology.

Whatever the true origin of polystomes, the central theme on their phylogenetics indicates that several clades arose during the break-up of Gondwana, with ancestral parasite co-divergence following continental drift, and numerous duplication and host-switching events occurring during the diversification of the Polystomatidae (Badets *et al.* 2011; Héritier *et al.* 2015; Veneau *et al.* 2002).

1.3.4. Polystome life cycle

Monogeneans usually infect the gills or skin of actinopterygian and chondrichthyan fish, but the Polystomatidae infect the urinary bladder, oral region or conjunctival sacks of their respective hosts. Polystomatids have a direct life cycle and the only exception is a slight deviation, generally referred to as a neotenic cycle, mostly occurring in anuran polystomes of the genera *Polystoma* and *Metapolystoma* (Badets *et al.* 2009; Badets & Verneau 2009; Llewellyn 1957; Murith 1981b ; Prudhoe & Bray 1982).

A normal life cycle for anuran polystomes is distinctly synchronised with the life cycle and reproductive strategy followed by the host. The adult parasite will usually reproduce actively as soon as the reproductive system of the host has been activated (Du Preez & Kok 1992b). As an example of an anuran infecting polystome, a simplified life cycle of the genus *Polystoma* is illustrated in Figure1.3: Synchronized life-cycle of . However, it should be noted that other genera may have very different life cycles, with some migrating to

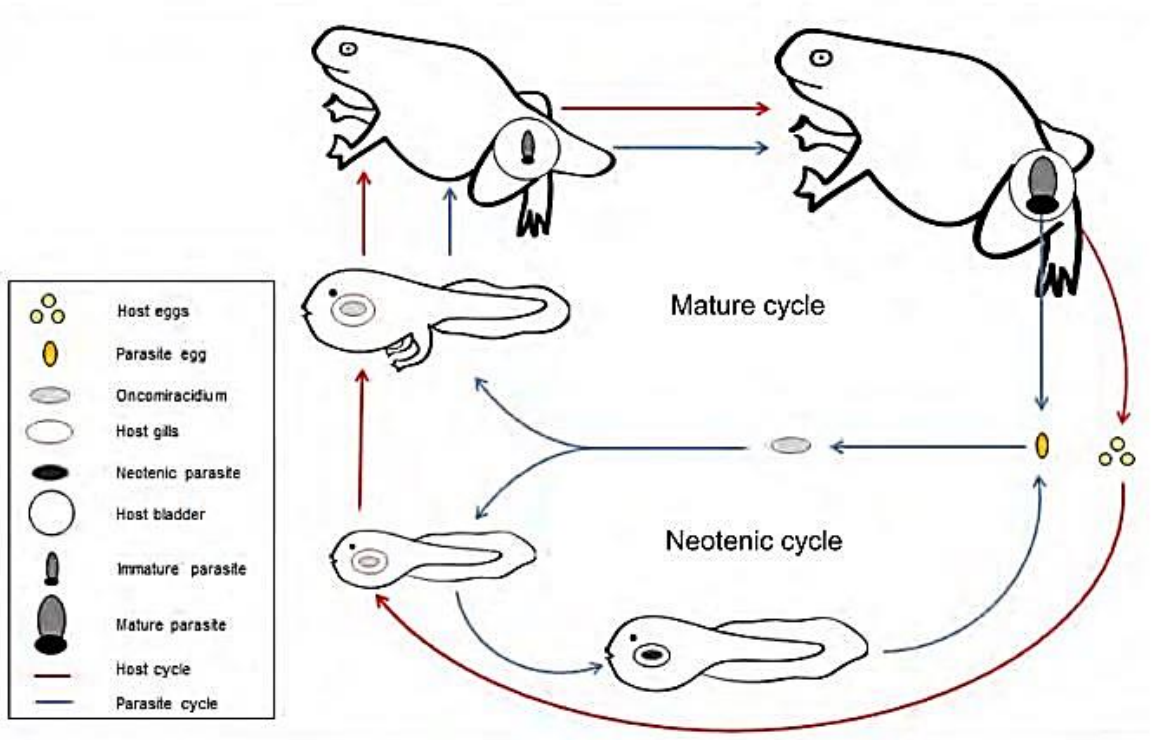

Figure1.3: Synchronized life-cycle of *Polystoma* and their anuran hosts (Theunissen 2014)**, as an example of an anuran polystome.**

several other organs. *Protopolystoma*, for example, directly infects the host through the cloaca and migrates to the kidney, where it develops. They then migrate to the urinary bladder where they mature and reproduce (Theunissen *et al.* 2014; Tinsley & Jackson 1998b; Tinsley & Owen 1975).

A neotenic cycle is present in the life cycles of both *Polystoma* and *Metapolystoma.* It is a rapid cycle on the gills of the tadpoles, with the polystomes reaching reproductive maturity and commencement of egg laying after about 16 days. During the normal cycle, the polystomes may only reach maturity in the next reproductive season of the host. Several authors have found that any hatched oncomiracidia of a species practising the neotenic cycle, may mature in either one of the mature forms and that the choice of developmental path depends on chemical signals emitted by the tadpole concerning its age (Badets *et al*. 2013). If the tadpole is close to metamorphosis, the parasite will patiently

wait on the gills until it can safely migrate to the cloacal opening and enter it. However, if the tadpole is still young, the neotenic maturation process will take place rapidly (Murith 1982; Williams 1960).

Some authors maintain that the neotenic cycle found in some polystomes may be remnants of their ancestors, specifically the fish parasites (Sinnappah *et al.* 2001; Verneau *et al.* 2009a). Williams (1995) observed that *Protopolystoma* has essentially the same morphology as the neotenic adults from *Polystoma* species. However, it inhabits the same site as the normal adults. This has led her to believe that *Protopolystoma* may have been one of the original polystome genera to have evolved, as suggested by Tinsley (1981) and Llewellyn (1957).

The morphology of neotenic parasites is often different to the normal parasite since the complete cycle is accelerated and incomplete. Differences include changes in the female genital duct, with neotenic forms having no uterus or vaginae, but only an ootype (Bychowsky 1961; Williams 1961). In addition to the differences in morphology, the lifespan also differs, with normal individuals found to live up to six years in the host's bladder, while the neotenic parasite usually lives only for one and a half to two months (Bychowsky 1961).

The polystome inhabiting *Ptychadena longirostris* shows three alternative reproductive strategies: the neotenic cycle, the normal cycle, and an internal vesicle cycle (Murith 1981b). This internal cycle resembles the *in situ* reproduction found only in members of the genus *Eupolystoma*. This cycle is of particular interest since all members in other genera require some aquatic medium for host to host transmission by the free-swimming larvae. However, with this *in situ* strategy, *Eupolystoma* are able to exploit a host species who briefly and infrequently visit water (Tinsley 1978b), occupying a completely different, arid niche.

Chelonian polystomes may inhabit one of three sites on their host, namely the urinary bladder, conjunctival sacs, or oral area. Although these sites differ, the same simple and direct life cycle is followed by parasite species inhabiting the respective sites. The life cycle can be seen in Figure 1.4.

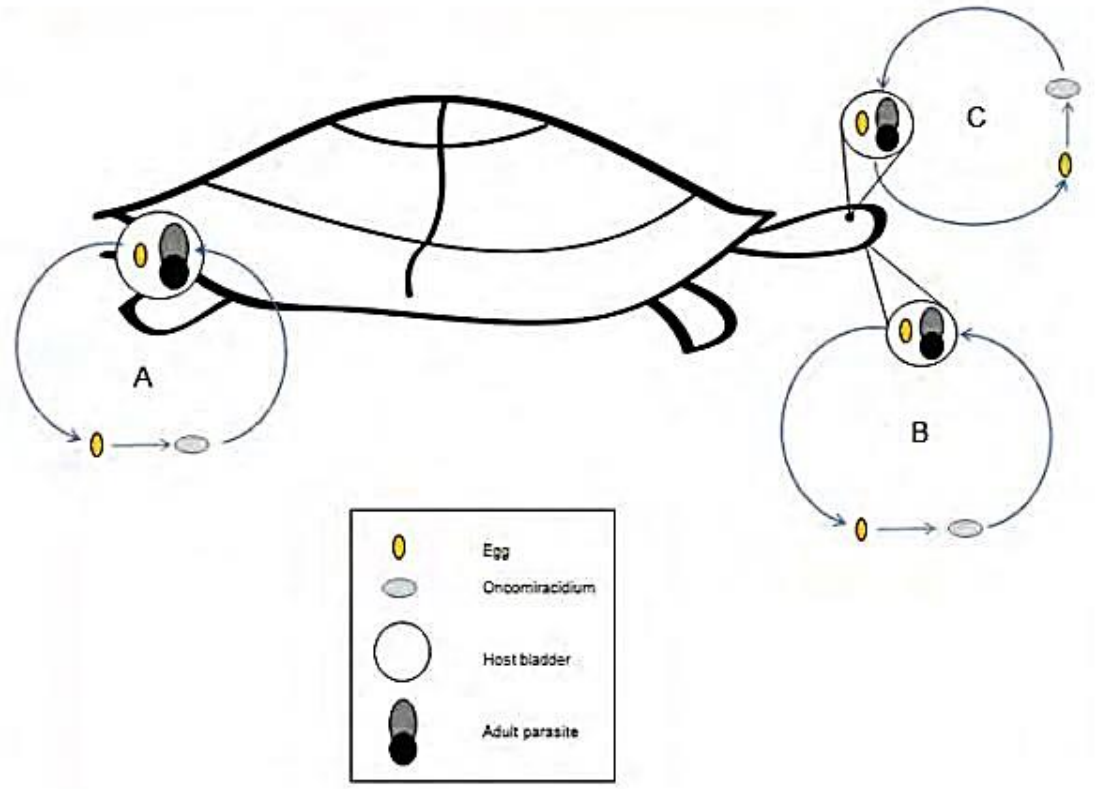

Figure 1.4: Life cycle of chelonian polystomes in their respective micro-habitats (Theunissen 2014).

1.4. Morphological plasticity

1.4.1. General introduction

Individuals within a species are roughly centred around a single morphological pattern. However, variations in this morphological pattern do occur, and may be found between groups (interspecies variation), or between individuals (intraspecies variation, or plasticity) (Prudhoe & Bray 1982).

Phenotypic plasticity occurs throughout nature and has been well-studied in all major groups of biology, from plants (Adler & Karban 1994; Agrawal 2001; Dudley & Schmitt 1996; Pigliucci 2005), to invertebrates (Brönmark *et al.* 2011; Lüning 1992; Mladineo 2013), and the largest mammals (Jones 2012; Miner *et al.* 2005). It can be defined as the ability and potential of an organism with a single genotype to produce more than one alternative phenotype, physiology, development and/or behaviour in response to different environmental conditions (Agrawal 2001; DeWitt *et al.* 1998; Miner *et al.* 2005; West-

Eberhard 1989). However, since the description of Mendel's laws until the early 1980s, the environmental effect on plasticity has been seen as little more than a nuisance. Hereafter, it formed an important part of scientists' understanding of an organism's ecology (Pigliucci 2005).

This ability to change provides an organism with a mechanism for adaptation to temporally or spatially changing environments or ecosystems. It was suggested as early as the time of Lamarck's first law, which said '*that organisms acclimate to their environment to improve their performance'* (Agrawal 2001; Badets *et al.* 2009; Dudley & Schmitt 1996). Murith (1981b) confirmed the morphological adaptations occurring due to environmental factors, and added that these adaptations may also be related to the habitat and biology of the host, in the case of parasites, for example, hosts living in arid environments, compared to those living in tropical climates. These morphological adaptations, as a type of phenotypic response, are generally canalised and usually cannot return to the state it was in before the adaptation was necessary (Agrawal 2001).

Although some authors (Agrawal 2001; DeWitt 1998; Mladineo *et al.* 2013; Pigliucci 2005) said that morphological variation and plasticity may lead to speciation, Poisot *et al.* (2011) claimed that it may be due to several factors, including an ongoing speciation process (if the plastic traits are inherited by future generations), host-induced polymorphism within the population, inter-individual variation, or a combination of these (Badets *et al.* 2013; Poisot *et al.* 2011).

1.4.2. Benefits, costs and limits to plasticity

There are several costs, benefits, and limits to having plastic traits which influence the ecology and food-chain structure of the organisms harbouring the plastic traits (Agrawal 2001; Miner *et al.* 2005). One of the benefits includes the formation of mutualistic relationships between organisms. The relationship between leguminous plants (from the family Fabaceae) and nitrogen-fixing bacteria (rhizobia) is an excellent example. The bacteria, located near the roots, produce lipo-oligosaccharides. The plants possessing plastic traits are able to adapt their roots to produce tubules around these bacteria, which in turn fix atmospheric nitrogen for the use of the plant (Agrawal 2001).

Another advantage of demonstrating plastic traits, i.e. for defence, is indicated by, among other, the relationships between mussels and crabs (Leonard *et al.* 1999), and plants and herbivores (Adler & Karban 1994; Agrawal 2001). Adler and Karban (1994) explain several defensive methods in plants, such as the 'moving-target' model, whereas Agrawal (2001)

mentions that plants are able to emit volatile substances that attract the predators of the herbivores preying on them. Crabs with larger claws typically feed on mussels possessing an extra strong adductor muscle. These traits, the claw and the adductor muscle, were shown through experimental studies, to change depending on the presence of the other trait in the same waterbody (Leonard *et al.* 1999). The defensive responses in both plants and animals may lead to a better immunity in that organism, including harbouring fewer parasites (Agrawal 2001). Van Buskirk and McCollum (2000) described the influence of predators on the morphological and behavioural plasticity in tadpoles. In a laboratory experiment, tadpoles exposed to predators typically had reduced feeding-habits, as well as larger and more colourful tails, compared to those that had no predators.

DeWitt (1998) stated that natural selection generally favours organisms that are able to change their development based on the changes in their environment. This enables the individuals possessing certain plastic traits to enter into novel habitats and this has been confirmed in a study done by Kaci-Chaouch *et al.* (2008) on the gill parasite of sparid fish, *Lamellodiscus*. They found that morphological plasticity enabled this species to colonise new hosts and that the larger variety of hosts inhabited, the more its intraspecific variance increased.

Agrawal (2001) found that organisms in new locations generally possessed traits explained by phenotypic plasticity, rather than by genetic change. At first, the changed phenotype will result in different morphotypes of one species in different localities (Poisot *et al.* 2011). However, after some time has passed since entering into this new environment, this plasticity may lead to genetic differentiation, while other factors, like for example allopatry or induced preference, may cause a restriction in gene flow to the original habitat (Agrawal 2001; Mladineo *et al.* 2013; Pigliucci 2005). This genetic differentiation, due to a high degree of plasticity, may then lead to species becoming specialists, as opposed to generalists, in this new habitat, which may then lead to different degrees of host-specificity (DeWitt 1998; Kaci-Chaouch *et al.* 2008). The plastic organism is able to adapt to changing environments, therefore producing a better match between their phenotype and the environment than would be possible if they had only one phenotype in all environments (DeWitt 1998). However, Vignon *et al.* (2011) suggest that this variability among species is of phylogenetic origin, rather than due to environmental influences such as host-specificity or geographic distribution.

Even though the benefit of being able to inhabit new and diverse habitats is quite significant, it can be reduced if the development thereof produces an impaired developmental range, developmental instability, or extreme energetic costs (DeWitt *et al.*

1998). The costs of plasticity are typically fitness shortages associated with plastic genotypes compared to fixed genotypes that may lead to the same phenotype in a certain environment. In contrast, the limits are only functional restraints that may reduce the benefit when compared to perfect plasticity (the best possible phenotype-environment match in all circumstances) (DeWitt 1998; DeWitt *et al.* 1998; Pigliucci 2005).

The costs to plasticity, according to DeWitt *et al.* (1998) and DeWitt (1998) are summarised as follows:

- Maintenance costs – being able to maintain the sensory and regulatory mechanisms that lead to plastic traits;
- Production costs – creation of extra phenotypes leads to higher costs compared to those paid by fixed genotypes to produce the same phenotype;
- Information acquisition costs – finding a compatible host or environment during environmental sampling;
- Developmental costs – instability that may result in variable plastic development compared to fixed development;
- Genetic costs – for example, the linkage of plastic genes with genes having a low fitness, this includes pleiotropy and epistasis.

The limits include (DeWitt 1998; DeWitt *et al.* 1998):

- Limited information reliability – information about environmental cues triggering plastic traits that don't reflect the true state of the environment;
- Lag time limits – where there is a delay in sensing and responding to environmental information;
- Developmental range limits – which occur if plastic development is incapable of producing extreme phenotypes that can be created through fixed development;
- An epiphenotype problem – where add-on phenotypes may be less effective than developing the phenotype during early ontogeny.

These costs and limits may have a profound effect on the degree of plasticity found in organisms, and organisms will only show plasticity when the benefits exceed these costs (Alcock 2009).

1.4.3. Plasticity among polystomatids

The intra- and interspecies variation in morphological traits among polystomes has been known for some time, even though the intraspecies variation may be significantly more than the interspecies variation (Aisien & Du Preez 2009; Du Preez *et al.* 2002; Du Preez & Maritz 2006).

Among the monogeneans, the haptoral structures, used for attachment to their hosts, are possibly the most influenced by phenotypic plasticity in generalist species (Poisot *et al.* 2011). Williams (1960) and Tinsley (1974) explained that there were various *"malformations"* in the hamuli of polystomatids (including curved shafts). Tinsley and Jackson (1998b) also noticed that sub-Saharan African *Protopolystoma xenopodis* showed a significant variation in the size of their genital spines based on their geographical distribution. Except for these three, studies of the plasticity of the haptoral parts of polystomes has been rather scarce. However, several other haptoral studies on some other monogenean species have been conducted. Olstad *et al.* (2009) studied the effect of varying temperatures and host species on the size and shape of the haptoral hard parts in *Gyrodactylus* species and noted that some species showed a temperature-based plasticity, while others did not. However, these differences were only reported for the ventral bars, which are absent in polystomes, and the marginal hooks and hamuli showed no differences due to the increased temperature. The marginal hooks and hamuli also showed no size difference when the parasite grew on secondary hosts, rather than on primary hosts, but had, in fact, shape differences (Olstad *et al.* 2009). To study the plasticity in the shape of the marginal hooks and hamuli, Teo *et al.* (2013) suggest using a 3D model rather than the traditional morphometric method under a light microscope. The changes seen in the study by Olstad *et al.* (2009) are somewhat in contrast to previous studies, which indicated that fluctuations in salinity and temperature had an effect on the morphology of gyrodactylid marginal hooklets. However, Du Preez and Maritz (2006) and Murith (1981a) claim that this effect has not been seen in amphibian polystomes.

Another form of plasticity among polystomes is developmental plasticity found in the neotenic and normal forms that depend on the morphological stage of the host in which it is infected (Badets *et al.* 2009; Badets *et al.* 2013; Badets & Verneau 2009; Williams 1995). Neotenic gill parasites' morphology often differs from the normal parasite as it is accelerated and incomplete, for example, the female genital duct, which shows no uterus or vaginae (Bychowsky 1961; Williams 1961). Badets *et al.* (2009) found that the neotenic development strategy followed by *Polystoma gallieni* was influenced by tadpole-derived chemicals in the water, which revealed the host physiological stage without the requirement for physical contact, confirming that the plasticity depends on environmental cues and host ecology (Badets *et al.* 2011; Badets *et al.* 2013; Williams 1995).

Polystome eggs have been found to show some form of plasticity, especially when it came to shape and time of hatching. Eggs of neotenic parasites are characteristically rounder, while bladder parasites of *Polystoma* are oval in shape (Du Preez 2013). Among chelonian

polystomes, those found in the conjunctival cavity are characterized by their spindle-shaped eggs, compared to round to oval-shaped eggs found in other infection sites (Du Preez & Moeng 2004; Du Preez & Morrison 2012). Warkentin (2011) suggested that the timing of hatching events can be seen as a form of plasticity, as it may be influenced by environmental cues. Jackson *et al.* (2001) studied the inter- and intraspecific variation of egg development and hatching of *Protopolystoma xenopodis* and *Protopolystoma occidentalis* in different temperatures. They found that *Pr. occidentalis* was more sensitive to the cold conditions, with no eggs hatching at 15°C, while some *Pr. xenopodis* did hatch 49-88 days post-collection. Optimal hatching occurred at 25°C for both species after 18-26 days (*Pr. xenopodis*) and 27-37 days (*Pr. occidentalis*).

The oncomiracidia of polystomes also show some intergeneric plasticity. In *Madapolystoma*, there are no free-swimming, ciliated oncomiracidia, as has been suggested to be a distinct characteristic of the family Polystomatidae (Du Preez *et al.* 2010). In contrast, *Eupolystoma* species have shown that there are two different types of oncomiracidia. One is heavily ciliated and destined for external release as a free-swimming larva to infect new hosts, while the other is unciliated and therefore remains inside the host in order to boost the existing infrapopulations. For this reason, *Eupolystoma* is known for very high parasite intensities (Du Preez *et al.* 2003; Du Preez 2015). In their description of *Polystoma dawiekoki*, Du Preez *et al.* (2002) showed that this species, and *P. grassei*, also host large parasite intensity levels. One or two eggs remain *in utero* and the oncomiracidia are released upon immersion in the water, which enables them to immediately locate a host, which may be the one they were just expelled from.

