Madagascan polystome (Monogenea) diversity and taxonomy

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ABSTRACT AND KEY TERMS

Amphibians serve as hosts to all the major parasite groups including polystomes (Monogenea). Although Madagascar has more than 350 frog species of which all but two are endemic, the known polystome (Monogenea: Polystomatidae) diversity parasitising Malagasy frogs is restricted to three species of *Madapolystoma* Du Preez, Raharivololoniaina *et al.*, 2010, one species of *Kankana* Du Preez, Raharivololoniaina *et al.*, 2011 and one *Metapolystoma* Combes, 1976. Investigating the anuran parasite diversity at selected Malagasy localities led to the discovery of polystomes unknown to science. Two *Madapolystoma* species were discovered from respectively *Blommersia domerguei* Guibé, 1974 and *Mantella expectata* Busse and Böhme. For *Metapolystoma*, two new host genera are reported with the description of six new species from *Boophis* Tschudi, 1838 treefrogs, *Boophis albilabris* (Boulenger, 1888), *Boophis doulioti* (Angel, 1934), *Boophis luteus* (Boulenger, 1882), *Boophis madagascariensis* (Peters, 1874), *Boophis occidentalis* Glaw & Vences, 1994, and *Aglyptodactylus madagascariensis* (Duméril, 1853). These descriptions are supported with morphology and, where possible, with phylogenetic analyses and genetic divergences. For *Madapolystoma*, advanced *in utero* development was reported following the observation of well-developed hamuli and two pairs of haptoral suckers in developing embryos of the newly described species. Inside some of these *in utero* embryos an early F2 generation embryo was also observed. This is the first report of true viviparity among polystomatid flatworms. Twenty-six percent of the 311 Malagasy frogs for which red listing assessment has been completed are classified as Endangered and 7% as Critically Endangered. Consequently, it will be difficult to describe further polystomes that were retrieved from frogs now listed as Threatened. For some of these only a single specimen is available, and the Threatened conservation status would prohibit any further collection. The full extent of the effect that polystomes have on their hosts is not fully understood. In numerous anuran species the parasite-host interactions are at best poorly documented. Polystomes provide insights into the biogeographical origin of their amphibian hosts and ultimately aid in their conservation. The need for parasite surveys in Madagascar is, therefore, emphasised to better conserve its biodiversity. Using the criteria proposed by Smyth and Wakelin (1994) for 'ideal parasites', amphibian polystomes have been found to meet all the listed requirements necessary to be considered 'ideal parasites'.

Key terms:

Aglyptodactylus, *Blommersia*, *Boophis*, Madagascar, *Madapolystoma*, *Mantella, Metapolystoma*, Ovoviviparity, Polystomes, Parasites.

OPSOMMING EN SLEUTELTERME

Amfibieë dien as gashere vir al die hoof parasitiese groepe, wat onder andere polistome (Monogenea: Poystomatidae) insluit. Alhoewel daar meer as 350 paddaspesies in Madagaskar voorkom, waarvan slegs twee nie endemies is nie, is die bekende polistoomdiversiteit tot drie *Madapolystoma* Du Preez, Raharivololoniaina *et al.*, 2010 *spesies*, een *Kankana* Du Preez, Raharivololoniaina *et al.*, 2011 en een *Metapolystoma* Combes, 1976 spesie beperk. ’n Parasitologiese studie in Madagaskar by geselekteerde lokaliteite op amfibieërparasiete het tot die ontdekking van verskeie polistoomparasiete, wat onbekend is tot wetenskap, gelei. Twee *Madapolystoma* spesies is in *Blommersia domerguei* Guibé, 1974 en *Mantella expectata* Busse and Böhme gevind. Verder, word twee nuwe gasheergenera vir *Metapolystoma* gerapporteer met die beskrywing van ses nuwe spesies uit boompaddas, *Boophis albilabris* (Boulenger, 1888), *Boophis doulioti* (Angel, 1934), *Boophis luteus* (Boulenger, 1882), *Boophis madagascariensis* (Peters, 1874), *Boophis occidentalis* Glaw & Vences, 1994*,* en *Aglyptodactylus madagascariensis* (Duméril, 1853)*.* Hierdie beskrywings word met morfologiese bewyse en waar moontlik ook filogenetiese analise en genetiese divergensie gerugsteun. Vir *Madapolystoma* word gevorderde *in utero* ontwikkeling gerapporteer op grond van die ontwikkeling van goed ontwikkelde hamuli en twee pare haptorale suiers in ontwikkelende embrio’s van nuut beskryfde spesies. In sommige van die *in utero* embrio’s was ’n vroeë F2 generasie embrio waargeneem, en is hierdie ook die eerste rekord van ware viviparie onder polistoomplatwurms. Ses en twintig persent van Madagascar se 311 paddas waarvoor rooilysevaluerings voltooi is, word as Bedreig geklassifiseer, ’n verdere sewe persent word as Krities bedreig geklassifiseer. Gevolglik sal dit moeilik wees om polistome te beskryf wat destyds vanuit paddas versamel is en nou as Bedreig geklassifiseer is. Vir sommige van hierdie spesies is slegs ’n enkele eksemplaar beskikbaar en verhoed die bewaringsstatus van die gashere die versameling van meer materiaal. Die volle omvang van die effek wat polistome op hulle gashere het, is nie heeltemal duidelik nie. In vele Anura-spesies is parasietgasheer-interaksies, op die beste, swak gedokumenteer. Polistome voorsien ’n blik op die biogeografiese oorsprong van hul amfibieërgashere en dra uiteindelik ook tot hul bewaring by. Die noodsaaklikheid vir parasitiese opnames in Madagascar word ook beklemtoon om so ook uiteindelik bewaringsstrategieë te bevorder. Verder het Smith en Wakelin (1994) kriteria vir die “ideale parasiet” voorgestel. Amfibieërpolistome word hier geëvalueer volgens hierdie kriteria, wat dan ook daarop dui dat polistome aan al hierdie vereistes voldoen, en sodoende as “ideale parasiete’ beskou kan word.

Sleutelterme:

Aglyptodactylus, Blommersia, Boophis, Madagascar, *Madapolystoma*, *Mantella Metapolystoma* Ovoviviparitеit, Parasitete, Polistome.