The vitelline follicles are also quite variable (Enabulele *et al.* 2012). In some individuals of *Polystomoides bourgati* from the same locality, these follicles were either 'fine' or 'coarse'. In *Madapolystoma*, there is also no vitellaria, and it is suspected that the ovary is a germovitellarium, as is found in fish monogeneans (Du Preez *et al.* 2010).

There is significant variation (plasticity) to be found in the intestinal arrangement in *Polystoma africanum.* This variation can especially be seen in the same species occurring in different areas/localities (Aisien & Du Preez 2009; Tinsley 1974). Sub-species were created based on specimens closely resembling each other but differing in one or two morphological traits (Tinsley 1974). Tinsley (1978b) found that there was a high level of variation in the intestinal caecae of *Eupolystoma anterorchis*, with some individuals having no lobes or branches, while others had prominent diverticula of various lengths. These diverticula were frequently short, but occasionally they had formed a post-ovarian transverse inter-caecal anastomosis (Tinsley 1978b).

Apart from these traits mentioned above, there is still some uncertainty as to the degree of morphological plasticity found in polystomatids. Badets *et al.* (2013) mentioned that our knowledge of this subject is solely based on morphological species descriptions, and according to Tinsley (1974) there are no real comprehensive data available on this subject. He noted, however, that the degree already known sheds some additional uncertainty on the validity of the described species. To assess phenotypic plasticity further, Badets and Verneau (2009) suggested using hox-genes, as they play a fundamental role during the early stages of development.

1.5. Host- and site-specificity of polystomes

1.5.1. Host-specificity

Host-specificity can be defined as the situation in which a species of parasite is restricted to a singular host or group of related hosts (Prudhoe & Bray 1982). There are two types of host-specificity, phylogenetic and convergent. In phylogenetic specificity, the host and parasite have evolved together for a long time, but it may not have happened at a similar rate. In convergent specificity, the relationship between the host and parasite is still relatively new (Prudhoe & Bray 1982).

The mechanisms for host-specificity are still mostly unknown. Among the Monogenea, it is suspected to be, at least in part, mediated through host chemical signals (Badets *et al.* 2009). Hargis (1957) suggested that it may be either physiological, genetic or ecological, or even a combination of these factors. Fischthal (1955) found a definite correlation between the habitat, life cycle and host-specificity. However, there have still been reports of instances where similar numbers of the parasite were present in the host, no matter what stage of the life-cycle was involved (Prudhoe & Bray 1982). Several authors also mentioned that the host's ecology, diet or breeding conditions may have a significant effect on the specificity of the parasite, and immunological of physiological factors may not be as significant (Bourgat & Salami-Cadoux 1976; Kaci-Chaouch *et al.* 2008; Murith 1979; Prudhoe & Bray 1982). Llewellyn (1957) proposed that the specific morphology and/or physiological adaptations of a parasite species, such as the attachment structures, may influence its compatibility with a host species, which may lead to specificity. The relative size of the host may also have an influence (Sasal *et al.* 1999). However, as Hargis (1957) forewarned, knowledge concerning life histories, physiology and ecology of the parasite species must first be understood well enough before any definite conclusions can be reached concerning host-specificity.

Agrawal (2001) links ethology with host-specificity when questioning whether the ability to learn and associate certain chemical or environmental cues with particular hosts can influence the choice of a new host and a chance to specialise in that new host. Kaci-Chaouch *et al.* (2008) echo this as they suggest that host-specificity could be influenced by interspecific variation due to environmental factors.

According to Prudhoe and Bray (1982) platyhelminth parasites in amphibians generally belong to groups that are strictly host-specific. However, it is also common among the platyhelminths for a species to show a certain preference for a host, without it being specific to that host.

Host-specificity among monogeneans has been studied for some time, and according to Hargis (1957) there are two aspects to consider, namely infra-specificity and supra-specificity. Infra-specificity is the occurrence of a single parasite species on members of a single host taxon. This term includes species-specificity, and the specificity may be physiological, ecological, or a combination thereof in nature. Supra-specificity, on the other hand, is the restriction of a group of parasite species to a group of host species (Hargis 1957). For example, the Polystomatidae are not supra-specific, as they occur in a variety of host taxa (amphibians, turtles, hippos, etc.), but they are supposed to show a certain degree of infra-specificity, with several species being strictly host-specific (Aisien & Du Preez 2009; Badets *et al.* 2011; Bychowsky 1961; Hargis 1957; Héritier *et al.* 2015; Jackson *et al.* 1998; Kaci-Chaouch *et al.* 2008; Llewellyn 1957; Verneau *et al.* 2011).

According to Hargis (1957) 89% of his collection of Monogenean parasites were strictly species-specific. 88% of the remaining 11% were genus-specific. Comparison of marine and fresh-water species indicated that the latter was less specific than the former.

Several monogenean species have been studied with regards to this subject, and the degree of host-specificity was highly variable in these studies. In the studies of Llewellyn (1956b) on fish parasites, he found that there was generally a high degree of host- and site-specificity. Meyer *et al.* (2015) indicated that several host switches occur with chelonian polystomes, indicating a relatively low host-specificity. There was, nevertheless, a high degree of site-specificity among these polystomes (Verneau *et al.* 2011).

Regarding amphibian polystomatids, there is also a varying degree of host-specificity. One of the traditional characterising features of polystomatids, and especially the genus *Polystoma*, are their apparent strict host-specificity (Aisien & Du Preez 2009; Bentz *et al.* 2006; Du Preez & Kok 1993; Du Preez & Kok 1997; Hargis 1957; Héritier *et al.* 2015; Kok & Du Preez 1987). Prudhoe & Bray (1982) reported that specificity varied among different

regions. For example, *Polystoma intergerrimum* was found in only one specimen of *Rana esculenta* in central Germany (out of 255 specimens sampled), whereas it was found regularly in two different *Rana* species elsewhere in Europe (Prudhoe & Bray 1982). Murith (1981a) mentioned that the neotenic parasite is able to infect non-specific hosts, in direct contrast to the strong host-specificity found in the bladder parasite. Du Preez (1994) studied host-specificity among polystomatids and concluded that southern African polystomes were host-specific, although there were some exceptions.

The genus *Eupolystoma* has shown a low level of host-specificity, with *E. alluaudi* reported from at least seven host species, representing four genera and two families (Du Preez 2015). However, this might be a complex of cryptic species. Tinsley (1978b) also questions the host-specificity of members of *Eupolystoma.* In a cross-infection experiment, *Eupolystoma vanasi* were not found in substitute hosts after a 14-day period after exposure to oncomiracidia (Du Preez *et al.* 2003). This may be because of the lack of involvement of the tadpole when the polystome infects the host, as in the case of *Polystoma*, where the presence of the tadpole is crucial for identifying and infecting the host (Du Preez 2015).

According to Tinsley and Jackson (1998a; 1998b), *Protopolystoma* also has a variable degree of host-specificity. Four species are reported to be restricted to a single host species, while two more occur in more than one host taxon. They believe that the specificity among this genus is determined by the ability of the polystome to complete its development in the kidneys of its host.

Strict species-specificity has traditionally been assumed to be critical for the systematics of African polystomes, but Tinsley (1978b) has suggested that more experimental evidence is needed for this. Murith (1981a) explained that there were, in fact, a lot of exceptions to host-specificity where young tadpoles were concerned. She suspected that this might have been due to the fact that the defence system of these tadpoles was still being formed (Murith 1981a). Du Preez & Kok (1997) also indicated that, although oncomiracidia of Southern African polystomes displayed a strong partiality to their natural hosts, the same degree of host-specificity was not indicated by all of them. Bourgat and Salami-Cadoux (1976), Murith (1979), and Bentz *et al.* (2001) proposed that neotenic gill forms might develop on non-specific tadpoles, while the normal vesicular forms may rather exhibit a strict host-specificity.

Among adult polystomes some species can be found in multiple hosts, for example, *Polystoma channingi* found in *Cacosternum boettgeri* and *C. nanum* (Du Preez 2013), *Polystoma australe* found in *Kassina senegalensis* and *Semnodactylus wealii* (Kok & Du Preez

1987). It has been suggested that in instances where closely-related host species occur sympatrically, the oncomiracidia may infect either one or both (Du Preez 2013).

Since monogeneans were generally thought to be strictly host-specific, and this has been assumed for polystomatid species as well. However, there are now so many exceptions to this "rule" that there is a need for a re-evaluation thereof. This has already been suggested by Bentz *et al.* (2001), who proposed that some polystomes were generalist species, rather than specialists. However, if the findings concerning plasticity leading to specialisation on a host (Agrawal 2001; Kaci-Chaouch *et al.* 2008; Poisot *et al.* 2011) are true, it may be that the earliest polystomatids were generalists, and as plasticity helped them to invade new hosts, they specialised to their specific host species, leading to speciation.

1.5.2. Site-specificity

Most anuran polystomes usually only occur in the bladder of their respective hosts, with the exception of neotenic forms establishing themselves on the gills of tadpoles. However, among other polystomatids, especially those infecting freshwater turtles, the adults have a specific site preference within the host. While most of these parasites mainly stay in the preferred sites, it may be possible for some species to establish themselves on a different site, if necessary. An example of this is an intestinal trematode being able to survive in all areas within the host's intestine (Prudhoe & Bray 1982). Among fish parasites it has also been found that parasite species prefer certain gill lamellae to some other, or another site altogether (Llewellyn 1956b; Perkins *et al.* 2009).

For polystomatid species, Rohde (1975) mentions that individuals of *Protopolystoma xenopi* have certain "inborn" site preferences within their host during certain life stages. They usually enter the cloacal opening, after which they travel to the kidneys, where they mature before migrating to the cloaca once again (Rohde 1975).

Site-specificity among polystomes is most profound in those infecting freshwater turtle species with species being highly specific to one of three sites (or microhabitats) (Du Preez & Lim 2000; Du Preez & Morrison 2012). These three sites include the urinary bladder, the conjunctival sacs and/or the pharyngeal cavity. Host-specificity among chelonian polystomes is of a lower degree compared to their site-specificity, as some host species have had polystomes in each of the three different microhabitats (Verneau *et al.* 2011). Congeneric species inhabiting the same microhabitat in different hosts have been found to be of closer relationship than those inhabiting different sites in the same host (Littlewood *et al.* 1997). This may indicate that speciation between these species most

likely did not occur from another species within the same host, but rather from another polystome inhabiting the same site in another host.

Strict site-specificity among turtle polystomes is further supported by Tinsley and Tinsley (2016), who described a new genus among these polystomes for individuals occurring solely in the bladder of their hosts (*Uropolystomoides*).

1.6. Monogenean morphology regularly used in taxonomy and systematics

As explained before, traditional taxonomy and systematics relied heavily on morphological differences between species (Mayr 1942; Tinsley 1978b). It is often found among polystomatids that the genera can be easily distinguished based on morphology alone, but when attempting species discrimination below genus-level, morphological characteristics are often not sufficiently discriminative. In these cases, factors like ecology, biology (including the behaviour, migratory status pairing habits and seasonal occurrence) and, in the case of host-specific parasites, host-class or -species may also be vital (Berthier *et al.* 2014; Du Preez 1994; Kok & Du Preez 1987; Mayr 1942; Pichelin 1995).

For the morphological description of species, Mayr (1942) and Prudhoe and Bray (1982) described that one should use a combination of several traits (as characters may influence each other) from a representative number of individuals, rather than using only one or two specimens. They warned that fewer individuals and traits will lead to misclassification and/or the development of synonym species.

Polystomatids, as all flatworms, have soft, highly flexible bodies, which makes the use of body measurements of limited value, since fixation may cause unnatural flattening (Du Preez & Morrison 2012; Platt *et al.* 2011; Vignon *et al.* 2011). In this regard, Mayr (1942) and Tinsley and Jackson (1998b) suggested that taxonomists should attempt to find relatively stable, phylogenetically conservative traits that are unaffected by fixation.

In flatworms, it seems that the parts consisting of scleroproteins (marginal/larval hooklets, hamuli, and genital spines, collectively referred to as sclerites) remain relatively constant in size, ratio, shape, position and numbers to be used for taxonomy (Du Preez & Maritz 2006; Fairfax 1990; Vignon *et al.* 2011). Such is the value thereof that Llewellyn (1957) suggested a classification of monogenean larvae based mainly on the larval hooks and the occurrence and structure of the eyes. Vignon *et al.* (2011) also found in their study that there is a strong phylogenetic signal for the presence and shape of the sclerites.

Several authors have commented on the low intra- and high interspecific variation found in sclerites of polystomatids (Bychowsky 1961; Combes & Rohde 1979; Du Preez & Maritz 2006; Llewellyn 1956a; Tinsley & Jackson 1998b). This is demonstrated in that, for example, the shape of the hamuli of *Polystoma africanum* was found to be consistent when collected from three different countries (Aisien & Du Preez 2009; Tinsley & Jackson 1998b). Tinsley & Jackson (1998b) also found that the point of the hamulus reached its maximum size while the parasite was still in its juvenile stages in the kidney. The presence or absence of the hamulus can also be a valuable feature since some genera may have none, one or two pairs respectively (Bychowsky 1961). The length of the hamuli versus sucker diameter ratio was recently used in the description of a new genus, *Uropolystomoides* (Tinsley & Tinsley 2016).

The number, shape and arrangement of the genital spines have also been indicated as valuable for taxonomy (Combes & Rohde 1979; Tinsley & Jackson 1998b). Fairfax (1990) noted that, although there seemed to be some variation in their number, it can still make a significant contribution when used in combination with some other traits.

The marginal hooks are the most significant sclerotized feature for the taxonomy of polystomatids (Bychowsky 1961; Combes & Rohde 1979; Du Preez & Maritz 2006; Kok & Van Wyk 1986; Murith 1981a). These hooks are used by the larvae for attachment to the tadpole. Even though they lose this function as the polystome matures, being replaced by strong suckers and large hamuli, the hooks are retained (Du Preez & Maritz 2006; Murith 1981a; Williams 1995). Their value has been indicated on several occasions, with primary protocols for their measurement being proposed by Murith (1981a). This protocol was later on amended by Du Preez and Maritz (2006) to be more objective and with a greater classification potential.

All of these sclerites seem to be quite significant features, but Prudhoe and Bray (1982) warn that focusing solely on the presence or absence, as well as the number, thereof, may be misleading. They claim that these spines may be rapidly lost as soon as an individual dies or is fixated.

The haptoral suckers may also prove to be a significant trait for differentiating between species. Pichelin (1995) described three types of suckers for polystomatids. The first type consists of muscle only, with no sclerotized features. This can be found in species of amphibian polystomes, including *Polystoma, Protopolystoma, Metapolystoma, Mesopolystoma, Parapolystoma, Parapseudopolystoma, Pseudopolystoma, Diplorchis, Neodiplorchis, Pseudodiplorchis* and *Riojatrema* (Pichelin 1995). The second type of sucker

has primarily muscle, with a lining of soft keratin and can be found in chelonian polystomes (*Neopolystoma, Polystomoides, Uropolystomoides* and *Polystomoidella*), as well as the hippopotamus polystome *Oculotrema*. The third and last type of sucker has extremely sclerotized skeletons with much-reduced muscle and can be found only in *Concinnocotyla*, from the Australian lungfish (Pichelin 1995).

The haptoral and sclerotized parts, although some of the most important, are not the only traits to take into consideration when approaching the problem of classification. Other morphological features of importance include several organ systems, for example, the protonephridial or nervous systems (Rohde 1975). Some of the organs may be highly variable for one family while proving to be constant in another (Prudhoe & Bray, 1982). An example of this is the genital structures. Prudhoe and Bray (1982) argued that for the platyhelminths in general, the use of the gonads for taxonomic distinction should rather be avoided, since the appearance and size could be altered depending on the age and reproductive activity of the organism. However, this was found in some genera of trematodes, and although the testis shape of *Polystomoides bourgati* showed some geographic variation (Enabulele *et al.* 2012), this is generally not the case for polystomatids. While using the testis as a trait to describe *Polystoma testimagna*, Du Preez & Kok (1993) mentions that it is unfortunate that there is such little information available regarding the size and position of the testis in polystomes in general.

The position of the female gonads may, however, be considered as plastic depending on the season, but there is still no proof of this being the case (Du Preez 2015; Du Preez & Maritz 2006). Until this can be investigated, the female genital duct is also used to distinguish among especially genera. Permanently neotenic genera, like *Protopolystoma*, as well as some other genera (*Polystomoides, Neopolystoma* and *Polystomoidella*) do not possess a uterus and vaginae. And although *Oculotrema* have a uterus, the vaginae are still absent (Williams 1961). This absence is regarded as an ancestral trait by some authors (Llewellyn 1957; Tinsley 1976; Tinsley 1981; Williams 1995). Enabulele *et al.* (2012) echoed Prudhoe and Bray (1982) in claiming variation in the appearance and distribution of the vitelline follicles. In some individuals from the same species and area, they found that some follicles appeared fine, while other were grainier.

The arrangement of the intestine, the presence or absence of intestinal branching, as well as the number and position of anastomoses within the intestinal caeca have proven to be of significant taxonomic importance (Aisien & Du Preez 2009; Du Preez *et al.* 2003). Tinsley (1974) and Tinsley and Jackson (1998b) claim this to be one of the '*principle systematic characteristics for polystomes*'.

According to Tinsley (1976, 1981) the number and distribution of ciliated cells in the oncomiracidia, have been found to be relatively constant in the polystomatid taxa he has so far examined, and claims that this exclusively larval characteristic has proven to be of great taxonomic importance. However, in modern species descriptions, this trait has not been mentioned.

Although not a morphological trait, Combes and Ktari (1976) suggest using host-specificity as character for new species description. However, Du Preez (1994) warns that a new species should not be described solely based on the identity of its host.

In conclusion, several characteristics have proven significant for the use of taxonomy. The most important traits have been discussed in this section, although several others may still prove valuable depending on the genera. Rohde (1965) indicated that the only morphological characters of taxonomic significance in the genus *Polystomoides*, other than those discussed, were the relative sizes of the oral sucker and the pharynx. In his thorough study of *Polystoma integerrimum*, Williams (1960) fully described each of the following systems: external features of the adult and oncomiracidia (length eyes, pores, ducts and openings, hooks, etc.), behaviour and relationship with the host, the structure of the opisthaptor, integument, muscles, suckers and their associated muscles, all hooks and their associated muscles, the mesenchyme and contained cells, the alimentary canal, nervous and sensory systems, the excretory system, the genital system (male and female) including insemination and the eggs. This description by Williams (1960) may seem excessive, but it may also be a valuable reference for future descriptions.

1.7. Problem statement, general aim and objectives

Polystomatid flatworms represent a diverse, geographically widespread group of monogenean parasites. While some genera are restricted to single continents or even islands, others like *Polystoma* Zeder, 1800 are found in all zoo-geographical realms, except for the Australian realm. Of the 64 known *Polystoma* species 32 are from Africa. A high diversity of other polystomatid genera are also found in Africa.

Traditionally morphology and morphometrics played a defining role in determining the identity of polystomes (Du Preez & Maritz, 2006). Although molecular tools are now widely used in systematic studies, the value of morphology can never be over-estimated. For classification purposes, measurement consistency, reliability and uniqueness are very important and several authors have commented on morphometric and classification protocols (Du Preez & Martiz, 2006; Murith, 1979).

Polystomatids are soft-bodied animals and consequently, the degree of flattening during fixation greatly affects the body measurements. For this reason, great emphasis has been placed on the morphometrics of sclerites such as the marginal hooklets, genital spines and the large hamuli (Du Preez & Morrison 2012; Platt *et al.* 2011; Vignon *et al.* 2011). The primary function of marginal hooklets is to enable the oncomiracidium to get a firm hold on its host. Even though they lose this function as the parasite matures into an adult, these hooklets do not disappear but remain embedded in the haptor (Du Preez, 2011; Du Preez *et al.*, 2002; Theunissen *et al.* 2014). Some intraspecies variation does occur, but inter-species differences are often profound and hooklets differ among species (Du Preez & Maritz, 2006).

Tinsley (1974) suggested that there is a definite need for a more comprehensive study of the morphological variation found in each polystomatid genus. Therefore, this study aims to determine the extent of morphological plasticity in some of the polystomatid flatworm genera, including the amphibian polystomes *Protopolystoma*, *Polystoma*, and *Metapolystoma*, and the chelonian polystomes *Polystomoides, Uropolystomoides* and *Neopolystoma*.

The general objectives of the present study are:

1. To discover whether chemical fixatives might have an effect on the measurements of marginal hooklets (Chapter 3)
2. To determine the validity of *Metapolystoma* Yamaguti, 1963 based upon genetic and morphological studies (Chapter 4)
3. To re-evaluate the taxonomy of chelonian polystomes based on morphological evidence and infection site on the host (Chapter 5)

More specific objectives will be discussed in each chapter.

CHAPTER 2

General materials and methods

Morphological plasticity is a very diverse topic that may include combinations of several subject fields (such as genetics, morphology, ecology, physiology, and behaviour) and therefore often uses very variable methods (Agrawal 2001; DeWitt *et al.* 1998; Miner *et al.* 2005; West-Eberhard 1989). In this study, we explored plasticity by using comparative morphology, morphometric and molecular tools for different aspects studied. The specific methods for each aspect will be discussed in the respective chapters to follow. However, some general materials, such as the hosts of the different polystomes, and methods, i.e. the collection and keeping of the anuran hosts, will be discussed briefly.

2.1. Parasite and host species used during this study

2.1.1. *Protopolystoma xenopodis* ex *Xenopus laevis*

Xenopus laevis (Pipidae) is a permanently aquatic frog that only sporadically will leave the waterbody to find new habitats. Although introduced populations occur in several other countries, like the UK, Chile, France, and the USA, the genus originally only occurred in Africa. Twenty-nine species of *Xenopus* are currently known, with four in Southern Africa, namely *X. laevis, X. gilli*, *X. muelleri*, and *X. poweri* (see Frost *et al.* 2016). Except for a small area along the North-Eastern border of South Africa and Mozambique, *X. laevis* occurs throughout the country in both man-made and natural waterbodies. During this study, individuals of *X. laevis* were collected in and around Potchefstroom, North-West Province. *Xenopus* is host to a large variety of parasites, including two monogenean species namely *Gyrodactylus gallieni*, inhabiting the mouth, and *Protopolystoma xenopodis*, inhabiting the urinary bladder and kidneys of the host (Tinsley 1981).

Due to the continuous reproductive output *Protopolystoma xenopodis* is an ideal polystome species for laboratory studies (Jackson & Tinsley 1988; Jackson & Tinsley 2001). This is due to the host permanently living in the water, and the parasite, therefore, has no need to retain eggs within a uterus (Jackson & Tinsley 1998). A ciliated, free-swimming oncomiracidium hatches from the egg after ±22 days at 20°C, and infects the host via the cloaca (Jackson & Tinsley 1998; Theunissen *et al.* 2014). Juveniles develop in the kidneys, from where they later migrate to the urinary bladder. They will reach maturity 3 to 4 months after the infection, and the eggs are expelled from the host with the urine, making it easy to harvest the eggs. This parasite has been well-studied and therefore an ideal species to use during morphological plasticity studies.

2.1.2. *Polystoma* and *Metapolystoma* ex *Ptychadena* spp.

The frog genus *Ptychadena* (Ptychadenidae) is known to be very suitable hosts for several polystomes (Bentz *et al.* 2001; Du Preez 2011; Du Preez & Kok 1992b). There are currently fifteen known species of polystomes occurring in the host genus *Ptychadena* (Du Preez 2011). Of these three are *Metapolystoma*, and the remainder belong to *Polystoma*.

Ptychadena is commonly known as grass frogs, due to their incidence in especially lower areas of the savanna bushveld in most of sub-Saharan Africa, including the banks of the Nile, but excluding South-Western Africa. They also occur in Madagascar, the Seychelles and the Mascarene Islands (Du Preez & Carruthers 2009). Their wide distribution is only one of several reasons for their successful hosting of polystome parasites. The other reasons include that (1) they are still in the active phase of evolution (Du Preez & Kok 1992b), (2) their habitat types include marshy swamps to dry woodland, occurring at sea level to altitudes of up to 2400m (Du Preez & Kok 1992b), and (3) morphological adaptations of the gills and gill chambers allowing up to three fully grown neotenic polystomes in a single tadpole (Du Preez & Kok 1992b). Breeding occurs in stagnant or very slow-flowing water, for example, temporary pools, pools, ditches, vleis, drainages, swamps, and dams, throughout the rainy season. The particular morphology, physiology and especially ecology of *Ptychadena* species are probably very suitable for completion of the life cycle of polystomatids, even though they may have very different life-history strategies, as is the case for *Polystoma* and *Metapolystoma*. (Du Preez & Kok 1992a). Du Preez (2011) has stated that there is a definite host-parasite co-evolution between *Ptychadena* sp. and polystomes.

During this study, attempts were made to collect *Ptychadena porosissimae* from the areas surrounding Sodwana and St Lucia in KwaZulu-Natal.

2.1.3. Chelonian polystomes

Chelonian polystomes (*Neopolystoma, Polystomoides, Uropolystomoides,* and *Polystomoidella*) are globally dispersed among pleurodires and cryptodires of distinct families having a close affinity with water (Bychowsky 1961). They have colonised several micro-habitats within the host, i.e. the urinary bladder, conjunctival sacs and pharyngeal cavities. However, it is also known that multiple infections can occur, with up to three polystomes infecting one host in each distinct microhabitat (Meyer *et al.* 2015; Héritier *et al.* 2015; Verneau *et al.* 2011).

2.2. Collection and keeping of hosts

For this study, there was no collection of new chelonian polystome specimens, and therefore the collection and keeping of turtles will not be discussed. However, attempts were made to locate *Ptychadena porosissima* and *X. laevis*. Protocol for the collection and care of the latter will be discussed. A permit for KwaZulu-Natal (Permit number: OP 4374/2015) was obtained from KZN Wildlife while permit HQ18/05/16-088 NW was obtained from North West Parks and Tourism. All handling and dissecting of frogs were done in accordance with NWU ethical standards (Ethics number: NWU-00006-14-A3).

Several individuals of wild *Xenopus laevis* were collected from various sites in Potchefstroom, South Africa. 20 L bucket-traps fitted with an inward directed funnel was baited with chicken liver enclosed in gauze to prevent ingestion thereof by the frogs (Jackson & Tinsley 1998; Theunissen *et al.* 2014). Traps were placed in a slow-moving or stagnant water body with about a quarter of the trap above the water level, to allow the captured frogs enough oxygen. They were left overnight and retrieved the following afternoon. Captured clawed toads were placed individually in 500ml plastic containers half-filled with fresh water and left overnight in an undisturbed area. The following morning the frogs were individually transferred to clean containers and water while the previous water was put aside, allowing the suspended debris to settle. The water containing the debris was scanned for the distinct *Protopolystoma xenopodis* eggs (Figure 3.2). Frogs found to be infected were separated from uninfected frogs and kept in an aquarium, while the uninfected frogs were released at the collection site. The water in the aquarium was replaced every 48 hours to collect *Pr. xenopodis* eggs. The captive frogs were fed regularly and returned to the collection site as soon as the egg production was lower than 50 eggs/48hours. The same method was then followed again to capture frogs from a different site to avoid the recapturing of individuals.

For molecular studies of *Pr. xenopodis*, some infected *X. laevis* were dissected to collect adult parasites in the urinary bladder. Dissected individuals were also used for another study assessing the total parasite load of clawed toads in Potchefstroom. Prior to dissection individuals were euthanized by placing them in individual containers containing chlorobutanol. Individuals were dissected and all the organs, including the excretory bladder, liver, digestive system, and kidneys were removed. Removed organs were placed in glass Petri dishes with saline to keep them from desiccation. The kidneys and urinary bladder were inspected for the presence of polystomes. The parasites were removed and fixed immediately in 70% analytical grade ethanol.

Several attempts to collect *Ptychadena porosissima*, the host species for *Metapolystoma porosissimae* and *Polystoma sodwanensis*, were not successful due to the prolonged drought in Northern KwaZulu-Natal, South Africa, during the 2015-2016 season.

CHAPTER 3

Fixatives and sclerite morphometrics

3.1. Introduction

Taxonomy of polystomatids rests fundamentally on the morphology and morphometrics of structures largely unaffected by fixation (Mayr 1942; Tinsley & Jackson 1998b). The sclerotized hooks of polystomes (hamuli, genital spines and marginal hooks) show relatively large inter-, but restricted intra-specific morphological variation (Tinsley & Jackson 1998b), and have therefore been proven valuable for taxonomical studies of these soft-bodied flatworms (Du Preez *et al.* 2002; Du Preez & Maritz 2006; Llewellyn 1963). In evolutionary studies, the size, shape, position and/or numbers of the hooks provide information of relationships, specificity, specialisation and reproductive isolation in the organisms over time (Fairfax 1990; Llewellyn 1970; Tinsley 1974; Vignon & Sasal 2010; Vignon *et al.* 2011).

There are some reports that sclerites of some groups may change their shape or size due to external factors such as temperature and/or age as reported for gyrodactylids and dactylogyrids (Kearn 1968; Kearn 1999; Olstad *et al.* 2009). However, in polystomatids, oncomiracidia emerge from egg capsules with a set of 16 fully formed marginal hooks, while the hamuli and suckers only develop in the post-oncomiracidial stages (Llewellyn 1963; Llewellyn 1968). The shape and size of these marginal hooklets are species-dependent and unaffected by external factors such as temperature and parasite life stage (Du Preez & Maritz 2006; Theunissen *et al.* 2014).

Marginal hooklets are primarily used during the larval phase of polystomatids for attachment to their host tissue. It functions well for initial attachment, while the suckers used by adults are more adapted to life in an enclosed environment, like the branchial chamber or urinary bladder (Bychowsky 1961; Du Preez & Maritz 2006; Theunissen *et al.* 2014; Williams 1995). In other monogeneans like gyrodactylids, the function is partially preserved to support the other attachment structures during stressful conditions (Bychowsky, 1961; Llewellyn, 1963). In polystomes, hooklets 1 and 2 are located posterior-most between the posterior pair of suckers, hooks 3-5 respectively inside suckers 1, 2, and 3, and hooklets 6-8 are located between the two anterior-most suckers (Theunissen 2014). The posterior-most pair of marginal hooklets, referred to as the C1 hooklets (Murith 1981a), is known to be somewhat larger than the other marginal hooklets in species of *Polystoma* and *Metapolystoma*, but not in other polystomatid genera (Llewellyn 1963; Theunissen 2014) (Figure 3. 1).

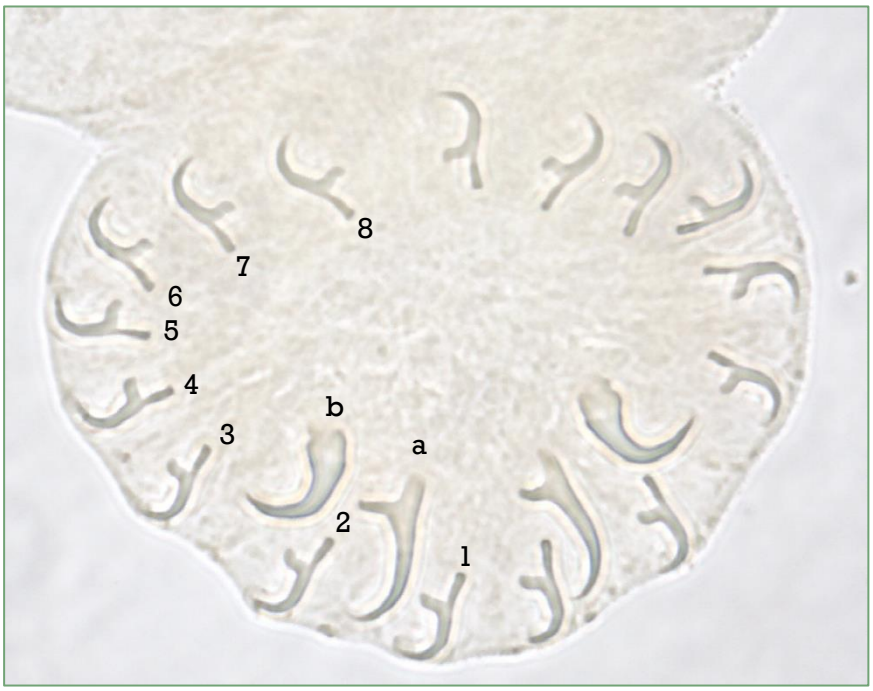

Figure 3.1: Haptor of a *Polystomoides* oncomiracidium showing the 8 pairs of marginal hooklets (1-8) and 2 pairs of developing hamuli (a & b).

In 1956a, Llewellyn noticed that the marginal hooklets showed some intra-specific variation between species. Malmberg (1970) was the first to measure these hooklets on a species of *Eupolystoma*, but his account of the hooklets was confusing, to say the least. According to Kok and Van Wyk (1986), the most valuable contribution to the morphometrics of marginal hooklets was that of Murith (1979; 1981a; 1981b). She suggested a protocol for measuring the marginal hooklets of polystomes, where the C1 hooklets are used for classification. Using this protocol, Tinsley and Jackson (1998b) claimed that the dimensions of the marginal hooklets of *Protopolystoma* species were not of particular value. This led Du Preez and Maritz (2006) to note additional problems, leading to subjectivity in taking the measurements according to Murith's protocol. They set to work developing a new, objective and easy to use protocol for measuring marginal hooklets with a high repeatability and high classification potential (Figure 3.3). However, they cautioned that the hooklet should be on the same focal plane in order for the measurement to be absolutely accurate (Du Preez & Maritz 2006).

The main concern of using hard, sclerotized parts as taxonomical characteristic is that the shape of these parts can be distorted by preparation techniques and by the way the specimens are orientated on the slides (Teo *et al.* 2013). Several authors have suggested that the flattening of parasites during the fixation (i.e. under cover slip pressure) could influence their morphology and morphometrics (Platt 2011; Prudhoe & Bray 1982). Platt

(2011) proposed that some specimens should be fixed without flattening them, but those used for sclerite measurements ought to be flattened under coverslip pressure for accurate measurements.

Cox *et al.* (2006) studied the effects of fixatives on tissue morphology and RNA integrity of rat liver. They found that it had an important effect, with some fixatives preserving the morphology well while compromising the integrity of the RNA, and vice versa. Regarding polystomes, Tinsley and Jackson (1998b) also found fixatives to affect the sclerites of *Protopolystoma* individuals. According to their study, specimens fixed in different chemical conditions from preserved hosts showed a slight deviation from the data reported in previous studies when it came to the morphometrics of sclerites. Those obtained from fresh hosts, fixed in 4% formal saline and mounted in Canada balsam had morphometrics similar to those of previous studies (Tinsley & Jackson 1998b).

Besides this latter study, there has been no other mention of the effects of fixatives on the sclerites of polystomatids. Therefore, the aim of this study was to assess whether different fixatives may have an effect on the morphometrics of marginal hooklets. This was primarily done by determining the effect of different, commonly used fixatives on their morphometrics. Secondarily, the sizes of the marginal hooks found in adults and oncomiracidia were compared, in order to confirm that these hooks do not, in fact, change during maturation. It is suspected that neither fixatives nor life stage of the parasite, will have an effect on the marginal hooklet. Hence, it will remain a valuable structure for classification.

3.2. Methods

To determine the effect of fixatives on the marginal hooklet measurements, oncomiracidia of *Protopolystoma xenopodis* and *Polystoma channingi* were used. The influence of parasite life-stage was measured using adults, oncomiracidia, bladder-destined parasites still situated on the tadpole's gills, and neotenic forms of *Metapolystoma porosissimae*, *Polystoma sodwanensis*, *Polystoma channingi* and *Protopolystoma xenopodis*. The latter two life-stages depended on the availability.

Most specimens measured were pre-fixed and obtained from the collection of L.H. du Preez. However, newly hatched oncomiracidia of *Protopolystoma xenopodis* were also used due to the easy accessibility and availability of the host, *Xenopus laevis*, and the continuous reproductive output of the adults (Jackson & Tinsley 1988; Jackson & Tinsley 2001). According to Jackson (1982a), the daily rate of output was on average

10eggs/worm/day at 20°C. A minimum estimate of fertilization efficiency was gained from the proportion of larvae developing to the point of hatching. There was a 94.8 – 97.6 % mean viability for the eggs in her study (Jackson 1982b).

For the newly-hatched oncomiracidia, wild *X. laevis* were collected from various sites by using 20 L bucket-traps fitted with an inward directed funnel and baited with chicken liver (Jackson & Tinsley 1998; Theunissen *et al.* 2014). Traps were left in the pond overnight and retrieved the following afternoon. Captured clawed toads were placed individually in 500ml plastic tubs half-filled with water and left overnight. The following morning the frogs were transferred to clean tubs and water while the previous water was put aside, allowing the suspended debris to settle. The supernatant was decanted and the sediment and eggs, if present, remaining in the tubs were gently rotated to allow centripetal force to concentrate the heavier items, like the eggs, at the centre of the tub. A positively infected frog's water would contain the characteristic, golden fusiform eggs of *Pr. xenopodis* easily seen under a stereo-microscope (Figure 3.2.).

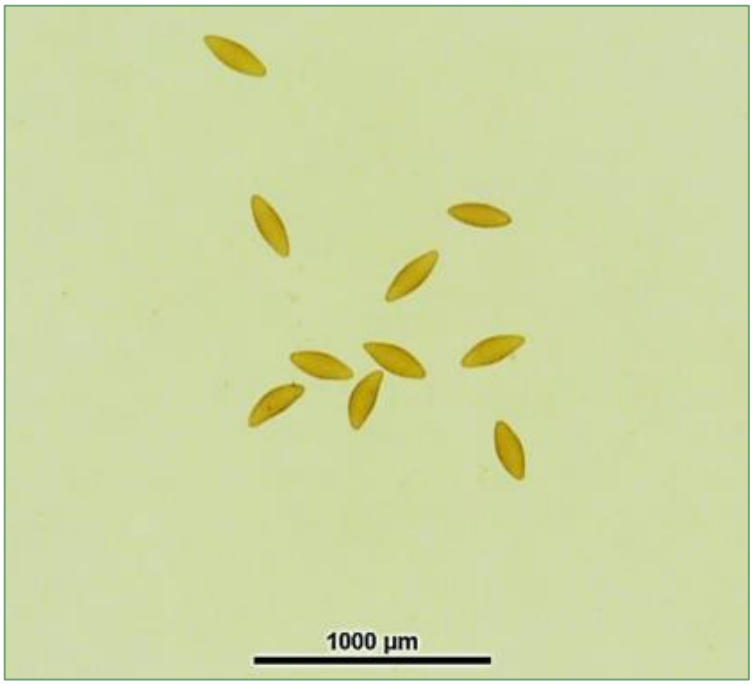

Figure 3.2: Golden, fusiform eggs of Protopolystoma xenopodis as seen under a stereo microscope. Photo: M. Theunissen (2014)

Infected frogs were kept in an aquarium, while the uninfected frogs were released at the collection site. The water in the aquarium was replaced every 48 hours. Eggs were collected by pouring the old water through two plankton sieves measuring 500um and 100um respectively and kept in an incubator at 22°C. Egg output in *Protopolystoma xenopodis* is highly sensitive to temperature fluctuation, as is the development of the oncomiracidia in the eggs (Jackson & Tinsley 1998; Theunissen *et al.* 2014). The incubation period for *Pr. xenopodis* is approximately 22 days (Theunissen *et al.* 2014). When the egg

production ceased, the *X. laevis* individuals were released in the pond where they had been caught, and frogs from another pond were obtained for further use.

The development of the oncomiracidia was monitored using a dissecting microscope. Developed eggs were put in direct sunlight for 30 seconds to initiate hatching (Theunissen *et al.* 2014; Tinsley & Owen 1975; Warkentin 2011). Immediately after hatching, the oncomiracidia were fixed using different fixatives. The initially proposed fixatives, included 70% ethanol, neutral buffered formalin (NBF), ammonium picrate glycerine, and lactophenol (Aisien & du Preez 2009; Du Preez 2011, 2013; Du Preez *et al.* 2002; Tinsley & Jackson 1998b). Due to unfortunate and unexplained problems with the development of oncomiracidia, only enough oncomiracidia hatched for fixation in the latter two fixatives. Sufficient oncomiracidia to fix in ethanol and NBF were not obtained. Previously fixed specimens from the collection were used instead.

Sclerites are traditionally analysed using a set of linear distance measurements, indicating only the size of the sclerites, (Vignon *et al.* 2011) with the use of geometric morphometrics, indicating also the shape, only emerging recently (Vignon & Sasal 2010; Vignon *et al.* 2011). However, for the study of polystomatids, the traditional, linear measurements (Du Preez & Maritz 2006) are adequate, since all polystomatid marginal hooks have the same basic shape, and the protocol was specifically designed for the morphometric measurements to give some idea of the shape of the hooklets (Du Preez & Maritz 2006). The most posterior-medial hooklets (C1 hooks) were measured. The measurements taken for adequate separation of species, as suggested by Du Preez and Maritz (2006), include (a) the length of the hooklet, (b) the aperture distance of the hook and (c) the width of the hooklet (Figure 3.3). All measurements were taken only if the marginal hooklet was flat on

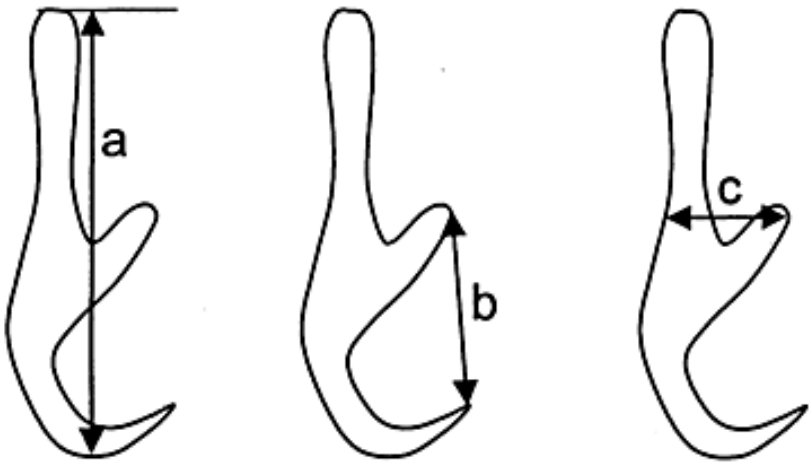

Figure 3.3: Measurements taken for C1 marginal hooklets of polystomatid flatworms. (a) length of the hooklet, (b) aperture distance of the hook and (c) width of the hooklet

the same focal plane, using a Nikon Eclipse E800 compound microscope. Marginal hooklet plots were then compiled by plotting a x b on the x-axis and a x c on the y-axis. Statistical significance was measured using StatSoft Statistica v.13.1.