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CHAPTER 1:
GENERAL INTRODUCTION



MADAGASCAR, A PARASITE'S PARADISE

When delving deeper into the study of parasitology, it is evident that parasitism is a very successful and common phenomenon and there are far more parasitic than non-parasitic organisms in existence (Roberts & Janovy, 2010). All living organisms are probably affected by parasitism sometime during their lifespan (Kennedy, 1984). In fact, Roberts & Janovy (2010) go as far as saying that if an organism is not a parasite, it probably acts as a host for parasites. Thus, parasites not only influence the functioning of populations and the ecosystems in which they occur, but also the evolution of all free-living, non-parasitic organisms (Kennedy, 1984). The parasitic lifestyle is highly successful, with its evolution reflected into nearly every independent phylum, including plants, animals, and protistans (Roberts & Janovy, 2010). As one of the major parasitic groups, flatworms (Platyhelminthes) infect frogs globally. Platyhelminthes comprises four classes, namely Turbellaria, Trematoda, Monogenea, and Cestoda (Hickman *et al.*, 2020). Except for the class Turbellaria which includes mostly free-living non-parasitic flatworms, the majority of flatworms are parasitic (Neodermata).

Amphibians are diverse with a globally fragmented distribution. Excluding Antarctica and a few other islands, both aquatic and terrestrial species can be found in various habitat types. The amphibious (from Greek '*amphi*'= both; '*bios*'= life) lifestyle allows these organisms to serve as host for both terrestrial and aquatic parasites (Raharivololoniaina *et al.*, 2011). Furthermore, they occupy an intricate position in the food chain, serving as both prey and predator for various species, favouring the transmission of parasites between different intermediate and final hosts. Frogs serve as hosts for all the major parasite groups, ranging from single-celled protozoans to large cestodes (tapeworms) (Smyth & Smyth, 1980). Some of these groups include monogeneans, trematodes, acanthocephalans, nematodes, leeches and mites (Smyth & Smyth, 1980). For example, *Xenopus laevis* (Daudin, 1802) (Common Platanna or African clawed frog) serve as host for more than 30 different parasite species from all the above mentioned groups, excluding only acanthocephalans in its native South African range (Crosgrove & Jared, 1974; Macnae *et al.*, 1973; Pritchard, 1964; Schoeman *et al.*, 2020; Tinsley, 1996). Among the parasite groups found in amphibians, the polystomatid platyhelminths (Monogenea, Polystomatidae) are of particular interest and will be the focus of this study. The study of amphibian helminth parasites was initiated in 1737, with the discovery of a lung helminth by Swammerdam (Lezama & Sarabia, 2002). It was only 43 years later, in 1758, when the first anuran monogenean (Greek: monos 'single' and genea 'generation') (Platyhelminthes) was discovered by Roesel von Rosenhof (Stunkard, 1917).

Monogenea, also known as monogenetic flukes, is the smallest of the four platyhelminth classes, containing approximately a tenth of the diversity of Digenea and less than half that of Cestoda (Caira & Littlewood, 2001). The term Monogenea was created by Carus (Yamaguti,

1963) and accepted at class level in 1978 (Boeger & Kritsky, 1997). Although Monogenea is recognised in most modern classifications of Platyhelminthes, there are some debates regarding its nomenclature. Although some authors still prefer to use the term Monogenoidea, this study will refer to Monogenea.

1.1 Polystome history and evolution

Monogenea are mostly parasitic on fish except for one family, the Polystomatidae (Polystomes) that radiated onto tetrapods (amphibians, chelonians, and the African hippopotamus) and the Australian lungfish (Dipnoi Müller, 1844) (see Pichelin *et al.*, 1991; Sinnappah *et al.*, 2001; Verneau *et al.*, 2002). The name Polystomatidae (from Greek 'poly'= many; 'stoma'= mouth) originated from a misidentification where early scientists identified the haptoral suckers as mouths. The Polystomatidae is one of the most diverse families of Monogenea (Badets *et al.*, 2011) with 30 recognised genera and more than 200 species (Fig. 1.1).

Polystomes radiated with their hosts and today we find them wherever their hosts occur. Thus, they can be found globally, on all continents, excluding only Antarctica and some islands (Du Preez *et al.*, 2008). They have co-evolved with their hosts and exhibit direct life cycles that led to extensive speciation (Héritier *et al.*, 2015; Hickman *et al.*, 2020; Tinsley & Earle, 1983). This co-evolution is especially evident in the reproductive cycle of polystomes, which is synchronised to that of both the reproductive cycle and behaviour of the host (Armstrong *et al.*, 1997; Cable *et al.*, 1998; Smyth & Smyth, 1980; Tinsley, 1999), and in the tracking of the evolutionary history of main vertebrates (Verneau *et al.*, 2002). These facts suggest an early origin of polystomes that may date back to the transition of aquatic vertebrates to terrestrial vertebrates (Verneau *et al.*, 2002). Verneau *et al.* (2002) suggest that polystomes most probably arose 425 million years ago, coinciding with the transition between actinopterygians and sarcopterygians. Moreover, Héritier *et al.* (2015) postulated that amphibian polystomes originated in the Middle-Late Devonian period accompanied by the ecological transition of monogeneans from fish to tetrapods and co-evolved with their hosts during the subsequent Mesozoic and Cenozoic periods. It is hypothesised that the Polystomatidae Gamble, 1896 originally diverged from anurans, after the monogenean transition from fish to amphibians, and radiated to freshwater chelonians (Boeger & Kritsky, 1997). Furthermore, it is suggested that polystome evolution amongst extant amphibians, semi-aquatic hippopotami, and aquatic chelonians indicate aquatic habitats in certain ancestral lineages during early evolutionary history (Héritier *et al.*, 2015).

With the exception of *Gyrdicotylus* Von Nordmann, 1832 (Gyrodactylidae Cobbold, 1864), polystomes are the only monogeneans known from terrestrial vertebrate hosts (tetrapods), which

display aquatic or semi-aquatic behaviour, except for the Australian lungfish which is exclusively aquatic (Héritier *et al.*, 2015).