3.3. Results

Attempts to successfully hatch *Protopolystoma* eggs in order to fix enough oncomiracidia in different fixatives failed to some extent. Although the collection and incubation methods described by Theunissen *et al.* (2014) were followed precisely, only about 40 eggs hatched during a period of about a year. Most of the eggs seemed to have failed to develop to the point where hatching should occur, while other eggs hatched after 40 days of incubation, which is double their normal incubation time of 22 days. Those that did in fact hatch were fixed and measured, and the results of the effect of fixatives on the marginal hooklets are shown in Figure 3.4.

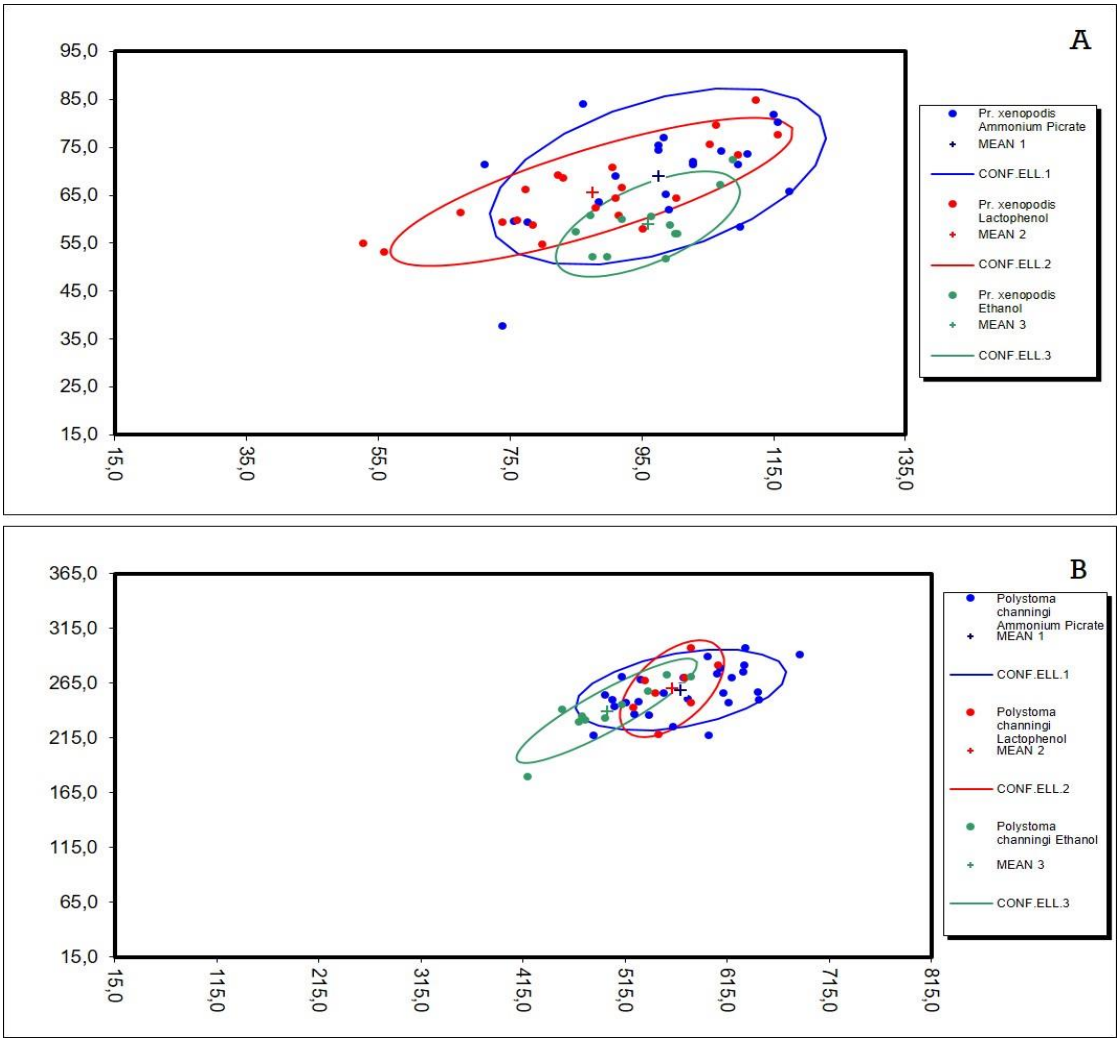

Figure 3.4. *Comparison between the marginal hooklet sizes of individuals fixed in ammonium picrate, lactophenol and ethanol respectively. (A) Protopolystoma xenopodis, and (B) Polystoma channingi.*

All confidence ellipses were drawn at Chi = 1.80, 80.2% percentile. For *Protopolystoma xenopodis* a total of 55 hooklets was measured for the creation of the marginal hooklet plots (21 ammonium picrate, 22 lactophenol and 12 ethanol). However, Figure 3.4A shows that all the hooklets are centred on the same point, with all three means close to each other. For the hooklets fixed in ammonium picrate, 81% of the points fell in the confidence ellipse, whereas for lactophenol and ethanol these numbers were 82.8% and 83.3% respectively.

A total of 48 hooklets were used in the analysis of *Polystoma channingi* from Potchefstroom, of which 30 were fixed with ammonium picrate, eight with lactophenol, and ten with ethanol. For ammonium picrate, 83.3% of the points were within the 80.2% percentile confidence ellipse, 70% for ethanol, and 100% for lactophenol. In Figure 3.4B the clusters for all these data combined are indicated. The means for ammonium picrate and lactophenol are very similar.

Table 3.1: Minimum, maximum and mean marginal hooklet length of polystomes of different life-stages

	P. marmorati			P. sodwanensis		P. channingi			M. porosissimae	
	Adult	Oncomi-racidium	Neotenic	Adult	Oncomi-racidium	Adult	Oncomi-racidium	Bladder on gill	Adult	Oncomi-racidium
Mean	40,16	40,99	37,89	34,83	35,96	32,92	33,61	34,56	31,4	35,27
Min.	38,07	35,57	32,74	31,31	30,27	28,05	30,18	30,79	25,62	32,19
Max.	42,86	44,79	40,98	36,71	42,11	35,7	35,89	37,13	34,88	37,81

The comparison between the marginal hooklet lengths of different life-stages is shown in Figure 3.5, and the average, minimum and maximum hooklet lengths for each group are presented in Table 3.1. The measurements of *Metapolystoma porosissimae* are indicated in Figure 3.5 A. Only 17 adults and 30 oncomiracidia were available for this analysis, with 76,5% and 80% of the points falling into the respective confidence ellipses. A T-test indicated that the differences between the adult and oncomiracidial hooklet sizes were statistically significant, with a p-value of 0.00000.

The clusters for the measurements of *Polystoma sodwanensis* are shown in Figure 3.5 B. Ten adults and 30 oncomiracidia were used. There was a clear overlapping of the points, with not much difference between the two means. 80% of the adult points were located within the confidence ellipse, compared to 86.7% for the oncomiracidia.

All the data for *Polystoma channingi* are relatively centred around the three means (Figure 3.5 C). There were 30 bladder-destined oncomiracidia, taken off the gills of the tadpoles, fixed and measured, and 83.3% were placed within the confidence ellipse. 84.2% of the 19 adults and 72% of the 25 oncomiracidia fell into this ellipse.

There was a significant difference in the relative sizes of the hooklets for *Polystoma marmorati* (Figure 3.5 D). A total of 25, 23, and 21 measurements were taken for neotenic

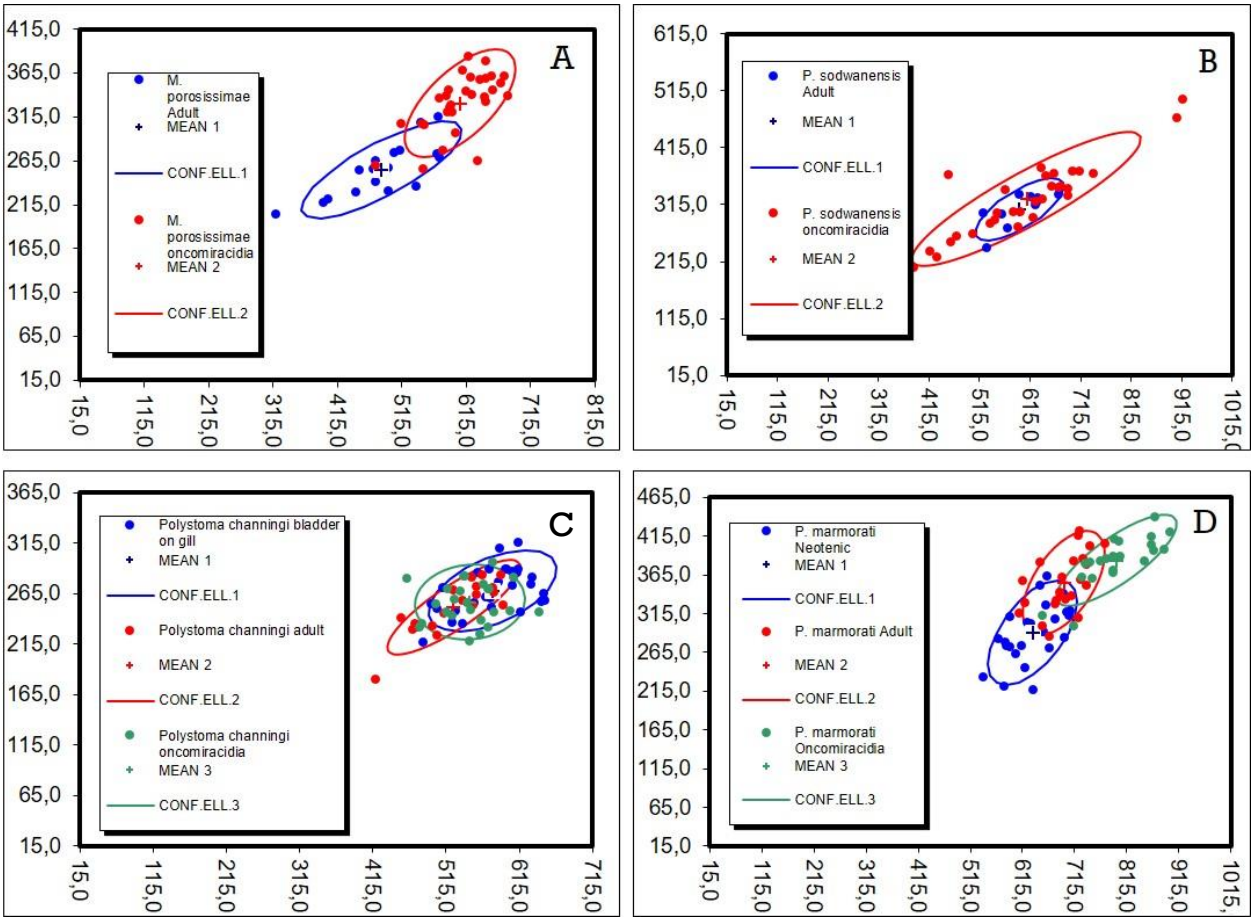

Figure 3.5: Comparison between the marginal hooklet sizes of normal and neotenic adults and oncomiracidia of different species. The latter adults were only measured if available. (A) Metapolystoma porosissimae, (B) Polystoma sodwanensis, (C) Polystoma channingi, (D) Polystoma marmorati

adults, normal adults and oncomiracidia respectively. For the neotenic adults, 84% of the points were in the confidence ellipse, compared to 69,6% for the normal adults, and 85.7% for the oncomiracidia. The p-value for the T-test comparing neotenic parasites with adults and oncomiracidia, were 0.000007 and 0.000001 respectively, making the difference very significant. The difference between normal adults and oncomiracidia were not at all significant (p = 0.1).

3.4. Discussion and conclusion

Our failed attempts to successfully hatch *Protopolystoma xenopodis* eggs are somewhat unexplained, although there are some possible explanations. Warkentin (2011) describes that periods of developmental stasis, or dormancy, may occur within some eggs. If environmental conditions directly limit development, this is considered quiescence. However, when dormancy is endogenously maintained, even when conditions permit development, it is referred to as diapause (Warkentin 2011). In our case, we experienced a certain case of diapause. However, it was not observed that the embryos ever restarted their development after this period of dormancy. After some time had passed after the regular incubation period of 22 days, the eggs were found to be dull and the embryos dead, since yolk amounts limit energy reserves and the structure of the egg capsule affects oxygen supply (Warkentin 2011). Tinsley and Jackson (1998a) showed that there was some variation in egg viability associated with eggs from concurrent infections. This reduced viability could have had an effect in our study since individuals of *Xenopus laevis* were sampled from different sites near each other. However, the probability of catching the same individuals more than once in different sites is quite low.

Warkentin (2011) explained that encapsulated embryos of parasitic flatworms are hardier and more tolerant of environmental variation than newly-hatched larvae. However, she also claimed that embryos were sensitive to cues from the environment, which may directly indicate, or be associated with host availability. In this study, the few eggs that did in fact hatch were incubated in water from the pond where the hosts had been caught. However, when using water from the same pond at a later stage, there was no development. Eggs were not incubated in the water collected from the tank where infected hosts were kept for the duration of this study. But this would make an interesting study, as there may be chemical cues in the water where the eggs were originally laid that disappear after some time.

Theunissen *et al.* (2014) found the marginal hooklets of *Pr. xenopodis* to be approximately 13-14µm in length. Our means for all three fixatives were also in that range, however, the minimum found was 11,8 µm, and the maximum 15,04µm, both fixed in ammonium picrate. The minimum and maximum lengths of the other two fixatives did not differ that much from this. Generally, the fixatives did not have a definite effect on the size of the marginal hooklets (Figure 3.4).

It is commonly assumed that the marginal hooklets remain the same size and shape during adulthood, even though they are functionally replaced by suckers (Du Preez *et al.* 2002;

Du Preez & Maritz 2006; Llewellyn 1970; Tinsley & Jackson 1998b). This was indicated in our study since *Polystoma sodwanensis* and *Polystoma channingi* showed little to no change in hooklet sizes over time (Figures 3.5 B and C). According to the measurements taken by Du Preez and Kok (1992a), the mean length of *P. sodwanensis* C1 marginal hooklets was 38µm, whereas our study found the mean at 36µm. Although the mean reported by this study was smaller, the range still included the mean reported by Du Preez & Kok (1992a), and the difference may only be credited to the number of specimens measured. For *P. channingi* the reported mean length of the C1 hooks is 34µm (Du Preez 2013), thus confirming our results (Table 3.1).

Surprisingly, there was a substantial difference between the sizes of hooklets on oncomiracidia and adults of *Metapolystoma porosissimae* (Figure 3.5A), as well as between normal and neotenic adults of *Polystoma marmorati* (Figure 3.5D). Du Preez and Kok (1992a) reported the length of *Metapolystoma porosissimae*'s C1 hooklets to be 35µm long, while our study found a similar mean for oncomiracidia, but not for adults (31.40µm; p = 0.000000) (Table 3.1). According to Van Niekerk, Kok and Seaman (1993) *Polystoma marmorati* had C1 marginal hooks 41µm long, whereas ours were 40.2, 41.0 and 37.9 for adults, oncomiracidia, and neotenic parasites respectively (Table 3.1) (p = 0.000001 / 0.000007). Kearn (1999) described that in some monogeneans, the marginal hooklets assisted the hamuli and suckers in adults for a firmer grip in extra stressful times, such as when a fish rapidly moves away from a predator. However, this is not known in polystomatids.

Kearn (1993) and Lambert (1981) noted that several changes take place once an oncomiracidium locates a host, for example, the loss of their cilia, or their eyes. It is assumed that chemical cues emitted by the pre-metamorphosed tadpole, causes the development of a neotenic parasite from the oncomiracidium (Badets *et al.* 2013; Kearn 1981; Murith 1981a). The reduction in hooklet length may, therefore, be one more change occurring after location of a host, brought about by the chemical signals from the tadpole. A reduced hooklet will still allow the neotenic parasite to adequately attach to the tadpoles' gills since the lamellae are thin and soft.

Kearn (1999) explained that in some gyrodactylids the marginal hooklets grow incessantly. However, contrary to this, our results (Figure 3.5 A) suggested that some of the polystomes' hooks reduce in size from oncomiracidium to adult. This could be expected, since these are the only structures available to the oncomiracidium for attachment (Llewellyn 1963; Theunissen *et al.* 2014). As the parasite matures and loses this function, it becomes reduced, vestigial features. In some fish monogeneans, like

Leptocotyle minor, the hooks are present in the oncomiracidium but absent in the adult. This is because of the site of infection. *L. minor* attaches to a hard surface, therefore impenetrable for hooks. They rather 'cement' themselves to the host. Several *Microcotyle* species are also known to gradually lose their marginal hooklets, with some degeneration occurring first (Llewellyn 1968).

Reduced hook size may also be a consequence of the miniaturization process: by reducing the size of the hooklets, the flexible haptoral suckers have more manoeuvrability, which causes a better grip on the host (Hanken & Wake 1993; Theunissen 2014).

In a molecular study, using COI and 28S genes (Chapter 4), *P. marmorati* and *Metapolystoma* spp. were placed next to, or close-by each other, on the tree (Figure 4.6). This may indicate near-relation and may be why these two species showed the same difference in marginal hooklet sizes. However, more study is needed on this.

The true reason and extent of the difference in marginal hooklet sizes of adults, oncomiracidia and/or neotenic forms of some polystomatids, remain to be discovered. Morphologists studying this group should, therefore, proceed with caution when using only marginal hooklets for species differentiation. Since we found no effect regarding the different fixatives used, it can be concluded that taxonomists may continue using whatever is most readily available to them.

CHAPTER 4

Results: *Metapolystoma* as taxon: Valid or misnomer

4.1. Introduction

The Polystomatidae consist of more than 200 species in 26 genera. Among those infecting amphibians, only four genera (53 species) occur in Africa. This includes four species of *Eupolystoma* Kaw, 1950, three species of *Metapolystoma* Yamaguti, 1963, 40 species of *Polystoma* Zeder, 1800, and six species of *Protopolystoma* Bychowsky, 1957. This chapter will mainly focus on the genus *Metapolystoma*.

Verneau *et al.* (2009b) suggested that *Metapolystoma* colonized Madagascar from Africa at ± 14 Mya, following a natural overseas dispersal of the anuran host, *Ptychadena*. The genus *Metapolystoma* was proposed by Combes (1976) for two species which were then classified as members of *Polystoma*. However, based on the elongated uterus, and supported by other aspects of their biology, Combes suggested that *P. brygoonis* (Euzet & Combes 1964) and *P. cachani* (Gallien 1956) be elevated to their own genus, namely *Metapolystoma* (Combes 1976). Du Preez and Kok (1992a) later described the third species in this genus, *M. porosissimae* from the host *Ptychadena porosissima*.

Based on molecular analyses, several authors have suggested that the genus may not be valid. The phylogenetic trees compiled by Bentz *et al.* (2001), Verneau *et al.* (2002), and Olson and Tkach (2005) all indicated *Metapolystoma* placed in the middle of *Polystoma* clades. Olson and Tkach (2005) placed them between the clades for African *Polystoma* and South African *Polystoma* species respectively. Bentz *et al.* (2001) boldly stated that they did not consider the genus to be valid. She further suggested that the elongated uterus may only be a homoplastic trait resulting from the ecology of the host. This supports Tinsley's (1974) statement that the uterine structure within *M. cachani* and *M. brygoonis* may have been obtained through convergence.

Another situation which casts a shadow over the validity of the genus is the simultaneous co-occurrence of *Polystoma sodwanensis* and *Metapolystoma porosissimae* in the same host individual of *Ptychadena porosissima* (see Du Preez 1992a). Polystomes of anurans are assumed to be very host-specific, and the occurrence of two species in one host is very rare, although not unheard-of (Aisien & Du Preez 2009; Badets *et al.* 2011; Bychowsky 1961; Hargis 1957; Héritier *et al.* 2015; Jackson *et al.* 1998; Kaci-Chaouch *et al.* 2008; Llewellyn 1957; Verneau *et al.* 2011). In the instances where two polystome species were found in the same anuran host species, there was either a distinct allopatric separation in the populations (Berthier *et al.* 2014), or it may have been the result of recent duplication (intrahost

divergence) events within the host (Badets *et al.* 2011). For example, Bourgat & Murith (1980) reported the sympatric occurrence of two species, *Polystoma lamottei* and *P. aeschlimanni*, in *Ptychadena pumilio* in Togo.

However, the co-occurrence between *P. sodwanensis* and *M. porosissimae* described by Du Preez and Kok (1992a), was rather unusual, since it was the first report of two genera occurring together in a single host individual. Different genera, and species within genera, have markedly different life-history strategies related to the ecology of their hosts (Murith 1981b; Du Preez & Kok 1992a). In this specific situation, *Metapolystoma* has a large egg storage capacity and intra-uterine development of eggs, which gives it the typical life-history strategy of a parasite living in a 'xerophilic' host (an indicator of a moderately arid environment). In contrast, however, *Polystoma* shows the typical life-history strategy found in the genus, i.e. to be very water-dependant (an indicator of a wet or tropical environment) (Du Preez & Kok 1992b). Therefore, finding both of these parasites, on opposite extremes based on life-history strategies, in one host is extremely unusual (Du Preez & Kok 1992a).

This situation adds to the confusion surrounding *Metapolystoma*, and it has been suggested that the genus may only represent ecologically induced morphological plasticity (Bentz *et al.* 2001). Therefore, the aim of this study was therefore to determine the validity of *Metapolystoma* (Yamaguti, 1963) by using both molecular and morphological methods. We hypothesize that *Metapolystoma* will indeed be a valid genus that may have originated from and is closely related to *Polystoma*.

4.2. Methods

Several attempts to collect new specimens failed due to the unavailability of the host (*Pt. porosissima*) during the prolonged drought experienced in Northern KwaZulu-Natal. Original mounted material for *P. sodwanensis* and *M. porosissimae* (adults and oncomiracidia) from 1992 was obtained from the collection of L.H. du Preez.

The mounted specimens were originally fixed in 70% EtOH and stained in Alum Carmine. Their sclerites measured according to the suggested protocols for marginal hooklets (Du Preez & Maritz 2006) and hamuli (Du Preez *et al.* 2010) respectively. Using a combination of these features has proven valuable for taxonomy, since using only one may result in overlapping and misclassification (Du Preez & Maritz 2006; Tinsley 1974).

Tinsley and Jackson (1998b) suggested using a different protocol for the hamuli, which were then plotted using a principal component analysis (PCA) for assessing inter-specific variation (Figure 4.1).

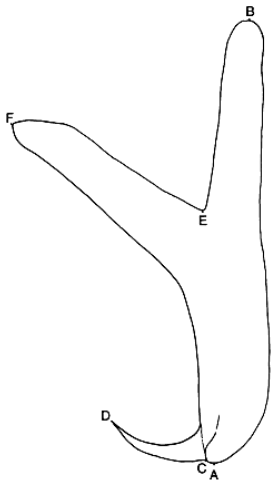

Figure 4.1. Hamulus morphometric characters as suggested by Tinsley & Jackson (1998b) A-B is the total length, C-D the point length, A-E the shaft length, E-B the dorsal root length, E-F the ventral root length, E-D the fork-point distance, and F-B the inter-root distance.

However, in a study conducted by Jackson and Tinsley (2007) all of these measurements, except for the point length, were found to increase in the adult. Therefore, we used the suggested measurements given in Du Preez *et al.* (2010), including the outer hamulus length (X), inner hamulus length (Y), and point length, since this is the most recent description of these measurements (Figure 4.2).

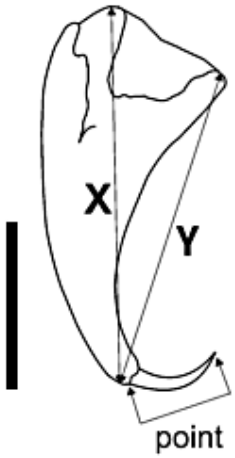

Figure 4.2. Measurements taken for the hamulus during this study, as suggested by Du Preez *et al.* (2010)

Several authors have found the marginal hooklet length and genital spine length to remain stable in adulthood (Du Preez & Maritz 2006; Jackson & Tinsley 2007; Llewellyn 1963; Llewellyn 1968; Tinsley 2016). The length and number of the genital spines were recorded, as well as the marginal hooklet measurements described in Figure 3.3 (Chapter 3). All measurements were made using a Nikon Eclipse E800 compound microscope.

To assess the relationships between *Metapolystoma* and *Polystoma*, two phylogenetic analyses of the mitochondrial (COI) and nuclear (28 rRNA) genes were conducted on *Polystoma channingi, Polystoma marmorati* and *Metapolystoma brygoonis*. The nuclear 28S rRNA gene is highly conservative and has been traditionally applied to explore the relationship between taxa on the suprageneric level, whereas the fast evolving mitochondrial COI gene was found to be more effective for distinguishing between closely related species. We, therefore, used both in our analyses.

Previously collected *P. channingi* were used for initial set-up of the protocol. Total genomic DNA was isolated from a single ethanol preserved worm using the standard protocol for the Kapa Express Extract kit (Kapa Biosystems, Cape Town, South Africa). Amplification of the mitochondrial COI gene was performed with the forward primer L-COIp (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and the reverse primer H-Cox1R (5'-AACAACAAACCAAGAATCATG-3') published by Littlewood *et al.* (1997). Partial 28S rDNA sequence was amplified using primer combinations LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3') and IR16 (5'-ATTCACACCCATTGACTCGCG-3') (Sinnappah *et al.* 2001; Verneau *et al.* 2009). Amplification was carried out following thermocycling profile: one initial step of 5' at 95°C for long denaturation; 30 cycles of 1' at 94°C for denaturation, 1' at 55 °C/58°C for 28S and COI annealing respectively, and 2' at 72°C for elongation; one final step of 10' at 72°C for terminal elongation. The PCR product was run through a 1% Agarose gel.

The PCR products obtained from each sample were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd. in Pretoria, South Africa) for purification and sequencing in both directions. Contiguous sequences were assembled and edited using Geneious Ver. 7.1 (created by Biomatters. Available from http://www.geneious.com). The partial 28S and COI sequences generated during this study were aligned with other African amphibian polystome species available on GenBank using MUSCLE implemented in Geneious Ver. 7.1; COI dataset was aligned with reference to the amino acid translation, using

the flatworm mitochondrial code, but analysed solely as nucleotides. Based on the topologies in the phylogenetic trees of the Polystomatidae published by Héritier *et al.* (2015), *Occulotrema hippopotami* (Stunkard, 1924) was used as the outgroup. Genbank accession numbers for all African amphibian polystome species used are shown in Appendix 1. The alignment was then trimmed to the length of the shortest sequence.

Phylogenetic analyses were carried out using Maximum Likelihood (ML) and Bayesian inference (BI) methods. Prior to analyses, the best-fitting model of nucleotide substitution was estimated using jModelTest 2 software (Darriba *et al.*, 2012). For the COI gene, this was the general time reversible model, with estimates of invariant sites and gamma distributed among-site rate variation (GTR + G + I), whereas it was the general time reversible model, with estimates of invariant sites (GTR + I) for 28S. BI analysis was performed using MrBayes software (ver. 3.2.3) (Ronquist *et al.*, 2012) run on the CIPRES portal (Miller *et al.*, 2010). ML analysis was performed using PhyML version 3.0 (Guindon *et al.*, 2010) run on the ATGC bioinformatics platform [http://www.atgc-montpellier.fr/ngs]. Nodal support in the ML analyses was estimated from 100 bootstrap pseudo-replicates. Trees were visualized using the FigTree ver. 1.4 software (Rambaut, 2012). Clades were considered to have high nodal support if Bayesian interference posterior probability was ≥0.90 and for ML bootstrap value was ≥ 70.

Fresh *Pr. xenopodis* were collected from dissected *X. laevis* and compared to previously collected samples, preserved in 70% molecular ethanol. Dissections to obtain the fresh specimens were done in accordance with NWU ethical standards (Ethics number: NWU-00006-14-A3; Permit number: HQ18/05/16-088 NW). Prior to dissection individuals were euthanized by placing them in individual containers containing chlorobutanol. Individuals were dissected and the urinary bladders and kidneys removed. If the individual hosted an adult *P. xenopodis*, it could be seen through the transparent walls of the urinary bladder. The collected *P. xenopodis* were fixed in 70% molecular ethanol and the frogs frozen until the remains could be discarded at a later stage.

Individuals of *Pr. xenopodis*, *P. channingi, P. marmorati* and *M. brygoonis* were subjected to either one or both of two DNA extraction methods, namely a longer method by Macherey-Nagel (NucleoSpin® Tissue, Genomic DNA from tissue – June 2014/Rev.14), and a shorter method by Kapa Biosystems.

In the light of the fact that we were unable to obtain fresh material of *Polystoma sodwanensis* and *M. porosissimae* we attempted to obtain DNA from ethanol-fixed stained specimens mounted on permanent microscope slides. If successful this would be a valuable tool in cases where obtaining fresh individuals is impossible or improbable due to logistic difficulties, as in our case, drought. Although the samples were more than ten years old, we expected that their 70% Ethanol fixation would allow for DNA-extraction, as Cox *et al.* (2006) found that this fixative was one of the top three methods to use as an alternative to formalin (which perfectly preserves the morphology, but compromises the RNA integrity) in order to maintain tissue morphology and RNA quality.

Specimens of *P. marmorati* ex *Hyperolius marmoratus* collected in December 1990 at Vernon Crooks, KwaZulu-Natal, South Africa, fixed in 70% ethanol, stained with Borax carmine, and mounted in Canada Balsam and Entellan were selected for this purpose. This species was selected due to availability of material. Individuals were manually removed from microscope slides. The extractions were PCR amplified and run through a 1% Agarose gel.

4.3. Results

4.3.1. Morphological analysis

The comparative general morphology of the sclerites is presented in Figure 4.3.

The results for all sclerites measured can be seen in Table 4.1, and the marginal hooklet plots are shown in Figure 4.4. For the marginal hooklet plots of *P. sodwanensis*, the number of points inside the 82% (Chi=1.80) confidence ellipses was 80% and 86,7% for adults and oncomiracidia respectively (Figure 4.4B). For *M. porosissimae* there were 76.5% and 80% of the points in these ellipses for adults and oncomiracidia respectively. Combining adults and oncomiracidia for each species, 93.3% of the points fell into the confidence ellipse for *P. sodwanensis*, compared to the 86.7% for *M. porosissimae* (Figure 4.4A). Although the marginal hooklet plots show a distinct overlapping of the two species, a t-test showed a significant difference in the marginal hooklet length ($p=0.00031$), genital spine length ($p=0.00002$) as well as hamulus length ($p=0.00664$). The hamulus shows increasing significance with when measuring the total length and point lengths ($p=0.000001$).

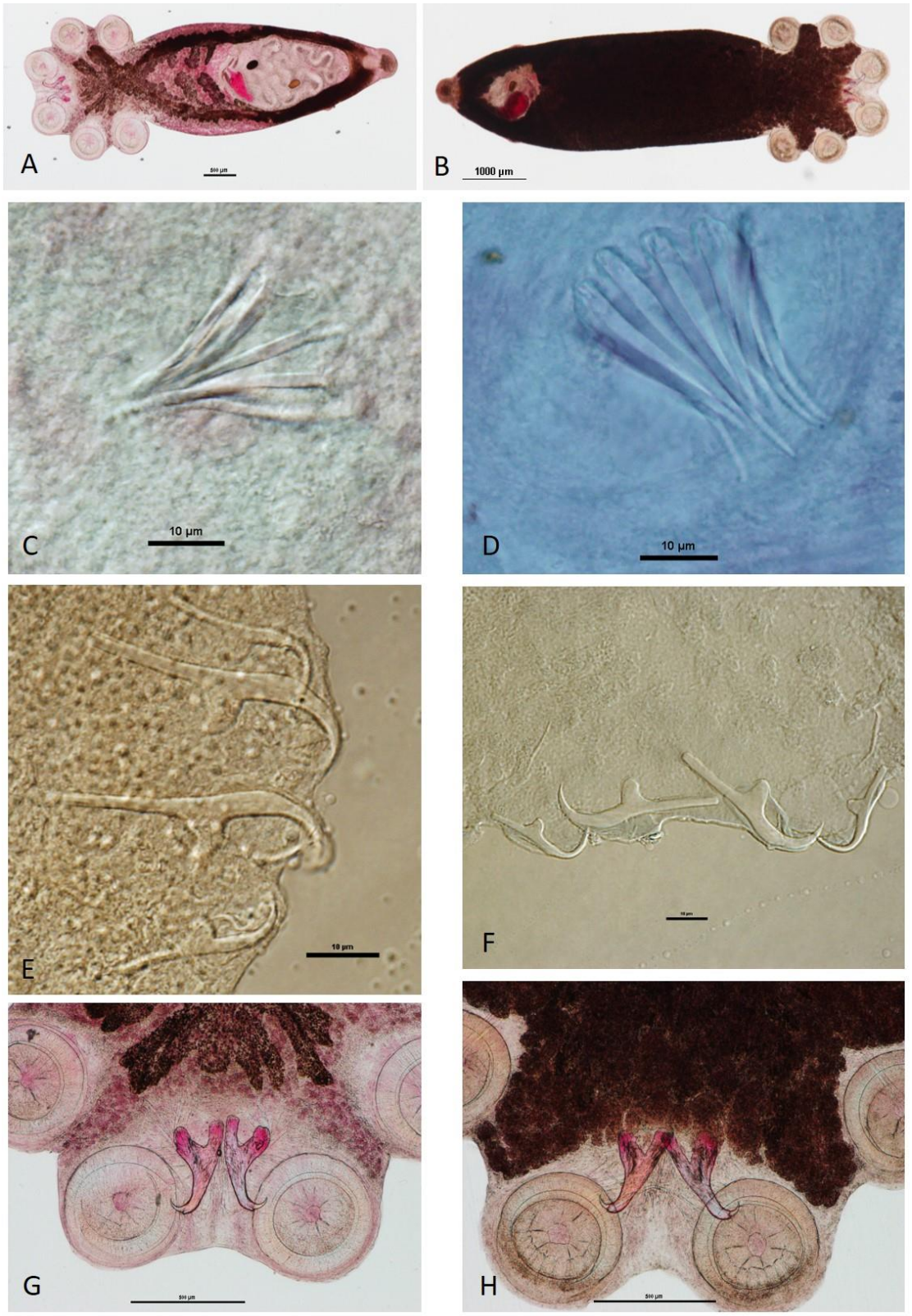

Figure 4.3. Comparative general morphology of *Metapolystoma porosissimae* and *Polystoma sodwanensis*. (A) Full *M. porosissimae* parasite – Scale: 500µm; (B) Full *P. sodwanensis* parasite – Scale: 1000µm; (C) Genital spines of *M. porosissimae*; (D) Genital spines of *P. sodwanensis*; (E) C1 marginal hooklets on the oncomiracidium of *M. porosissimae* – Scale: 10µm; (F) C1 marginal hooklets on the oncomiracidium of *P. sodwanensis* – Scale: 10µm; (G) Hamuli of *M. porosissimae* – Scale: 500µm; (H) Hamuli of *P. sodwanensis* – Scale: 500µm.

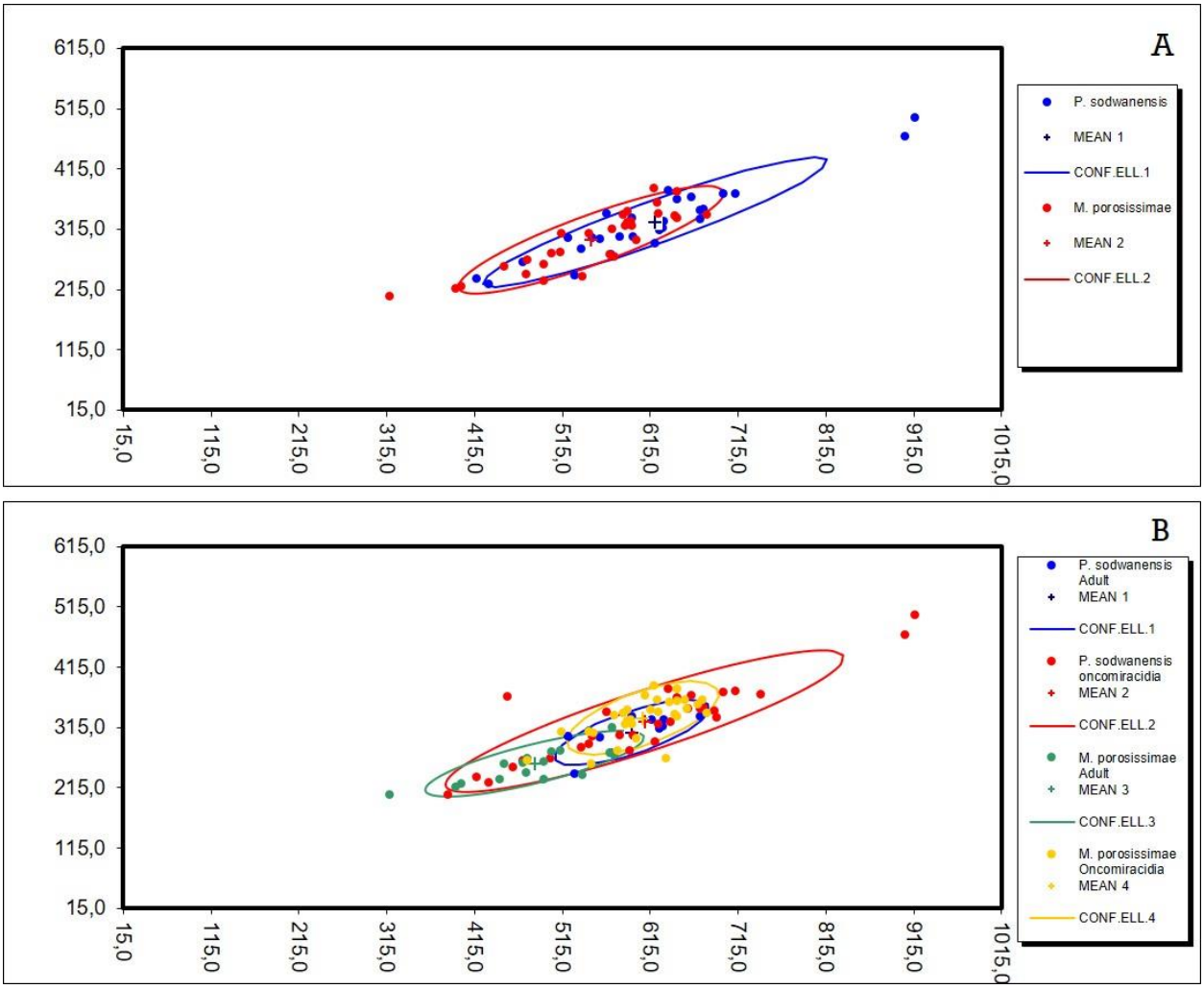

Figure 4.4: Marginal hooklet plots of *Metapolystoma porosissimae* and *Polystoma sodwanensis*. (A) shows not distinction between adults and oncomiracidia, while this distinction is visible in (B).

Table 4.1: Sclerite measurements for *Metapolystoma porosissimae* and *Polystoma sodwanensis*.

	Marginal hooklet length (µm)	Genital spine length (µm)	Hamulus length (X) (µm)	Hamulus length (Y) (µm)	Hamulus point length (µm)
	Metapolystoma porosissimae				
Mean	33,87	26,26	414,94	353,80	76,85
Minimum	25,62	23,36	298,33	301,70	64,60
Maximum	37,81	30,90	480,43	407,35	92,17
	Polystoma sodwanensis				
Mean	35,76	29,78	385,02	320,25	67,08
Minimum	28,36	27,57	343,08	277,72	57,35
Maximum	42,11	34,03	406,16	347,37	72,42

4.3.2. Molecular analysis

Successful amplification was achieved from eight representative specimens, viz. two *P. channingi* (one 28SrDNA and one COI), two *M. brygoonis* ex *Pt. mascariensis* (one 28SrDNA and one COI) and four *Pr. xenopodis* ex *X. laevis* (two 28SrDNA and two COI). Sequences for 28S rDNA obtained in the present study were represented by a short fragment (≈ 700 nt), since we did not succeed in amplifying the first fragment of the 28S gene with the primers reported in Héritier *et al.* (2015). The lengths of the sequences for COI were ≈ 400 nt.

The sequences from our PCR products were combined with sequences obtained from GenBank (Appendix 1) to create a consensus tree inferred from the Bayesian analysis. BI and ML analyses of the 28S (Figure 4.6) and COI (Figure 4.7) datasets depicted two strongly supported clades. The first clade comprised species of the *Polystoma* and *Metapolystoma* without clear separation into different genus level clades. The second clade comprised species of *Protopolystoma* and provide congruent strong support for a single haplotype known as *Pr. xenopodis*.