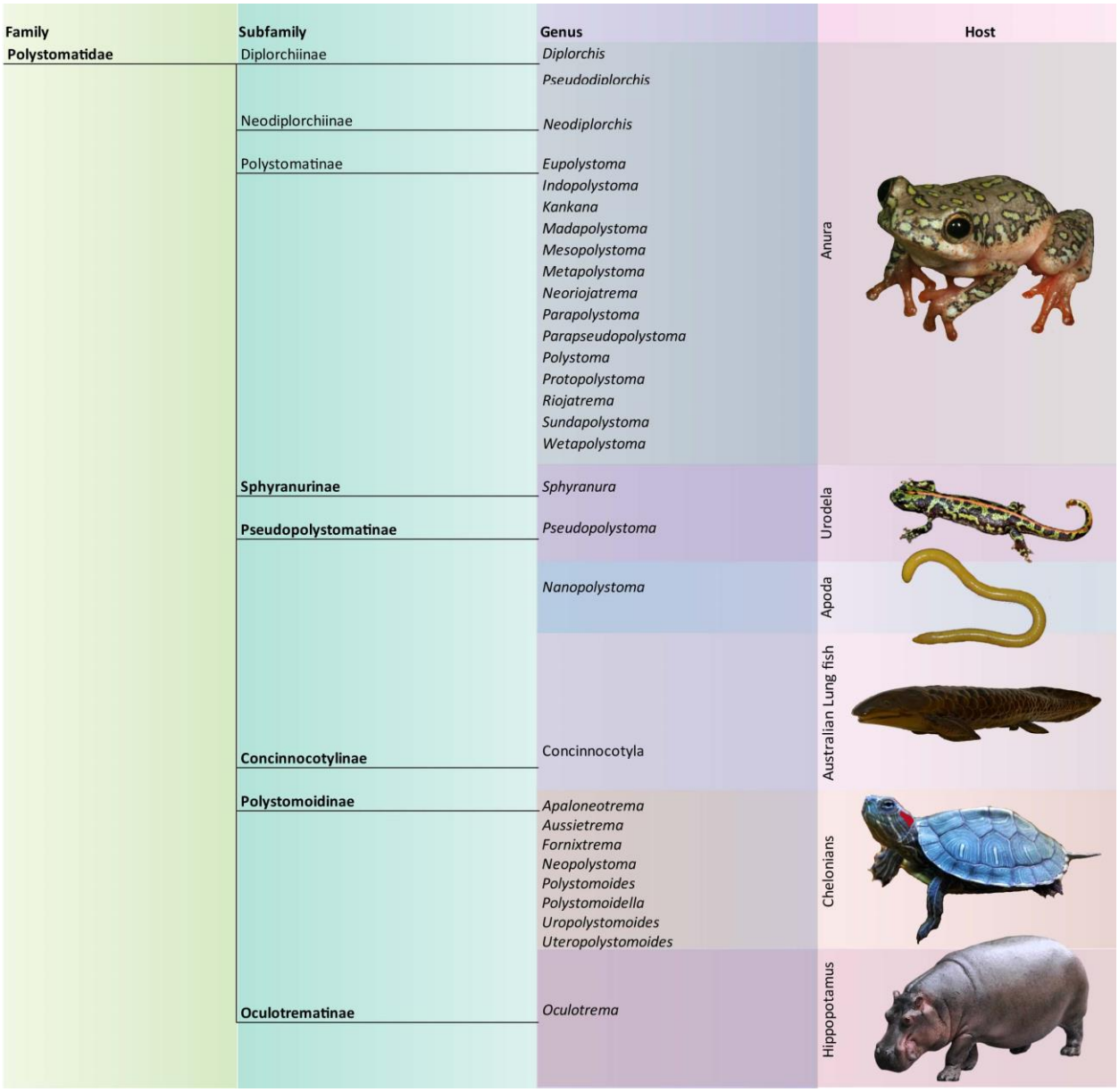


Figure 1.1: Subfamilies, genera, and host groups of the Polystomatidae.

Most monogeneans parasitising fish are found on the external surfaces and gills of chondrichthyan and actinopterygian fish, as well as in the internal cavities of sarcopterygian fish. Those monogeneans that occur in chelonians infect either the conjunctival sac, oral cavity, pharynx, or the urinary bladder of the host (Tinsley & Earle, 1983). Only one monogenean, *Oculotrema hippopotami* Stunkard, 1924, is found in mammals, where it occurs on the eyes of the semi-aquatic *Hippopotamus* Linnaeus, 1758 (Tinsley & Earle, 1983). Some anuran polystome larvae can be found on the gills of tadpoles, while others develop within the metamorphosed host's kidney, with all of the adult stages occurring in the urinary bladder of the host (Tinsley &

Earle, 1983). In all of these sites, the polystomes are protected from desiccation, but still maintain direct communication with the exterior of the host (Héritier *et al.*, 2015; Hickman *et al.*, 2020; Tinsley & Earle, 1983).

1.2 Polystomes, the 'Ideal parasites'

Smyth (Smyth & Wakelin, 1994) argues that the 'ideal parasite' must be able to: "recognise a host site suitable for establishment; maintain its position there; is adapted to the physico-chemical conditions of the host; utilises host nutrient in a manner compatible with host survival; presents a surface with a molecular configuration so that the host immune response is absent or minimized; and has a life cycle that is synchronised with that of the host." Such a model can be used to argue that amphibian polystomes, the focus group of this study, are 'ideal parasites'.