Our attempt to extract DNA from specimens permanently mounted on slides was not successful (Figure 4.5 C & D), thus indicating the importance of the freshly collected and ethanol-fixed material for the molecular studies.

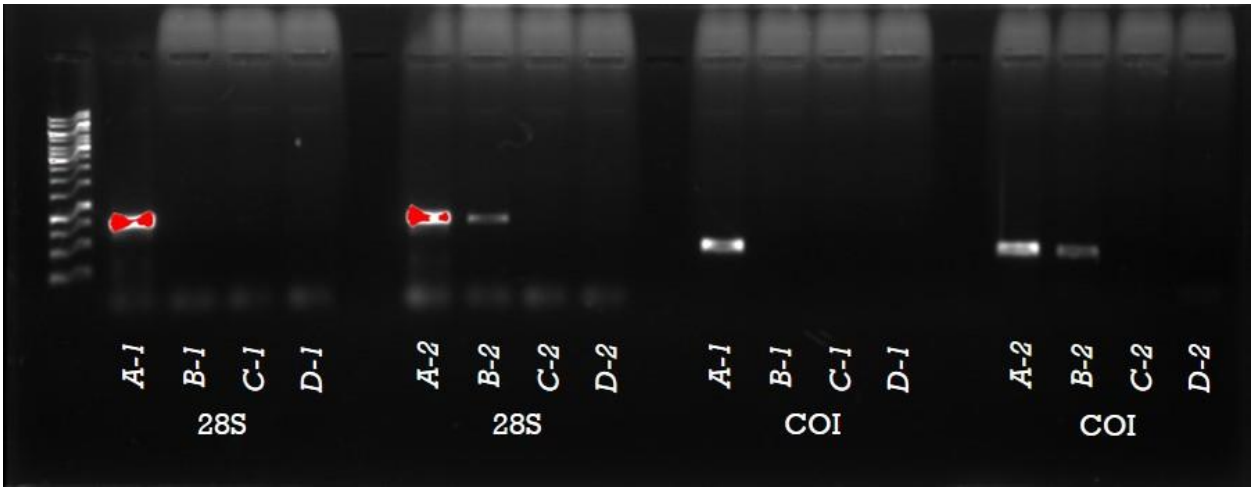

Figure 4.5: PCR amplification of *Protopolystoma xenopodis* and *Polystoma marmorati*. (A) represents all fresh specimens of *Pr. xenopodis* while (B) is the old representatives of this species. (C) is the specimens of *P. marmorati* mounted in Canada Balsam, and (D) is the same species mounted in Entellan. The numbers (1) and (2) indicates the short Kapa and long Macherey-Nagel extraction methods respectively.

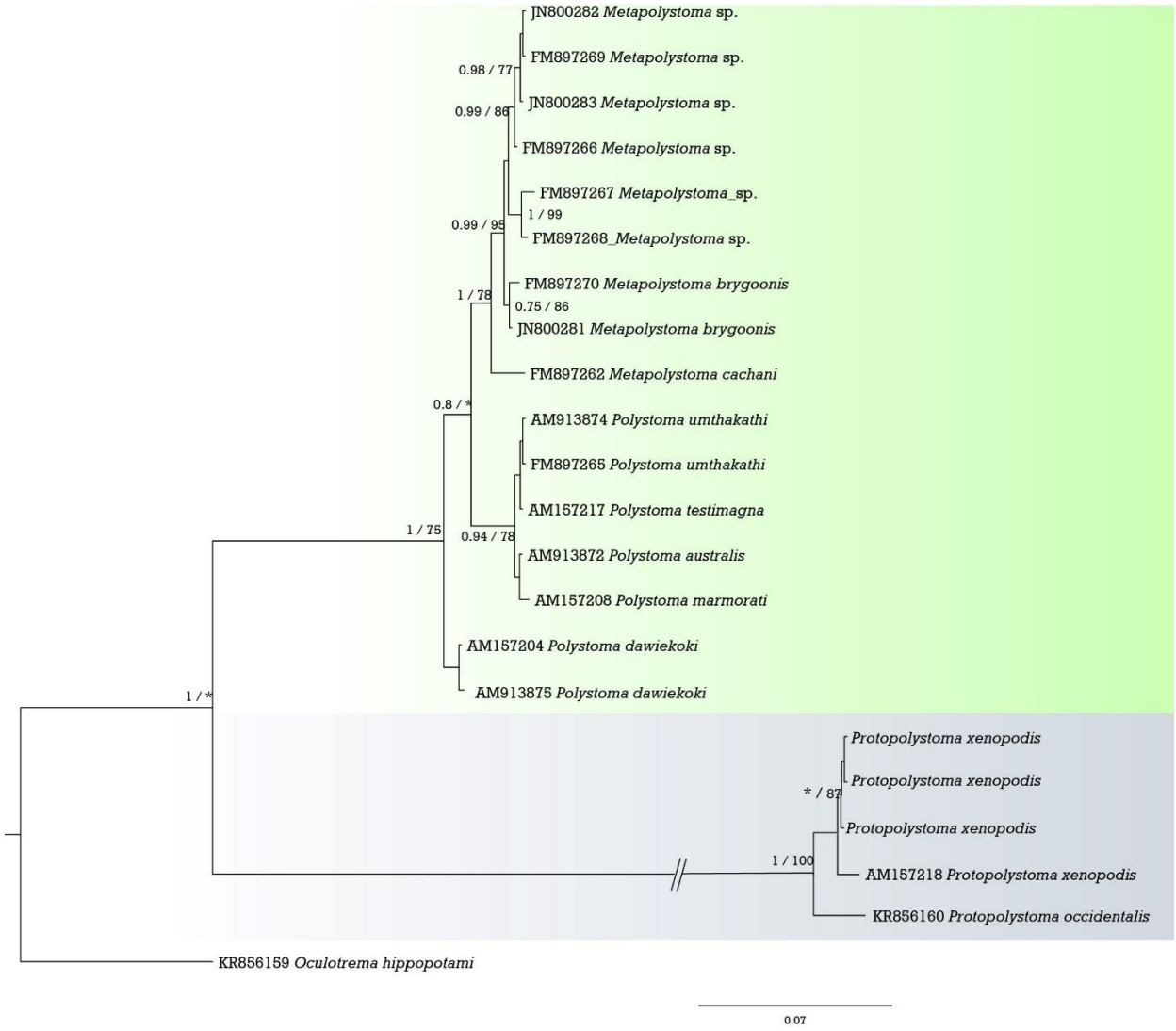

Figure 4.6: Phylogenetic relationships among Polystomatidae resulting from Bayesian inference (BI) and Maximum Likelihood (ML) analyses based on partial 28S rDNA data. Nodal support associated with the branches are listed as BI posterior probability/ ML bootstrap support; support values lower than 0.70 (BI) and 70 (ML) are indicated by an asterisk (*). The scale-bar indicates the expected number of substitutions per site. Sequences obtained in the present study have no Genbank accession number prior to the name.

When PCR amplifying the DNA of *Protopolystoma xenopodis*, the freshly-collected specimens amplified perfectly in all cases (Figure 4.5 A), while the older material amplified only with the short Kapa method of extraction, and then only faintly (Figure 4.5 B). This may prove important for future molecular verification of polystome species.

Figure 4.7: Phylogenetic relationships among Polystomatidae resulting from Bayesian inference (BI) and Maximum Likelihood (ML) analyses based on partial COI data. Nodal support associated with the branches are listed as BI posterior probability/ ML bootstrap support; support values lower than 0.70 (BI) and 70 (ML) are indicated by an asterisk (*). The scale-bar indicates the expected number of substitutions per site. Sequences obtained in the present study have no Genbank accession number prior to the name.

4.4. Discussion and conclusion

In their description of *M. porosissimae* and *P. sodwanensis*, Du Preez & Kok (1992a) described the differentiating morphological differences between the two species, with the main distinction being the length of the uterus and the position of the gonads. However, the marginal hooks have not been measured according to the currently proposed protocol. Du Preez and Maritz (2006) developed the current protocol on species of *Polystoma* but suggested that it may be useful for distinguishing species in other taxa as well. Our analyses of these hooklets have shown that, although the marginal hooklet plots overlap (Figure 4.4), there is a significant difference between the hooks (Table 4.1).

Our results also showed a significant difference in the hamulus lengths. Even more so was the difference between the hamulus points ($p = 0.000001$), which was suggested by Jackson and Tinsley (2007) to remain stable throughout the adults' life. Although both species had similar numbers of genital spines (8-9) the lengths were also found to differ significantly ($p = 0.000016$). Therefore, based only on the measurements of the sclerites of these species, it seems as though there is a clear distinction between *M. porosissimae* and *P. sodwanensis*.

The results of our molecular studies based on nuclear ribosomal and mitochondrial genes demonstrated the placement of *Metapolystoma* spp. within the genus *Polystoma*. This result is in agreement with previous studies by Bentz *et al.* (2001) and Verneau *et al*. (2009b), who emphasized the need for a future revision of the taxonomic validity of the genus *Metapolystoma*. Verneau *et al.* (2009b) studied species from Madagascar, including *Metapolystoma*. They found that all *Metapolystoma* and *Madapolystoma* specimens added to their study were distinct species.

Surprisingly, sequences for *Pr. xenopodis* generated during this study and those retrieved from Genbank (28rDNA and COI) seems like it may in fact represent three different species (Figure 4.6). However, additional study is needed to clarify this disagreement.

The morphological evidence for these two genera is seemingly clear, and although there is a significant difference between the lengths of the sclerites, the marginal hooklet plots (Du Preez & Maritz 2006) suggest that there are similarities. The difference between the hamulus lengths and genital spine length may only indicate the occurrence of two species in *Pt. porosissima*, rather than the suggested congeneric occurrence.

Prudhoe and Bray (1982) and Poulin (2002) suggested that congeneric monogeneans occurring in the same hosts may be due to sympatric speciation, resulting from an abrupt mutation or a small-scale radiation event. However, their suggestion fundamentally rests upon the assumption that the congeneric species are either very closely related or even sister-species, which may not always be the case in polystomatids. Duplication events may be able to result in the co-occurrence of several parasite species, from one monophyletic clade, in the same host. This may especially be true in parasites inhabiting the same, or very similar, microhabitats of their respective hosts, as is often the case in anuran polystomes (Verneau, Du Preez & Badets 2009). Bourgat and Murith (1980) and Du Preez and Kok (1992a) suggested that a temporary co-existence of two parasite species, as is the case for *P. sodwanensis* and *M. porosissimae*, may be the result of changes in either the host geographical range or even climatic changes. Based on this comment, one may pose the question of the influence of global climate change on host-specificity. However, this does not fall in the scope of the current study.

We therefore suggest that there is not sufficient evidence for the validity of *Metapolystoma.* However, sequences for *P. sodwanensis* and *M. porosissimae* are still necessary to conclude. The main distinguishing trait between the two genera, i.e. the elongated uterus in *Metapolystoma*, may be highly variable and dependent on the ecology of the host (Du Preez & Kok 1992a; Du Preez & Kok 1992b; Murith 1981b). Therefore, we suggest that the length of the uterus is only a plastic trait dependent on environmental factors, and should not be used for distinguishing between genera. It may, however, still be valuable for the differentiation between species, but further investigation is needed in this regard.

The present study demonstrates that the combination of molecular and morphological methods may further contribute to a better understanding of the diversity of the Polystomatidae in African wildlife. Future molecular studies with the application of multiple markers may discover unanticipated diversity within this family.

CHAPTER 5

Results: Taxonomic re-evaluation of chelonian polystomes

5.1. Introduction

5.1.1. Polystomes of chelonians

Polystomes parasitize a wide variety of hosts, including the Australian lungfish, amphibians, freshwater chelonians and the hippopotamus (Du Preez & Moeng 2004; Héritier *et al.* 2015). Polystomes of chelonians (subfamily Polystomoidinae Yamaguti, 1963), are globally distributed in the families of pleurodire and cryptodire turtles and are represented by four genera. These genera were originally distinguished mainly by the presence and absence of the hamuli, with *Polystomoides* Ward, 1917 having two pairs of hamuli of unequal length, *Polystomoidella* Price, 1939 possessing a single pair of hamuli, and *Neopolystoma* Price, 1939 without any hamuli (Bykhowsky 1961; Du Preez & Lim 2000; Morrison & du Preez 2011). However, Tinsley & Tinsley (2016) recently described a new genus, *Uropolystomoides*, based on the hamulus length vs. sucker diameter ratio for those with a pair of hamuli and situated in the urinary bladder.

The phylogenetic origin of chelonian polystomes is still a debated topic. Verneau *et al.* (2002) and Héritier *et al.* (2015) suggested during their phylogenetic studies that polystomatids of chelonians radiated *ca.* 191 Mya, following a switch from a presumably extinct aquatic amniote to freshwater turtles. However, Héritier *et al.* (2015) also mentioned that the switch may have happened from caecilians, as chelonian polystomes are genetically much closer related to *Nanopolystoma tinsleyi* infecting an aquatic caecilian.

Chelonian polystomes are assumed to be relatively host-, (Héritier *et al.* 2015) and site specific (Du Preez & Lim 2000; Du Preez & Morrison 2012). They generally inhabit one of three distinct sites, namely the urinary bladder and cloaca, conjunctival sacs, and the oral and pharyngeal cavities. Prudhoe and Bray (1982) suggested that Platyhelminth species show a certain preference for their infection site, but their physiology and morphology may not necessarily limit them to that site alone, and Meyer *et al.* (2015) suggested that chelonian polystomes may only change hosts when invading a new site. Multiple infections of different polystomes within their respective sites in one host species are common among chelonian polystomes (Berthier *et al.* 2014). *Neopolystoma spratti* Pichelin, 1995 and *Neopolystoma chelonidae* MacCallum, 1918 are, for example, two sister polystome species infecting the conjunctival sac and urinary bladder respectively, in the same host, *Chelodina longicollis* (Shaw, 1794) in Australia (Héritier *et al.* 2015). Littlewood *et al.* (1997) indicated that

congeneric species in different sites within the same hosts were not as closely related to each other, compared to congeneric species infecting the same site in different hosts. The latter statement was later on supported by Verneau *et al.* (2011).

Tinsley (1988) reported that the chaetotaxy of *Oculotrema* oncomiracidia is similar and may imply a close relatedness to that of mouth-inhabiting *Polystomoides* spp., whereas the bladder-dwelling *Uropolystomoides* spp. are closer in morphology to *Protopolystoma* found in the aquatic Clawed Frog. The clear differences are found in both African and North-American polystomes, and suggests the evolutionary divergence of microhabitats have happened before the fauna of Africa and America were separated in the Cretaceous era (Tinsley 1988, 1976). However, these morphological differences between individuals have not been adequately studied since Tinsley noted them in 1988.

5.1.2. Morphological vs. molecular evidence for phylogeny

Phylogenetic relationships can be either monophyletic (all descendants from one common ancestor), paraphyletic (only some members are from a common ancestor), or polyphyletic (the most recent common ancestor is not included in the clade). In evolutionary studies, the former two may be acceptable, with monophyly being preferable, whereas the latter is rejected (Hickman *et al.* 2008). The use of molecular or morphological methods to recreate the evolutionary history of a group of organisms has been a subject of discussion between morphologists and molecular systematists since the invention of molecular tools. However, in order for a proposed phylogenetic tree to be widely accepted as the most probable, molecular and morphological data on the specific group of organisms should complement each other (Berthier *et al.* 2014; De Leon *et al.* 2016; Hickman *et al.* 2008; Hillis 1987).

According to Tinsley (2013), the morphology of chelonian polystomes might have been relatively unchanged since the Jurassic era. Mayr (1942) suggested that characters for classification should be phylogenetically conservative and stable. Morphological characters of the Polystomoidinae may thus be regarded as of potential importance for phylogenetic studies. With morphological variation taken into account, the collective features of adult morphology may provide insights into the classification of genera (Mayr, 1942; Perkins *et al.* 2009; Prudhoe & Bray, 1982).

Rohde (1965, 1975) has suggested several traits to be of phylogenetic importance among species of *Polystomoides*. The protonephidial, nervous and reproductive systems, as well as

the ratios of the size of the hamuli relative to the haptoral sucker, the testis compared to the pharynx, and the oral sucker to the pharynx were found to be valuable. The morphology and morphometrics of the marginal hooklets and genital spines were also taken into consideration. Rohde (1965) and Vieira *et al* (2008) both found these characteristics to be valid for use in classification of species within the genus *Polystomoides*.

The current protocol in use to distinguish between the genera of chelonian polystomes is quite basic. And although Mayr (1942) suggested that there is often a genetic basis for basic morphological traits, the current knowledge on these polystomes' morphology has been inadequate to support genetic evidence.

Several molecular phylogenetic studies (Figure 5.1.) have indicated that there could be a problem with the current classification of chelonian polystomes (Héritier *et al.* 2015; Littlewood *et al.* 1997; Olson & Tkach 2005; Verneau *et al.*, 2011). Initially, this problem was pointed out by Littlewood *et al.* (1997) (Figure 5.1 c) who explained that the morphology of the genital spines of *Polystomoides asiaticus* and *Polystomoides renschi* more closely resembled those of two *Neopolystoma* species (*N. chelonidae* and *N. spratti*), rather than two other *Polystomoides* species. This was supported by the 28S analyses during the same study. However, contradicting these results, the COI analysis indicated a monophyletic clade including all *Polystomoides* and *Uropolystomoides* species, with *Neopolystoma* appearing non-monophyletic (Littlewood *et al.* 1997). This was the first indication that the genera were not clearly differentiated.

The next evidence came when Olson and Tkach (2005) reported that among the Polyopisthocotylea, the two genera (*Polystomoides* and *Neopolystoma*) are paraphyletic (Figure 5.1. a).

In 2011, Verneau *et al.* found that polytomies of chelonian polystomes were formed due to their early origin and divergence. They found the parasites clustering into distinct clades associated with their respective microhabitats, even though these sites did not cluster together. The genera were also shown to be non-monophyletic (Figure 5.1. b).

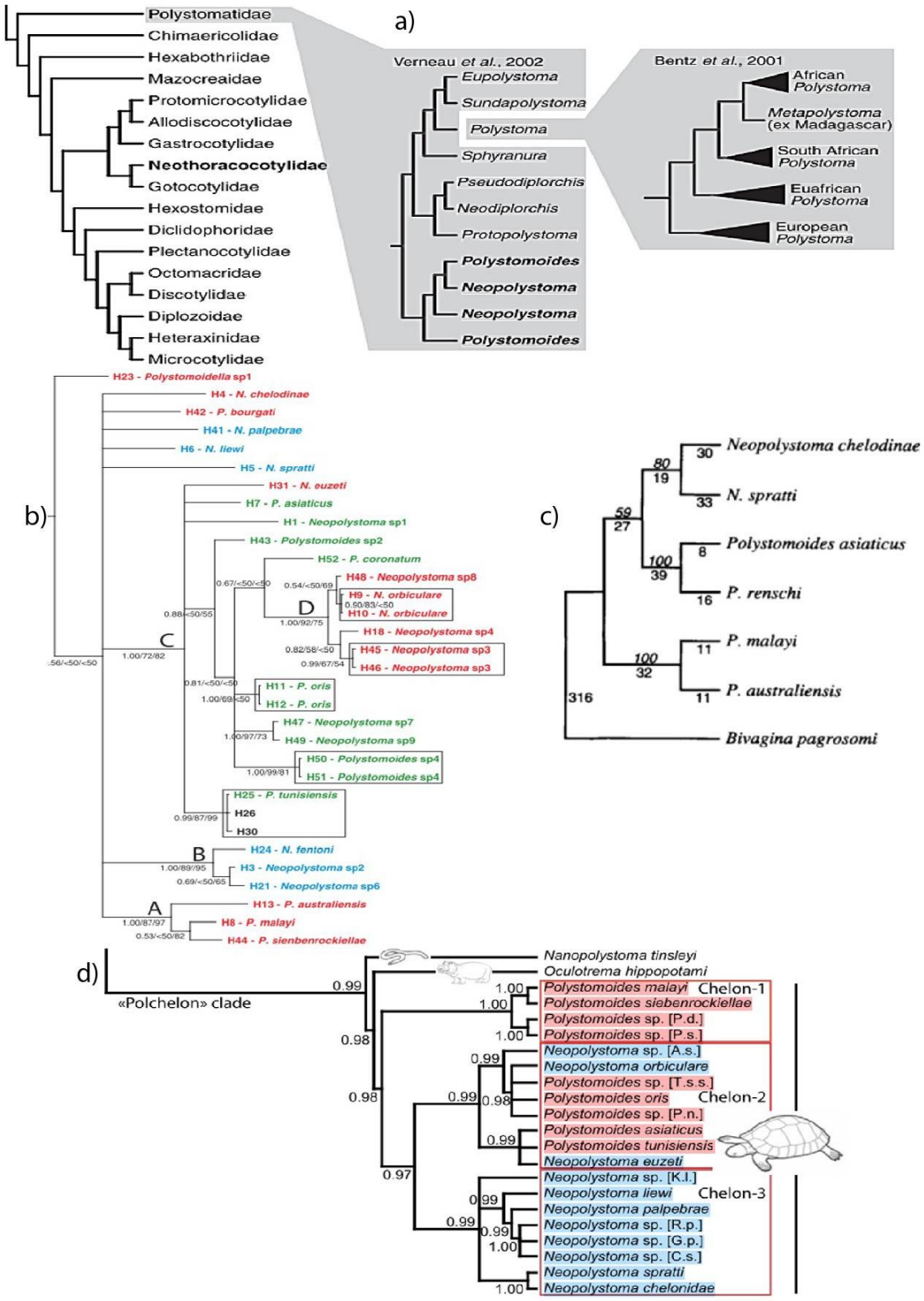

Figure 5.1. Previous molecular studies, showing the non-monophyly of the genera *Polystomoides* and *Neopolystoma*. a) Olson & Tkach (2005); b) Verneau *et al.* (2011); c) Littlewood *et al.* 1997; and d) Héritier *et al.* (2015)

A deep evolutionary study, using mitochondrial and nuclear genes, conducted by Héritier *et al.* (2015) also supported the non-monophyly indicated in previous studies. It added that the microhabitats were also generally not monophyletic when showing one clade on the phylogenetic tree consisting primarily of parasites that inhabit the conjunctival sacs, except for one specimen *Neopolystoma chelonidae*. Another clade has mostly pharyngeal parasites, with some occurring in the bladder weaved in between (Figure 5.1. d).

Hillis (1987) suggested that conflict between morphological and molecular evidence with regard to phylogenies may be an indication of the existence of theoretical or procedural problems in either one or both analyses. Alternatively, it may indicate the need for additional data in order to resolve the phylogenetic relationships. When looking at the morphological traits contradicting each other as well as, to some extent, the molecular evidence, it is clear that there is inadequate data available to rectify the current problems with chelonian polystome classification.

5.1.3. Problem statement, aim and objectives

Chelonian polystomes (Monogenea: Polystomatidae) inhabit three different locations on their respective hosts, namely the conjunctival sacs under the eyes, oral region, and pharyngeal pouch, and the urinary bladder (Du Preez & Lim 2000; Du Preez & Morrison 2012). Although chelonian polystomes form a monophyletic group, the genera *Polystomoides* and *Neopolystoma* are, according to published molecular studies, not monophyletic (Héritier *et al.* 2015; Littlewood *et al.* 1997; Olson & Tkach 2005; Verneau *et al.* 2011). This non-monophyly places a question mark on the validity of the current description of these genera. Lenis and Garcia-Prieto (2009) suggested the re-evaluation of the composition of *Polystomoides* based on the current knowledge of morphology and genetics, and Littlewood *et al.* (1997) implied that the species may have to be re-arranged according to the microhabitat they inhabit.

Therefore, our aim was to re-evaluate the morphology of the genera within chelonian polystomes, in order to rectify the current taxonomical issues.

The objectives of the current study were:

- to study the morphology of chelonian polystomes based on the species descriptions;
- to determine whether species cluster according to shared morphological characters or combinations thereof; and

- to compare these morphological clusters according to sites where species are found on the host and published molecular evidence.

5.2. Methods

The descriptions of all *Polystomoides* and *Neopolystoma* species were studied and summarised in a spreadsheet. The scaled diagrams of the type specimens were also studied and any trait not mentioned in the description, but present in another description, was measured and/or recorded from these diagrams, if possible. All measurements were converted to and recorded in micrometers (µm). However, several of the older species descriptions had relatively vague diagrams, and also might not have mentioned several traits mentioned in more recent descriptions.

The completed table was then simplified in order to shorten the descriptions of the traits. Ranges were set for the continuous (measurable) data, like the number or size of a feature, respectively, and replaced in the table with a given range value. Categorical (non-measurable) data were also quantified and replaced with a given value.

The morphological characteristics used included site on host (oral, urinary bladder or conjunctival sacks); hamulus presence; hamulus shape, testis shape (spherical or lobed); vitellaria distribution (throughout the body or in two lateral fields); ovary position; mouth shape and size; egg shape; egg size (larger/smaller than haptoral sucker). Numerical data recorded includes haptor length as a percentage of the body length; number of genital spines; marginal hooklet length; marginal hooklet length as a percentage of the body length; ovary length as a percentage of the body length, egg length as a percentage of the body length; and sucker diameter as percentage of body length.

Several analyses were performed, including multivariate analyses and statistical significance tests. For multivariate analyses, Canoco v.5 was used to create constrained RDA and unconstrained PCA analyses, where the coded traits were plotted against the microhabitat each species occur in.

The significance of the differences between traits was obtained through several statistical and practical significance tests. According to Ellis and Steyn (2003) and Rosenthal *et al.* (2000) both these tests are vital, since statistical significance does not automatically imply practical significance. Statistical tests usually rely heavily on a low *p*-value to indicate significance, but

for small sample sizes, it may be misleading. Therefore, practical significance (defined as a difference large enough to have an effect in practice (Ellis & Steyn, 2003; Rosenthal *et al.*, 2000)) by means of effect sizes may be a better indication. Effect sizes are independent of units, as well as sample size, but still relate it to the spread of the data (Ellis & Steyn, 2003).

In this study, a combination of statistical and practical significance tests was used. The statistical tests were performed using Statistica v. 13 (Dell Inc. 2015). The categorical and numerical data were separated and tested accordingly. All numerical data were tested for normal distribution and ANOVA, Kruskal-Wallis and Levene's Tests were performed. For the categorical data, a two-way contingency table was compiled.

To test the practical significance by means of effect sizes, two tests were done, one for each type of data. For the categorical data, Cramér's V test was done. Values of 0.1 are not considered significant, medium significance is assumed from values around 0.3, and values exceeding 0.5 are considered highly significant (Ellis & Steyn, 2003). Cohen's D test was done for the practical significance of numerical data. This test states the standardized difference between two means, and values higher than 0.8 is considered very significant, whereas values of 0.5 and 0.2 indicate medium and low significance respectively (Cohen, 1988; Ellis & Steyn, 2003).

Table 5.1: The morphological traits used, as well as their ranges and codes given for each morphological presentation of the trait

Morphological trait	**Codes**						
	0	**1**	**2**	**3**	**4**	**5**	**6**
Categorical data							
Site on host	Bladder	Mouth	Eye				
Hamulus	Absent	Present					
Hamulus shape	Absent	Solid	Split				
Testis shape	Spherical	Lobed					
Vitellaria distribution	Equally distributed	In two lateral fields					
Ovary position	Anterior third	Between anterior third and midline	Midline	Posterior third			
Mouth size	Small	Large					
Mouth shape	Round	Elongated					
Caeca length	To anterior part of haptor	Extending into haptor	Ending well before haptor				
Egg shape	Oval	Spindle/Diamond	Round				
Egg larger/smaller than haptoral sucker	Larger	Smaller					

Numerical data

Haptor length as a percentage of the body length	10 - 19,99	20 - 29,99	30 - 40	>40			
Number of genital spines	0 - 10	11 - 20	21 - 30	31 - 40	41 - 50	51 - 100	>100
Marginal hooklet length	11 - 20	21 - 25	26 - 30	>30			
Marginal hooklet length as a percentage of the body length	0,1 - 0,39	0,4 - 0,59	0,6 - 0,79	0,8 - 1,0	>1,00		
Ovary length as a percentage of the body length	1 - 4,99	5 - 10	>10				
Egg length as a percentage of the body length	1 - 4,99	5-10	>10				
Sucker length as a percentage of the body length	1 - 4,99	5 - 10	>10				

5.3. Results

For both multivariate analyses done, the total variation was 400.4. The unconstrained PCA with supplementary variation (Figure 5.2 a) done in Canoco v.5 had a 25.2% supplementary variation. The explained variation for one axis was 33.9%, whereas the cumulative explained variation for four axes were 76.3 %. The pseudo-canonical correlation was 0.691 for Axis 1, gradually decreasing to 0.167 for Axis 4.

For the constrained RDA analysis (Figure 5.2 b), the explanatory variation was 25.2%, with pseudo-F being 5.2 and a *p*-value of 0.002. The explained variation for Axis 1 was 20.3 %, with a steady increase in the cumulative explained variation for four axes at 68.2 %. The pseudo-canonical correlation for Axis 1 was 0.818, and for Axis 2 it was 0.6.

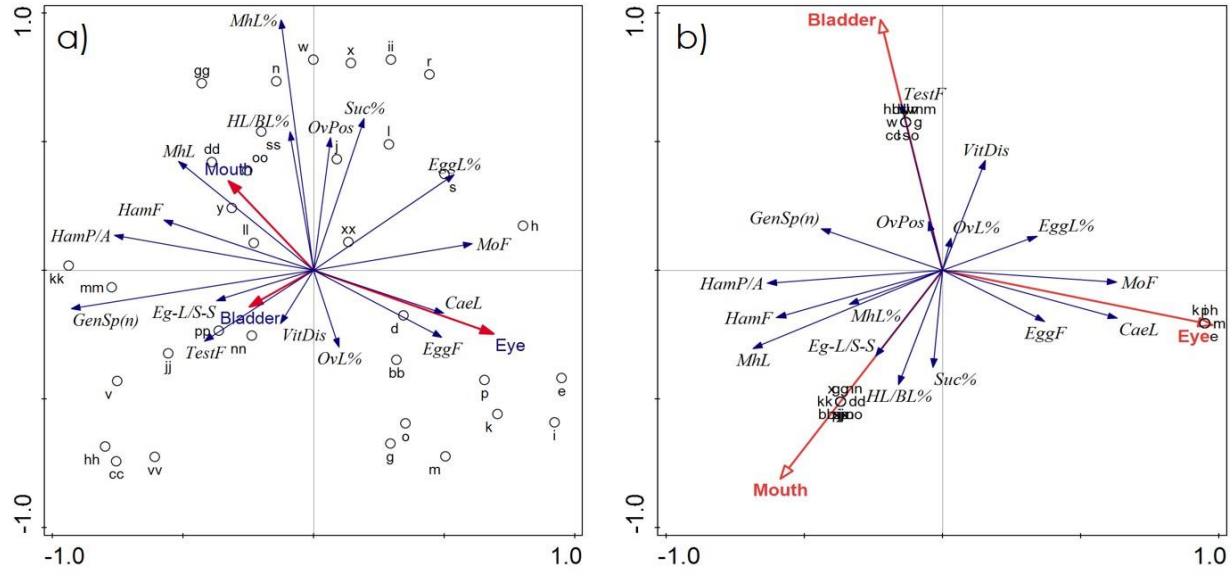

Figure 5.2: Multivariate analyses indicating the morphological traits plotted against the microhabitat of the polystome. Species are indicated as individual circles. a) Unconstrained PCA; b) Constrained RDA

Since the analyses included both categorical and numerical data, these low correlations could be expected. Although these correlations are relatively weak, it is still clear enough to see some traits associated with certain habitats. The characteristic most associated with bladder parasites, is the shape of the testis, whereas the most important features for eye parasites are the caeca length and egg shape. The mouth parasites showed no definite correlation with any trait when combining both analyses.

The statistical significance tests supported the above-mentioned traits to be both statistically and practically significant. The results of the ANOVA, Kruskal-Wallis, and Levene's tests can

be seen in Table 5.2. The ANOVA test indicated that marginal hooklet length is statistically the smallest in the eye, compared to bladder and mouth parasites, the number of genital spines is statistically the least in the eye parasites, compared to the bladder parasites only, and the sucker diameter as a % of the body length is statistically smaller in the bladder parasites, compared to eye and mouth parasites. The Kruskal-Wallis test echoed these findings, with the only difference being in the case of the number of genital spines, where it was found to be the least in the eye parasites, compared to both other micro-habitats (Table 5.2). The Kruskal-Wallis test is a non-parametric test, and therefore we can deduce that even if our data were not normally distributed, which it is, these characters would still be statistically significant. The numerical traits that were practically significant are the haptor length as percentage of the body length (HL/BL%), the number of genital spines, the marginal hooklet length, egg length as a percentage of the body length (Egg L %), and the sucker diameter as a percentage of the body length (Sucker %). The results can be seen in Table 5.3.

The categorical traits that proved most significant statistically are the presence and shape of the hamuli, as well as the testis and egg shapes respectively, the vitellaria distribution, and the caeca length (Table 5.4). The practical significance of these traits is shown in Table 5.5. The presence of the hamuli was also practically significant, as could be suspected since the current differentiation of the genera is based solely on this character. All eye-parasites are from the genus *Neopolystoma*, and thus have no hamuli. The testis shape is significant, with a Cramér's V-value of 0.71 (Table 5.5). This is indicated in that there are no lobed testes in either the mouth or eye parasites while being present in over 65% of bladder parasites (Table 5.4). The vitellaria distribution is shown to be of medium practical significance. Once again, the bladder parasites are the exception, with almost half of them having the vitellaria distributed in two lateral fields, compared to the 20% in the eye parasites. The caeca length is also somewhat significant, with about 70% of the eye parasites' caeca not extending into the haptor, whereas within both the mouth and bladder parasites the majority enter the anterior part of the haptor (Table 5.4). The egg shape and size compared to the sucker, although indicated as relatively significant, had several missing values due to gaps in the species descriptions. However, the differences in the egg shape, with those polystomes found in the conjunctival sacs being the only species with spindle/diamond shaped eggs, compared to the round-oval eggs of polystomes in other microhabitats, are supported by the observations of Pichelin (1995), Du Preez and Moeng (2004), Verneau *et al.* (2009a) and Du Preez and Morrison (2012). The position of the ovary was not significant at all. Mouth size and shape were

added to the initial analyses but were excluded due to subjectivity and the degree of flattening during fixation that may have influenced the result.

The morphological evidence supported by statistical analyses stated herein indicates that several characters may be of taxonomic value and may aid to resolve the taxonomic uncertainties surrounding chelonian polystomes. If combining the statistically and practically significant trait, those characters that show great potential to be of taxonomic importance include the presence or absence of the hamuli, the testis shape, the egg shape, the size of the marginal hooklets, and the number of genital spines. These results also correlate well with the findings of the PCA and RDA (Figure 5.2).

Based on the traits showing both practical and statistical significance, similarities were noted correlating with the different microhabitats of the polystomes. A morphological key (Table 5.7) was subsequently created, separating species primarily on the microhabitat they inhabit, and secondarily on the significant morphological features associated with that microhabitat (see Table 5.6).

We divided the group into three broad groups based on the site in the host. The eye polystomes is divided into two groups based on the marginal hooklet length with those having marginal hooklets smaller than 15 μm as Group A and those longer than 15 μm as group B (Table 5.6). Among the urinary bladder polystomes we group those with a spherical testis as Group C and those with a lobular testis were subdivided as those having hamuli as Group D and those without hamuli as Group E (see Table 5.6). Among the polystomes found in the pharyngeal and oral cavities those with hamuli form Group F and those without are divided in two groups based on the number of genital spines. Those with more than 20 genital spines are grouped together as Group G and those with less than 20 genital spines as Group H (see Table 5.6).

Table 5.2: Results of the ANOVA, Kruskal-Wallis, and Levene's tests done for measurable traits in each microhabitat

	Means ± Standard deviation			MSE (Error)		MSE (Effect)		p-value		
	Bladder	Eye	Mouth	Levene's Test	ANOVA	Levene's Test	ANOVA	Levene's Test	Kruskal-Wallis	ANOVA
Haptor length/Body Length %	23.26 ± 4,73	30.77 ± 11,57	33.24 ± 6,38	18.98	49.79	91.78	541.45	0.012208	0.00000	0.000127
Number of Genital Spines	38.91 ± 22,43	14.20 ± 7,10	32.50 ± 24,92	228.08	460.08	554.06	2135.06	0.098864	0.00120	0.014371
Marginal Hook Length	22.29 ± 4,74	14.30 ± 4,74	25.56 ± 3,93	6.19	16.44	3.86	396.10	0.541042	0.00000	0.000000
Marginal Hook Length % of Body Length	0.65 ± 0,31	0.71 ± 0,45	0.72 ± 0,22	0.02	0.10	0.14	0.03	0.005971	0.55860	0.774174
Ovary Length % of Body Length	6.57 ± 2,74	6.67 ± 1,79	5.99 ± 3,18	3.57	7.64	2.10	2.17	0.559760	0.44180	0.754076
Egg Length % of Body Length	7.83 ± 3,85	9.49 ± 2,94	6.40 ± 2,97	4.29	11.45	1.54	23.03	0.700752	0.10800	0.149427
Sucker diameter % of Body Length	6.64 ± 1,94	10.85 ± 4,02	9.97 ± 2,58	1.92	7.11	12.32	86.29	0.003389	0.00010	0.000054

Table 5.3: Results of the Cohen's D test for the effect size of numerical data, stating the standardized difference between two means. Value 0.2 = small, 0.5 = medium, 0.8= large) Highlighted values are significant.

Effect sizes (Cohen's D)

	HL/BL%	n Gen. Spines	M. Hook L	M. Hook L %	Ovary L %	Egg L %	Sucker %
Eye – Bladder	0.648268	1.10171	1.686411	0.132079	0.03678	0.429791	1.048038
Eye – Mouth	0.213409	0.734232	2.864145	0.040494	0.212247	1.041213	0.220013
Bladder – Mouth	1.562398	0.257304	0.689486	0.246099	0.180533	0.372195	1.289903

Table 5.4: Results of the 2-way contingency table: summary of observed frequencies for categorical data

2-Way Summary Table: Observed frequencies

	Bladder %	Eye %	Mouth %
Hamulus absent	34.78%	100%	11.11%
Hamulus present	65.22%	0.00%	88.89%
Hamulus none	34.78	100	11.11
Hamulus solid	65.22	0	66.67
Hamulus split	0	0	22.22
Testis lobed	65.22	0	0
Testis spherical	34.78	100	100
Vitellaria equally distributed	56.52	80	100
Vitellaria in 2 ducts	43.48	20	0
Ovary between anterior 1/3 and half	8.7	20	5.56
Ovary half	4.35	10	5.56
Ovary anterior 1/3	82.61	70	88.89
Ovary posterior 1/3	4.35	0	0
Caeca to anterior haptor	69.57	30	77.78
Ceaca ending well before haptor	8.7	50	11.11
Caeca extending into haptor	21.74	0	11.11
Caeca to just behind testis	0	20	0
Egg shape oval	94.12	40	84.62
Egg shape: spindle	0	60	0
Egg shape: round	5.88	0	15.38
Egg larger than sucker	56.25	57.14	14.29
Egg smaller than sucker	43.75	42.86	85.71

Table 5.5. Results for the Cramér's V test stating the practical significance of categorical data. Value of 0.1 = not significant; 0.3 = medium significant; 0.5 = very significant). Highlighted values are significant.

Statistics: Site on Host vs. Various characteristics

	Hamulus present / absent	Hamulus solid / split	Testis lobed / spherical	Vitellaria distribution	Ovary position	Caeca length	Egg shape	Egg larger / smaller than sucker
Pearson Chi-square p-value	0.00002	0.00002	0.00000	0.00476	0.77031	0.00219	0.00017	0.03992
M-L Chi-square p-value	0.00000	0.00000	0.00000	0.00085	0.74961	0.00441	0.00021	0.03040
Phi	0.6516064	0.7287516	0.7122123	0.4579290	0.2543887	0.6350932	0.7481988	0.4172615
Contingency coefficient	0.5459341	0.5889531	0.5801194	0.4163509	0.2465366	0.5361119	0.5990766	0.3850831
Cramer's V	0.6516064	0.5153052	0.7122123	0.4579290	0.1798799	0.4490787	0.5290565	0.4172615

Table 5.6: Morphological characters used for the creation of the morphological key, as well as the proposed groups per microhabitat.