1.2.1 Recognizing a host site suitable for establishment

Amphibian polystomatids are generally considered to be host-specific (Berthier *et al.*, 2014). Various studies have been conducted on the host specificity of polystomes (Bourgat & Salami-Cadoux, 1976; Combes, 1966; Combes, 1968; Du Preez *et al.*, 1997; Kok & Du Preez, 1987). These experiments were conducted on *Polystoma* Zeder, 1800 species, including *P. integerrimum* (Frolich, 1798), *P. galieni* Price, 1938, *P. africanum* Szidat, 1932*, P. australe* Kok & van Wyk 1986*, P. marmorati* van Niekerk, Kok *et al.,* 1993, and *P. umthakathi* Kok & Seaman, 1987. Based on experimental infections and cross-infections using *P. australe*, *P. marmorati*, and *P. umthakathi* host recognition occurs at the stage of first contact between the parasite and the host-tadpole (Kok & Du Preez, 1987). This was observed when oncomiracidia (polystome larval stage) made contact with a preferred host tadpole and remained on its surface until infestation. Interestingly, those oncomiracidia that made contact with substitute hosts regularly break contact with the tadpoles (Kok & Du Preez, 1987). This recognition at the onset of contact between the polystome and the host, forms the basis of host-specificity (Kok & Du Preez, 1987). Although these studies demonstrated that some polystome species would, in some instances, infect a substitute host, they develop poorly or have reduced egg production (Combes, 1966). Nevertheless, in the evolutionary context, the parasite's ability to switch hosts is essential to radiate onto other species. These excellent adaptation strategies and the ability to recognise suitable hosts for establishment can be observed in polystomes infecting *Ptychadena* Boulenger, 1917 (grass frogs). For example, *Ptychadena mascareniensis* (Duméril and Bibron, 1841) is a probable vector for *Metapolystoma* Combes, 1976, which, after introduction to Madagascar, diversified into various *Metapolystoma* species (Verneau *et al.*, 2009a).

1.2.2 Maintaining a position on the host

Amphibian hosts present two major physical challenges for polystomes: i) In those polystomes with a neotenic phase, infective larval stages need to attach to the surface of the gills of tadpoles (Fig. 1.2A-B), which suggests that the oncomiracidium needs to make contact with the tadpole of the correct host species and then locate the gills; ii) the flexibility of the urinary bladder where the thickness of the wall can alter in a short time during the expulsion of urine. The sudden change in the physical structure of the urinary bladder wall might cause bladder-associated parasites to lose their grip and detach from the bladder wall (Tinsley, 1971). Thus, polystomes have employed several morphological and evolutionary counteractions to overcome the above-mentioned problems. These include structures such as larval or marginal hooklets, hamuli, and haptoral suckers. Oncomiracidia are armed with 16 marginal hooklets (Fig. 1.3A) positioned on the periphery of the haptor. These hooklets are used to attach to the gills of host tadpoles. In egg producing neotenic parasites or mature parasites these marginal hooklets become non-functional as their attachment function is taken over by haptoral suckers, but the hooklets remain embedded in the haptor. These hooklets are generally positioned as follows (Fig. 1.3C): four hooklets are placed between the posterior-most haptoral suckers (sucker pair one), one hooklet is found inside each haptoral sucker, and six more hooklets are positioned between the anterior-most haptoral suckers (sucker pair three). Furthermore, two large hamuli are found in most polystomes between haptoral sucker pair one (Fig. 1.3B), which also plays a role in the attachment of adult parasites (Theunissen *et al.*, 2014). Haptoral suckers are cup-shaped and positioned symmetrically on the haptor (Fig. 1.2C). They are muscular and are used to attach to the gills of tadpoles (in neotenic parasites) or the urinary bladder's wall (in mature parasites). In neotenic parasites the haptors are soft and flexible and the suckers can attach in any direction around the haptor. This is essential for attachment to the finger-like buds of the tadpole gill filaments (Kok & Du Preez, 1989). The haptor of the bladder parasite is rigid with the suckers positioned in a ring and directed ventrally. The suction from the suckers is so efficient that it is often hard to remove parasites from dissected hosts (see Figure 1.2D showing markings or buds on the bladder surface of the host after parasite removal).