	Group	Realm	Clade (Héritier *et al.* 2015)	Site on host	M. Hook L	Testis lobed/spherical	Hamulus present / absent	n Gen. Spines	Hamulus length	Sucker size (um)	Ham/S %
N. cribbi	A	AU		eye	17	spherical	absent	8		215	
N. queenslandensis	A	AU		eye	18	spherical	absent	25		211	
N. spratti	A	AU	3	eye	17	spherical	absent	23		202	
N. tinsleyi	A	AU		eye	17	spherical	absent	23		193	
N. elizabethae	B	NA		eye	12	spherical	absent	8		372	
N. fentoni	B	SA		eye	13	spherical	absent	8		265	
N. grossi	B	NA		eye	13	spherical	absent	7		241	
N. liewi	B	OR	3	eye	13	spherical	absent	11		217	
N. moleri	B	NA		eye	10	spherical	absent	13		280	
N. palpebrae	B	PA	3	eye	13	spherical	absent	16		340	
N. domitilae	C	NA		bladder	24	spherical	absent	20		330	
N. euzeti	C	ET	2	bladder		spherical	absent	34		355	
N. macleayi	C	AU		bladder	25	spherical	absent	12		305	
N. orbiculare	C	NA	2	bladder	16	spherical	absent	16		300	
N. terrapenis	C	NA		bladder	20	spherical	absent	16		190	
U. megaovum	C	PA		bladder	25	spherical	2 pairs present	13	102	150	0,682
U. ocadiae	C	PA		bladder	25	spherical	2 pairs present	53	640	295	2,169
U. scottae	C	AU		bladder	22	spherical	2 pairs present	73	632	347	1,821
N. chelonidae	E	NA	3 ?	bladder	25	lobed	absent	14		369	
N. cyclovitellum	E	NA		bladder		lobed	absent	16		233	
N. exhamatum	E	OR		bladder	12	lobed	absent	17		325	
U. australiensis	D	AU		bladder	25	lobed	2 pr. present	85	599	380	1,576
U. bourgati	D	ET		bladder	29	lobed	2 pr. present	28	279	163	1,712
U. chabaudi	D	ET		bladder	24	lobed	2 pr. present	34	268	150	1,787
U. chauhani	D	OR	1	bladder		lobed	2 pr.present	40	365	205	1,780
U. godavarii	D	OR		bladder	23	lobed	2 pr.present	65	447	313	1,432
U. kachugae	D	OR		bladder		lobed	2 pr.present	40	900	400	2,250
U. ludhianae	D	OR		bladder		lobed	2 pr.present	59	581	309	1,883

U. malayi	D	OR	1	bladder	26	lobed	2 pr. present	75	630	320	1,969
U. nabedei	D	ET		bladder	23	lobed	2 pr. present	38	324	156	2,077
U. siebenrockiellae	D	OR	1	bladder	23	lobed	2 pr. present	51	378	176	2,148
U. simhai	D	OR		bladder	12	lobed	2 pr. present	60	595	275	2,164
U. stewarti	D	OR		bladder		lobed	2 pr. present	36	195	245	0,796
N. kreffti	F	AU		mouth	26	spherical	absent	24		344	
N. novaeguineae	F	OR		mouth	23	spherical	absent	32		270	
P. brasiliensis	G	SA		mouth	32	spherical	2 pr. present	8	72	455	0,158
P. fuquesi	G	SA		mouth	26	spherical	2 pr. present	2	67	499	0,133
P. uruguayensis	G	SA		mouth	21	spherical	2 pr. present	9	52	315	0,165
P. asiaticus	H	OR	2	mouth		spherical	2 pr. present	37	140	344	0,407
P. coronatum	H	NA		mouth	20	spherical	2 pr. present	32	132	370	0,357
P. japonicum	H	PA		mouth	28	spherical	2 pr. present	35	110	325	0,338
P. magdalensis	H	SA		mouth	25	spherical	2 pr. present	32	137	400	0,343
P. microrchis	H	PA		mouth	23	spherical	2 pr. present	45	104	410	0,252
P. multifalx	H	NA		mouth	30	spherical	2 pr. present	122	200	430	0,465
P. ocellatum	H	EU		mouth	25	spherical	2 pr. present	32	58	318	0,182
P. oris	H	NA	2	mouth	30	spherical	2 pr. present	27	120	330	0,364
P. pauli	H	NA		mouth	27	spherical	2 pr. present	39	135	408	0,331
P. platynotae	H	OR		mouth		spherical	2 pr. present	29	104	393	0,265
P. renschi	H	OR		mouth	18	spherical	2 pr. present	23	88	250	0,352
P. rohdei	H	SA		mouth	30	spherical	2 pr. present	30	160	405	0,395
P. tunisiensis	H	ET	2	mouth	25	spherical	2 pr. present	27	105	400	0,263

Table 5.7: Proposed morphological key for chelonian polystomes

1.	a)	Site on host: Conjunctival sacs	2
1.	b)	Site on host: Urinary bladder	3
1.	c)	Site on host: Pharyngeal cavities	5
2.	a)	Marginal hooklet length > 15µm	Group A
2.	b)	Marginal hooklet length < 15µm	Group B
3.	a)	Testis spherical	Group C
3.	b)	Testis lobed	4
4.	a)	Hamulus present	Group D
4.	b)	Hamulus absent	Group E
5.	a)	Hamulus present	Group F
5.	b)	Hamulus absent	6
6.	a)	Number of genital spines < 20	Group G
6.	b)	Number of genital spines > 20	Group H

5.4. Discussion and conclusion

The results reported provide support to those of Rohde (1965) which indicated that the morphological characters that proved to be of taxonomic significance within the genus *Polystomoides* were the relative sizes of the oral sucker and pharynx, the shape of the testis, the length of the hamuli, and the number and length of the genital spines.

DeWitt (1998) suggested that natural selection will favour organisms that are capable of changing to allow for entering into novel habitats. Agrawal (2001) also claimed that phenotypic plasticity is influenced by environmental cues enabling species to adapt to many circumstances. Therefore, it can be reasoned that certain morphological traits will develop genetically based on the environment (in this case the bladder, eye or mouth) the parasites occur in. This study has supported this reasoning in that polystome species inhabiting different microhabitats have different associated morphologies. Since morphological traits

may evolve quicker than genetics, it may be that genetic change follows morphologically plastic traits, therefore specializing a parasite on its host or site (Agrawal 2001; Prudhoe & Bray 1982).

Among the hippo and chelonian polystomes, those occurring in the conjunctival sacs had especially unique traits, such as spindle-shaped eggs, an extremely firm grip on the host, and the ability to stretch enough to be able to stay attached to the host, while stretching to feed (Du Preez & Moeng 2004; Du Preez & Morrison 2012; Verneau *et al.* 2009a). Our findings supported the distinct egg shape, as opposed to the round-oval shape of pharyngeal or bladder polystomes, as well as a few other characters. The other distinguishing characteristics of eye polystomes include 1) the size of the marginal hooklets being significantly smaller than those found in other microhabitats, (mean 14µm, compared to bladder parasites' mean at 22 µm and pharyngeal parasites' mean 26µm); 2) the number of genital spines being significantly less, and 3) the majority of the caeca not extending into the haptor.

These distinct traits found in the eye-inhabiting polystomes support the molecular findings of Héritier *et al.* (2015) placing them in their own clade. However, the appearance of the bladder polystome, *N. chelonidae*, within this clade poses somewhat of a problem. We indicated that *N. chelonidae* has quite a different morphology from the other eye parasites. Considering the statement by Agrawal (2001) that morphology in parasites often foregoes genetic change, it may be considered that *N. chelonidae* are in the stages of entering a novel habitat (in this case, the bladder). Alternatively, it's phylogenetic placement could be explained by the specimen molecularly analysed being a mislabeled specimen. If taking into account that the species was already described in 1980 by Rohde and Pearson, the latter explanation for its placement is the most favoured.

Within the eye-inhabiting polystomes, there are also distinct morphological differences between those of Australian descent and those occurring in the rest of the world. Non-Australian eye polystomes have a distinct spindle-shaped egg, and marginal hooklets have a length of less than 15µm, whereas Australian parasites have oval eggs, resembling those of bladder and pharyngeal polystomes, and marginal hooklets having lengths of more than 15µm. With the exception of one species (*N. fentoni*), the non-Australian polystomes also had no caecae entering the haptor. Badets *et al.* (2011) suggested that it may be because of their isolated lineage since they appeared most basal in the tree. On the phylogenetic tree created

by Héritier *et al.* (2015), the clade consisting of polystomes of the conjunctival sacs, the Australian and non-Australian parasites also form two distinct clades (Figure 5.1).

There were also distinct characteristics separating bladder-inhabiting polystomes from the other two microhabitats. The sucker diameter as a percentage of the body length was found to be significantly smaller (mean 7%) than in both pharyngeal (mean 10%) and eye (mean 11%) parasites. The most obvious distinctive trait for bladder-inhabiting polystomes is the shape of the testis. The lobed testis shape was only found in bladder polystomes, compared to the spherical shape of the other species. However, not all bladder polystome species showed this trait. Enabulele *et al.* (2012) claimed that there was some interspecies variation in the testis shape of *Polystomoides bourgati* depending on the sampling site. In those recovered from Ossisa and Abeokuta, the shape was ovoid to round, whereas the shape for other localities (Sapele) was lobed and laterally elongated. However, if the diagrams are studied of the species with so-called "round" testes, it is still clearly irregularly shaped, and not a true spherical testis as can be found in, for instance, *Polystomoides coronatum* (Enabulele *et al.* 2012). More study on this trait, as well as another, more objective, quantification method for this feature is therefore needed. Tinsley and Tinsley (2016) also noted some differences between *Polystomoides* species occupying the bladder and those occurring in the mouth. They described the new genus *Uropolystomoides* for these bladder inhabiting species of *Polystomoides*. Their description was based on the difference in hamulus length vs. sucker diameter ratio. Individuals of bladder polystomes had a hamulus that was distinctly larger than the sucker, whereas the opposite was found in mouth polystomes (Tinsley & Tinsley 2016). However, since their results were only published at the end of my study, these conclusions could not be added to my analyses.

The mouth-inhabiting polystomes had the least distinctive features. Rohde and Pearson (1980) noted that the species of *Polystomoides* living in the pharyngeal cavities all had large hamuli, yet was shorter than the diameter of the haptoral suckers. Other than this, the reasoning behind the separation of this group, rests mainly on the absence of the traits differentiating the other two groups.

The groups proposed in this key correlate relatively well with clades on the phylogenetic tree of Héritier *et al.* (2015) (Figure 5.1d). Although there are a few inconsistencies, for example, the position of *Neopolystoma chelonidae*, the proposed key provides a better description of the molecular situation than the paraphyly suggested by the existing genera. Hillis (1987)

suggested that conflict between morphological and molecular evidence with regard to phylogenies may be an indication of the existence of theoretical or procedural problems in either one or both analyses. Alternatively, it may indicate the need for additional data in order to resolve the phylogenetic relationships. In the case of chelonian polystomes, the phylogenetic problem will only be adequately resolved with the addition of more genetic data.

CHAPTER 6

General Discussion & Conclusion

Knowledge on morphological plasticity among polystomes is mainly based on morphological species descriptions (Badets *et al.* 2013). Although several authors have commented on the inter- and intraspecies variation (Aisien & Du Preez 2009; Du Preez *et al.* 2002; Du Preez & Martiz 2006), there has not been many comprehensive studies done on this subject. The main aim of the present study was to determine the extent of the morphological plasticity displayed by some of the polystomatid flatworm genera. Tinsley (1974) suggested that a knowledge of the degree of plasticity, may shed light on some uncertainty on the validity of the described species. Our objectives were reached mainly through morphological, and to a lesser extent, molecular methods. The results from both methods may influence the known taxonomy and may warrant a re-evaluation of the species status of several polystomes.

6.1. Assessment of morphological plasticity

Studies on the degree of plasticity of marginal hooklets displayed by polystomatids are few and far between. Most of the studies conducted has focused on gyrodactylids (Kearn 1968; Kearn 1999; Olstad *et al.* 2009) or on other sclerites (Tinsley 1974; Tinsley & Jackson 1998b; Williams 1960). Although gyrodactylids are also monogeneans, findings of these studies are not always relevant to polystomatids (Du Preez & Maritz 2006; Murith 1981a).

It has always been assumed that the marginal hooklets of polystomatids remain constant throughout the parasite's life, thus making it a reliable taxonomical characteristic (Du Preez & Maritz 2006; Llewellyn 1963; Llewellyn 1968; Theunissen *et al.* 2014). Although our results provide support for the consistency of these hooklets' measurements irrespective of chemical fixative used, it was also found that in some species the sizes of the hooklets seemed to change depending on the age and life-stage of the polystome. However, this has not been found in previous studies on the morphology of polystomatids and our sample were too small to make a conclusive recommendation. The plasticity found in our study may, therefore, be an indication of specific life-history similarities between those species that have shown differences in their marginal hooklet sizes across different life-stages. Since Poisot *et al.* (2011) suggested that the haptoral attachment structures of monogeneans may be influenced mostly by phenotypic plasticity among generalist species, it may be worthwhile to further investigate the degree of plasticity in these structures of polystomatids. It may be valuable to measure the C1 hooklets of not only the oncomiracidium, but also the adult and neotenic forms, if available, for species descriptions. Whether or not these sizes change, as well as their respective

measurements, may be able to aid in the separation of species, by ensuring optimal results of the marginal hooklet plots proposed by Du Preez & Maritz (2006).

Several other traits previously used for species differentiation were found to display some form of plasticity, such as the egg shape and size, the intestinal arrangement, and the placement and appearance of the gonads (Aisien & Du Preez 2009; Du Preez *et al.* 2010; Du Preez & Moeng 2004; Du Preez & Morrison 2012; Enabulele *et al.* 2012; Tinsley 1974; Tinsley 1978a). Rohde (1965) stated that several morphological characteristics can be used to differentiate between species of the genus *Polystomoides.* Tinsley & Tinsley (2016) have shown even further how morphology may be useful for deriving evolutionary divergence, while separating genera.

Even though Prudhoe & Bray (1982) cautioned against the use of the reproductive organ morphometrics for species differentiation in Platyhelminthes in general, several polystomatid species, and genera, such as *Metapolystoma*, are still described based hereupon. The placement of gonads in the polystome body have always been assumed to be a good indicator of polystome species due to the lack of sufficient evidence suggesting otherwise (Williams 1961). However, recently some authors have suggested that the position and appearance of the gonads may be variable to some extent (Du Preez 2015; Enabulele *et al.* 2012).

Our results further suggest the cautionary use of the appearance of the gonads, and especially the uterus, for species and generic differentiation. By combining molecular tools with morphological evidence, we have indicated that the genus *Metapolystoma* may be a junior synonym to *Polystoma*, and that the main distinguishing character, namely the elongated uterus, may only be a plastic trait dependent on the host's ecology and behaviour.

Metapolystoma spp. occur in the east of Africa and Madagascar (Combes 1976). The three known species in this genus, *M. brygoonis*, *M. cachani*, and *M. porosissimae* occur in three species of *Ptychadena*, namely *Pt. mascariensis*, *Pt. longirostris*, and *Pt. porosissima* respectively. Since most *Ptychadena* species display similar ecological preferences, it may indicate that similar environmental factors may have a similar influence on the morphology. For example, *Ptychadena* species are mostly adapted to a terrestrial lifestyle, but require water for reproduction. Du Preez and Kok (1992b) suggested that this semi-terrestrial lifestyle may be one of the reasons they are such suitable hosts for polystomatids and may also explain the description of two polystomes with very different life-history adaptations from one host (Du Preez & Kok 1992a). *Metapolystoma porosissimae*

has the typical morphological adaptations allowing the parasite to thrive in more arid areas, whereas *Polystoma sodwanensis* are more adapted to wet climates. This may imply that temporal isolation and some plasticity in the shape of the uterus have played an important role in the speciation of these co-occurring species. However, there has not yet been sufficient molecular divergence to permit the generic separation of these species.

When using the appearance or position of the gonads for species differentiation, it is advisable that several other traits, as well as the currently available molecular tools, be used in combination (Hillis 1987). When studying the morphology of chelonian polystomes, the shape of the testis is a valuable trait for differentiation of species within various microhabitats. In the present study, several species occupying their host's bladder were found to have a somewhat lobed testis, while not the case in polystomes living in other microhabitats. However, this trait was only one of several other traits indicating a distinct difference between these bladder-inhabiting polystomes and others. Similar findings from an independent study by Tinsley and Tinsley (2016) supported our results and they described a new genus, *Uropolystomoides*, for all *Polystomoides* species occurring in the bladders of their respective hosts.

Our results further suggested that all chelonian polystomes from the genus *Neopolystoma* inhabiting the conjunctival sacs of their hosts were separate from other *Neopolystoma* species. The shape of the eggs, size of the marginal hooklets and genital spines, and the length of the intestinal caecae all supported their distinct molecular position on the phylogenetic tree of Héritier *et al.* (2015). Therefore, we propose that, although these traits may have been only plastic traits once, their plasticity could have allowed these species to adapt to a new micro-habitat. The spatial isolation may then have led to sufficient variation to cause possible generic separation.

6.2. Conclusion and recommendations

In this dissertation we have explored several examples of morphological plasticity among the polystomatids and conclude that there is a much larger degree of plasticity than previously assumed. Based on our results, we recommend:

- Measuring all available life-stages of amphibian polystomes' marginal hooklets before describing new species;
- Synonymising *Metapolystoma* to *Polystoma*;
- Undertaking further phylogenetic studies on *Protopolystoma xenopodis* to assess the molecular divergence reported in Chapter 4;
- Doing generic separation of polystomes inhabiting the conjunctival sacs of chelonians.

We urge that those studying the morphology of these flatworms do so with caution, and seek sufficient morphological and molecular evidence before assuming an existing species, or describing a new one.

There is still room for improvement of our knowledge of morphological plasticity among the polystomatids. Future studies could aim to discover the following:

- The environmental and chemical cues that initiate development and hatching of polystome eggs in the water;
- The extent and reason for the reduced size in marginal hooklets from oncomiracidium to adult based on larger sample sizes;
- The influence of global climate change on host-specificity and, therefore, also speciation events; and
- The true phylogeny of chelonian polystomes, where the molecular and morphological evidence lines up.

CHAPTER 7

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Appendix 1

Parasite	Gene	Genbank Accession Number	Host	Locality
Metapolystoma brygoonis	28S	JN800281; FM897270	*Ptychadena mascariensis*	Vohiparara, MG Ambatolampy, MG
Metapolystom brygoonis	COI	FM897300; FM897297; JN800285; JN800284; JN800289; JN800288; JN800287; JN800286	*Ptychadena mascariensis*	Ambatolampy, MG Antovontany, MG Ambatolampy, MG Ambatolampy, MG Makira, MG Makira, MG Vohiparara, MG Ranomafana, MG
Metapolystoma cachani	28S	FM897262	*Ptychadena longirostris*	Nigeria
Metapolystoma cachani	COI	JN800294; KR856163	*Ptychadena longirostris*	Nigeria
Metapolystoma sp.	28S	JN800283	*Boophis doulioti*	Ankarafantsika, MG
Metapolystoma sp.	28S	JN800282	*Ptychadena mascariensis*	Ankarafantsika, MG
Metapolystoma sp.	28S	FM897266	*Aglyptodactylus madagascariensis*	Sahasorotra, MG
Metapolystoma sp.	28S	FM897267	*Boophis madagascariensis*	Andasibe, MG
Metapolystoma sp.	28S	FM897268	*Boophis occidentalis*	Isalo, MG
Metapolystoma sp.	28S	FM897269	*Boophis doulioti*	Ankarafantsika, MG
Metapolystoma sp.	COI	JN800291	*Boophis doulioti*	Ankarafantsika, MG
Metapolystoma sp.	COI	FM897301	*Boophis occidentalis*	Isalo, MG
Metapolystoma sp.	COI	FM897299	*Aglyptodactylus madagascariensis*	Sahasorotra, MG

Metapolystoma sp.	COI	FM897298	*Boophis madagascariensis*	Andasibe, MG
Metapolystoma sp.	COI	JN800295	*Aglyptodactylus madagascariensis*	Madagascar
Metapolystoma sp.	COI	JN800293	*Boophis madagascariensis*	Andasibe, MG
Metapolystoma sp.	COI	JN800292	*Ptychadena mascariensis*	Andasibe, MG
Metapolystoma sp.	COI	JN800290	*Ptychadena mascariensis*	Ankarafantsika, MG
Polystoma australis	28S	AM913872	*Semnodactylus wealii*	South Africa
Polystoma australis	COI	AM913854	*Semnodactylus wealii*	South Africa
Polystoma umthakathi	28S	AM913874; FM897265	*Natalobatrachus bonebergi*	South Africa
Polystoma umthakathi	COI	AM913861	*Natalobatrachus bonebergi*	South Africa
Polystoma dawiekoki	28S	AM157204; AM913875	*Ptychadena anchietae*	South Africa Tanzania
Polystoma dawiekoki	COI	AM913857; AM913856	*Ptychadena anchietae*	Tanzania South Africa
Protopolystoma xenopodis	28S	AM157218	*Xenopus laevis*	South Africa
Protopolystoma xenopodis	COI	KR856172; KP979657; EF380008; EF380007	*Xenopus laevis*	South Africa South Africa
		EF380004	*Xenopus laevis victorianus*	Masindi, Uganda Masindi, Uganda Mukono, Uganda
Protopolystoma occidentalis	28S	KR856160	*Xenopus muelleri*	Togo

Protopolystoma occidentalis	COI	KR856179	*Xenopus muelleri*	Togo
Polystoma testimagna	28S	AM157217	*Strongylopus fasciatus*	South Africa
Polystoma testimagna	COI	AM913860	*Strongylopus fasciatus*	South Africa
Polystoma marmorati	28S	AM157208	*Hyperolius marmoratus*	South Africa
Polystoma marmorati	COI	AM913859; AM913858	*Hyperolius marmoratus*	South Africa
Oculotrema hippopotami (Outgroup)	28S	KR856159	*Hippopotamus amphibius*	South Africa
Oculotrema hippopotami (Outgroup)	COI	KR856178	*Hippopotamus amphibius*	South Africa