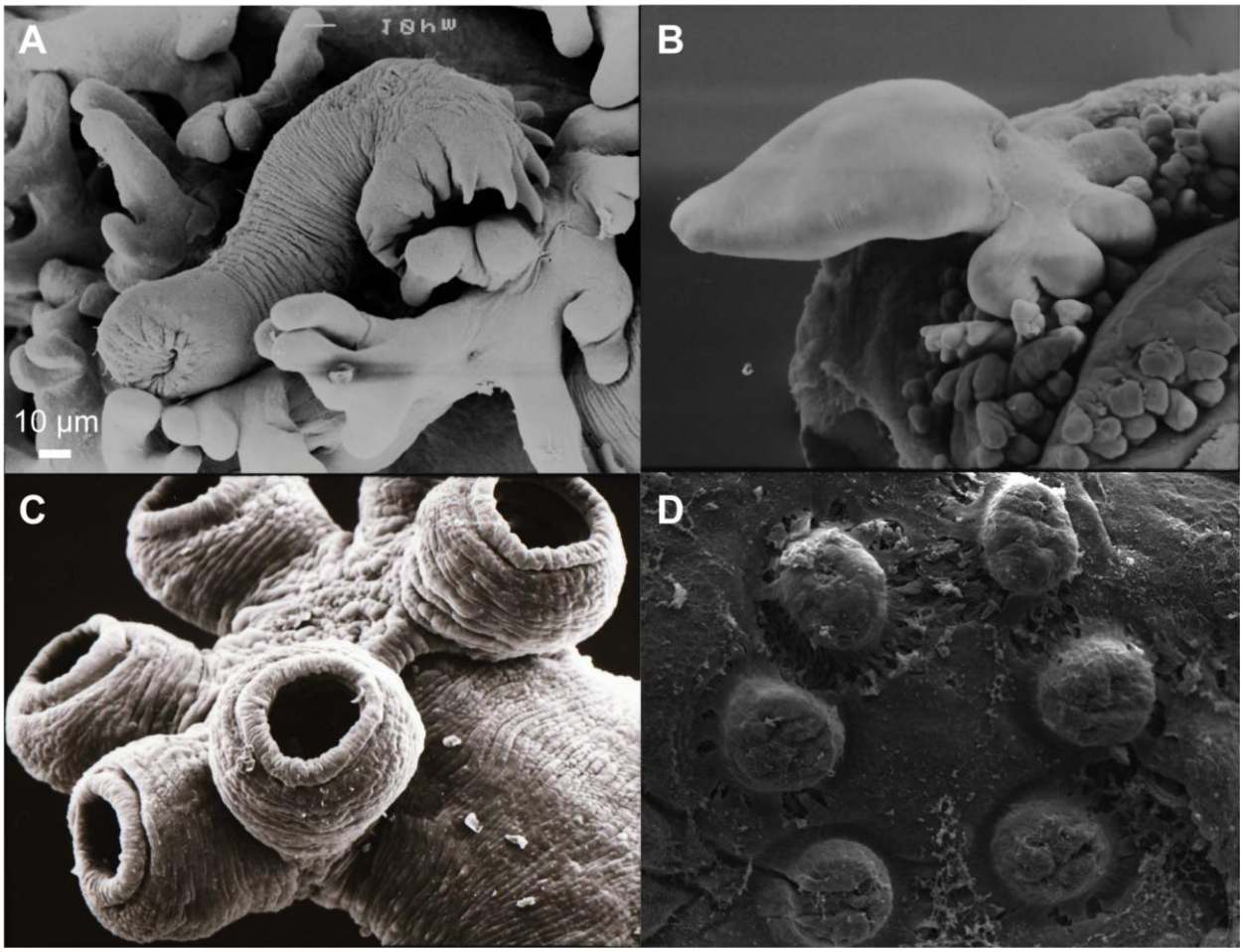


Figure 1.2: Scanning electron micrographs. (**A**) An oncomiracidium latched onto the gills of a tadpole with its marginal hooklets. (**B**) A neotenic polystome attached to the gills of a tadpole. (**C**) Neotenic polystome haptoral suckers. (**D**) Markings or sucker buds left on the urinary bladder wall of a frog after the removal of a polystome parasite. (Photo credit L.H. Du Preez)

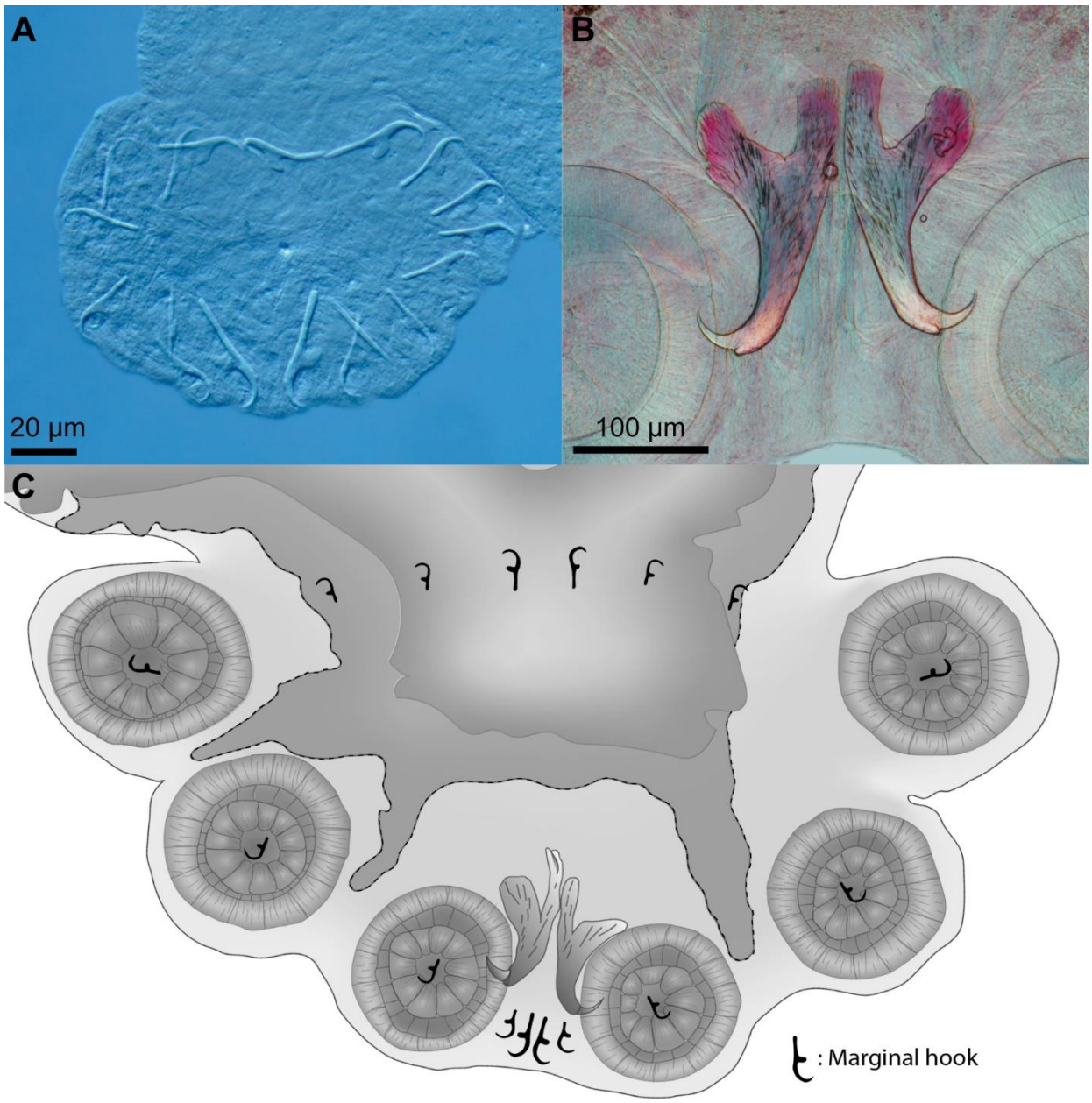


Figure 1.3: Light microscope micrographs (A & B). (**A**) Positioning of marginal hooklets on the haptor of a metapolystome oncomiracidium. (**B**) Hamuli between the posterior-most haptoral suckers of *Metapolystoma porosissimae*. (**C**) Diagrammatic illustration of the placement of marginal hooklets on a *Metapolystoma* species.

1.2.3 Adaptations to the physico-chemical conditions of the host

Polystomes are physiologically adapted to live in the environment provided by the host, and their maturation is linked to the level of sex hormones and maturation of the host (Miretski, 1951). For example, polystome parasite maturation can be induced by injecting immature host frogs with hormones that give them the physico-chemical appearance of mature frogs. Miretski

(1951) demonstrated this effect, where young frogs were injected with hypophysis extract that led to the maturation of juvenile polystomes within four to eight days. After the parasites matured, egg production started and continued over one week. This time corresponds to the average time spent by the hosts on spawning. The injection of the hypophysis extract induces an increase in both the gonadotrophins and gonadal hormones, each of which have the potential to elicit the maturation response from the parasite. Thus, the direct cause of the parasite maturation is as yet unknown.

Neotenic phenotypes of *Polystoma* and *Metapolystoma* develop when parasites attach to young tadpoles in pre-metamorphosis (Combes, 1968; Galien, 1935; Williams, 1961). In such a case, the neotenic parasite will attach to the gills of the tadpole, mature after two to three weeks, drop out and die as soon as the tadpole's front legs break out of the gill chamber during metamorphosis (Combes, 1968; Galien, 1935; Williams, 1961). However, if a larval polystome attaches to an older tadpole in pro-metamorphosis, when the hind legs of the developing tadpole bend, the parasite will develop normally into a mature reproductive phenotype. The oncomiracidium also attach to the gills but develop slowly, and when the front legs break free the bladder destined parasite migrates over the abdomen to the cloaca where it enters to establish itself in the urinary bladder (Badets *et al.*, 2009; Du Preez & Kok, 1999). In the bladder, the parasite reaches maturity a year later, or in some species after about two to three years (Combes, 1968; Galien, 1935; Williams, 1961). Thus, the parasite development following either a neotenic or mature reproductive cycle depends on the physiological stage of the tadpole to which the parasite attaches (Combes, 1968; Williams, 1961). Badets *et al.* (2009) presented evidence for chemical cues in the control of this reproductive plasticity. The authors illustrated parasite physico-chemical adaptations to the host in that the introduction of oncomiracidia to tadpoles of different ages at the same time resulted in more oncomiracidia developing into neotenic parasites, despite attachment to older tadpoles (Badets *et al.*, 2009).

1.2.4 Utilization of host nutrients in a manner compatible with host survival

Polystomes, especially those in mature cycles, depend entirely on their hosts for both food and oxygen. Since the mature cycle takes its course inside the frog's urinary bladder with no contact with oxygenated air or water, the parasite relies on the host for oxygen. This is not the case for neotenic parasites that have access to oxygenated water. A single frog can be infected with as many as 2 000 polystomes, as has been observed in *Eupolystoma* Kaw, 1950 (see Du Preez *et al.*, 2003). Although some monogenean species have adverse, detrimental effects on fish (Fajer-Ávila *et al.*, 2007; Montero, 2004; Rubio-Godoy & Tinsley, 2008), only minor pathological effects of these parasites have thus far been observed in amphibians. In the latter, polystomes with life stages utilizing either the lungs or kidneys of the host appear to be particularly

harmful. For example, *Pseudodiplorchis americanus* (Rodgers and Kuntz, 1940), which parasitises *Scaphiopus couchii* Baird, 1854, may cause lung epithelial scaring and possible chronic effects (Tinsley & Jackson, 2002). *Protopolystoma xenopodis* Bychowsky, 1957, parasitising *X. laevis* is suspected of causing some damage to the host kidneys (Fig. 1.4) during the parasite's two to three-month maturation period (Tinsley & Jackson, 2002). However, only high numbers of the blood-feeding *P. americanus* infecting lungs of a single host have been found to directly influence host survival (Tocque & Tinsley, 1994). This is due to the substantial blood loss caused by the parasite which drains the energy reserves of the frog preparing for hibernation. Although suction buds (Fig. 1.2D) have been observed at the site of the parasite attachment (Theunissen *et al.*, 2014), no epithelial damage or scarring from bladder parasites has thus far been reported (Tinsley & Jackson, 2002). Furthermore, even in heavily infected tadpoles, no apparent effects of neotenic parasites have been found, (Kok & Du Preez, 1989; Savage, 2009). However, since in numerous anuran species the parasite-host interactions are at best poorly documented, future studies will likely shed more light on this subject.

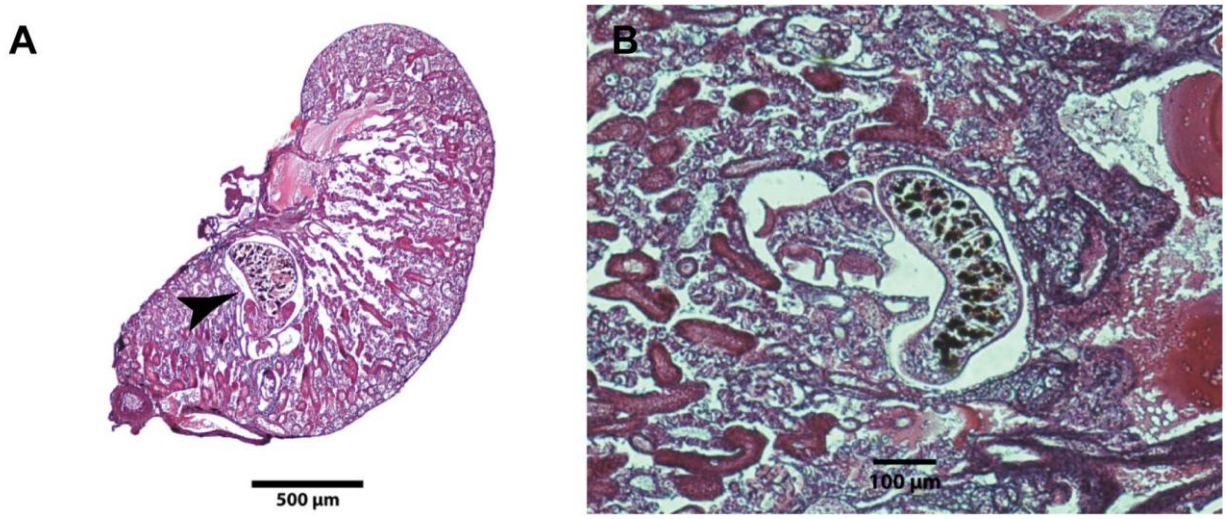


Figure 1.4: Light microscope micrograph of histology sections. (A) Juvenile *Protopolystoma xenopodis* (arrow) attached to the inside of the kidney of *Xenopus laevis*. (B) High magnification of *Protopolystoma xenopodis* in kidney. Photo credit (L.H. Du Preez).

1.2.5 Reduced host immune response

The immune systems of anurans comprise almost all the components of those of mammals (Carey *et al.*, 1999). These immune studies mainly focused on *Xenopus laevis* as a model and indicated the presence of T-cell receptors, B- and T-lymphocytes, and immunoglobulin receptors. The characterisation of lymph tissue, T-cell, and major histocompatibility complex-restricted cytotoxic responses have also been conducted for *X. laevis* (Carey *et al.*, 1999). This defence includes immunological memory (Carey *et al.*, 1999; du Pasquier *et al.*, 1996; Horton *et*

al., 1996). However, amphibian immune responses are suppressed by low temperatures (Maniero & Carey, 1997). This is true even for the functioning of memory cells that initiate responses based on the previously encountered pathogens (Maniero & Carey, 1997). For example, *Protopolystoma xenopodis* produced more eggs when its host was exposed to lower (6°C) and then higher temperatures (20°C). It was speculated that this might have been the result of the host's suppressed immune system which ceased to suppress parasite egg production (Tinsley & Jackson, 2002).

Furthermore, circumstantial evidence indicates the attributable control of polystomes by amphibian hosts (Tinsley, 1993). As has been shown by Tinsley (1995; 1999), infrapopulations of *Pseudodiplorchis americanus* are controlled by the host's immune response. As a result, 97% of larvae that have successfully invaded the host die before they can reproduce. It has also been shown that *X. laevis* employs acquired immunity to combat polystome parasites (Jackson & Tinsley, 2001). This has been demonstrated in laboratory-bred frogs that have been infected and subsequently reinfected with *P. xenopodis* after the first group of parasites died of natural causes (i.e. three years after the first infection). It was shown that in the reinfecting parasites the maturation was slower and the egg production less efficient than those observed during the primary infection. However, these experiments have only been conducted on two species of parasites infecting the same host species. Even though *P. xenopodis* elicits a potent immune response from its host, this parasite still has a 40% prevalence with a mean intensity of 1–2 reproductive worms and a maximum intensity of six reproductive worms per host (Tinsley & Jackson, 2002), which indicates that the host's immune defence is not efficient in eradicating all the parasites. This is especially evident in *P. xenopodis* that can live up to three years inside the host (Jackson & Tinsley, 2001), whereas other polystome species only reach maturity after two to three years. Since these parasites tolerate the immune system of the host for long periods without being eradicated, they must have some form of protection against the host's immune defence.

1.2.6 Synchronization of parasite and host life cycles

Monogeneans have direct life cycles with only a single host (Smyth & Wakelin, 1994). The life cycle of most known polystomes is characterized by a free-swimming ciliated oncomiracidium (Smyth & Wakelin, 1994). Polystomes have different reproductive strategies that co-evolved in synchrony to the maturation, reproductive cycle, and the behaviour of their host (Héritier *et al.*, 2015; Hickman *et al.*, 2020; Tinsley & Earle, 1983). This synchrony ensures that the parasite's eggs and embryos are released in the same environment and time as that of the host's offspring, providing new hosts for infection (Smyth & Wakelin, 1994). *Xenopus laevis* that regularly reproduces throughout the year, for instance, hosts *Protopolystoma xenopodis* which

continuously produces and releases one egg at a time (average nine eggs per worm per day at 20°C) during the hosts breeding season (Badets *et al.*, 2009; Jackson & Tinsley, 1998). At 20°C these eggs take about 22 days to develop and hatch (Jackson & Tinsley, 1998). Since hosts are readily available, they still have a considerable chance to find a host. At another extreme, *Pseudodiplorchis americanus* (Polystomatidae) adopted ovoviviparity with eggs that hatch with the initiation of host amplexus (Tinsley, 1983). This strategy is adapted to its host, *Scaphiopus couchii* Baird, 1854 (Desert-dwelling Couch's spadefoot toad), that only reproduce for one to three nights per year in harsh desert environments (Badets *et al.*, 2009). In situations like this, it is necessary for the parasite to have immediate infective larvae after egg deposition since suitable hosts will only be available for a very short period.

By taking these points into consideration, polystomes comply with all the requirements of being an 'ideal parasite', except for maybe some aspects of host immune elicitation. With this in mind, and the fact that polystomes are relatively common, widespread, and a well-studied parasite group, they serve as excellent resources and models for scientific experimentation and research.

1.3 Madagascan polystome diversity

As a biodiversity hotspot, Madagascar is ranked among the world's top three regions of conservation importance (Myers *et al.*, 2000). The high species diversity and level of endemicity is ascribed to the isolation of the island over an extended period of time (Galien, 1935; Glaw & Vences, 2007; Goodman & Benstead, 2005). Madagascar's separation from other landmasses began during the breakup of Gondwana about 156–165 million years ago (Rabinowitz *et al.*, 1983) and ended when it separated from India about 84–94 million years ago (Storey *et al.*, 1995). Globally, Madagascar is ranked as the country with the twelfth highest amphibian species richness (Andreone *et al.*, 2008). While the true number of anuran species was reckoned to be closer to 465 species (Glaw *et al.*, 2010; Glaw & Vences, 2006; Vieites *et al.*, 2009), 356 frog species are currently described from the island (Frost, 2020) with a near 100% endemicity. Only two non-native invasive species, *Hoplobatrachus tigerinus* (Daudin, 1802) and *Duttaphrynus melanostictus* (Schneider, 1799), have so far been recorded (Macnae *et al.*, 1973; Moore *et al.*, 2015). It is hypothesised that this rich amphibian diversity is mainly the result of dispersal from Africa, South America via Antarctica, and India (Case, 2002; Krause *et al.*, 1997; Noonan & Chippindale, 2006; Poux *et al.*, 2005; Tinsley, 1996; Vences *et al.*, 2003; Yoder & Nowak, 2006). Despite Madagascar's conservation status, the diversity and endemicity of the less prominent taxa are poorly known (Goodman & Benstead, 2005; Myers *et al.*, 2000). As can be expected, the high species richness is not restricted to the herpetofauna alone but also applies to their parasites (Junker *et al.*, 2010; Kuzmin *et al.*, 2013; Rocha *et al.*, 2012; Wohltmann *et al.*, 2007).

Madagascar remains strikingly underexplored for amphibian parasites in general, especially considering the local diversity of potential hosts. Until now, only 24 amphibian parasite species from the eight major parasite groups have been described from 28–29 frog species in Madagascar, with no described species for Acanthocephala (Table 1.1). At least five protozoan, 12 nematode, four acanthocephalan, 12 monogenean, four digenean, and two cestode morphotypes discovered by Louis du Preez from 23 frog species await formal description (Unpublished data). When taken into consideration the few frog species that were investigated for full parasite counts against the 356 known Malagasy frog species, the discovery of many more amphibian Malagasy parasites can be expected. With respect to polystome (Monogenea: Polystomatidae) diversity, the main focus of this study, four genera have been described from Madagascar: (i) *Uropolystomoides* Tinsley and Tinsley, 2016 with a single species *Uropolystomoides chabaudi* (Euzet and Combes, 1965) (Fig. 1.5A) from the chelonian host *Pelomedusa subrufa* (Lacépède, 1788) (ii) *Metapolystoma*, with a single species *Metapolystoma brygoonis* (Euzet and Combes, 1964) (Fig. 1.5B) from the anuran host *Ptychadena mascareniensis;* (iii) *Madapolystoma* Du Preez, Rahariviololoniaina *et al.*, 2010 with three species infecting frogs, namely *Madapolystoma biritika* Du Preez, Du Preez *et al.*, 2010 (Fig. 1.5C) from *Mantella madagascariensis* (Grandidier, 1872), *Madapolystoma cryptica* Berthier, Du Preez *et al.*, 2014 (Fig. 1.5D) and *Madapolystoma ramilijaonae* Berthier, Du Preez *et al.*, 2014 (Fig. 1.5E) from the same host *Guibemantis liber* (Peracca, 1893) and (iv) *Kankana* Raharivololoniaina, Verneau *et al.*, 2011, with a single species *Kankana manampoka* Raharivololoniaina, Verneau *et al.*, 2011 (Fig. 1.5F) from the frog *Cophyla pollicaris* (Boulenger, 1888). Among the amphibian polystomes, only *Metapolystoma* is not endemic to Madagascar. There are only three described species of *Metapolystoma,* namely *M. porosissimae* Du Preez and Kok 1992 (Fig. 1.6A) from *Ptychadena porosissima* (Steindachner, 1867) in South Africa; *M. cachani* (Gallien 1956) (Fig. 1.6B) from *Ptychadena longirostris* (Peters, 1870) in West Africa and *M. brygoonis* (Fig. 1.5E) in Madagascar.

As it has been shown that polystomes co-evolved with their hosts since their origin in the Palaeozoic age (Héritier *et al.*, 2015; Verneau *et al.*, 2002; Verneau *et al.*, 2009b), investigating polystome phylogeny can provide relevant insights into the diversification of amphibians over ancient and recent geological periods (Badets *et al.*, 2011). Of the 356 anuran species known from Madagascar (Frost, 2020), 86 species from a few selected localities were screened for polystomes (Verneau *et al.*, 2009a). Additional to the already described Malagasy polystomes, at least 20 more polystome morphotypes were identified from these amphibians, suggesting that, similar to the case of other parasite groups, a significant number of polystome species from Madagascar still await discovery. Therefore, it is important to study their systematics and evolution as this particular frog-polystome association may not only provide significant information

on the biogeographical origins of Malagasy frogs (Verneau *et al.*, 2009a) but also, ultimately, aid in their conservation.

Metapolystoma brygoonis was the only polystome known to occur in Madagascar until 2009 when two new endemic polystome genera were discovered, namely *Kankana* and *Madapolystoma*. During this time, various other undescribed species of *Metapolystoma* and *Madapolystoma* were reported from different hosts in Madagascar (Berthier *et al.*, 2014; Du Preez *et al.*, 2010; Verneau *et al.*, 2009b). The current study will focus on these two genera, shedding light on their reproduction, morphology, and taxonomy.

Table 1.1. Described amphibian parasite species from Madagascar, the parasite groups they are classified under, and the hosts they occur in.

Parasite Group	**Parasite sp.**	**Host sp.**	**Reference**
Protozoa	*Hepatozoon neireti*	*Ptychadena mascareniensis*	Laveran, 1905
Nematoda	*Aplectana brygooi*	*Mantella betsileo, Mantidactylus lugubris, Mantidactylus sp., Boophis luteus*	Baker,1980
	Aplectana courdurieri	*Ptychadena mascareniensis*	Chabaud & Brygoo, 1958
	Falcaustra golvani	*Boophis goudoti or B. madagacariensis*	Chabaud & Brygoo, 1957
	Harentinema ambocatca	*Boophis goudoti or B. madagacariensis*	Chabaud & Brygoo, 1957
	Madochotera alata	*Rhacophorus sp.*	Bain & Brunhes, 1968
	Madochotera landauae	*Rhacophorus sp.*	Prod'hon & Bain, 1974
	Madochotera pichoni	*Rhacophorus sp.*	Bain & Brunhes, 1968
	Paramadochotera guibei	*Gephyromantis redimitus*	Prod'hon & Bain, 1974
	Rhabdias blommersiae	*Blommersia domerguei*	Kuzmin *et al.*, 2013
	Rhabdias madagascariensis	*Ptychadena mascareniensis*	Chabaud *et al.*, 1961
	Rhabdias vencesi	*Boophis madagascariensis*	Junker *et al.*, 2010
Digenea	*Diplodiscus brevicoeca*	*Ptychadena mascareniensis*	Richard, *et al.*, 1968
Cestoda	*Nematotaenia dispar*	*Aglyptodactylus madagascariensis*	(Goeze, 1782) Lühe, 1899
Hirudinea	*Malagabdella niarchosorum*	*Boophis madagascariensis*	Rocha *et al.*, 2012
	Malagabdella fallax	*Boophis roseipalmatus*	Rocha *et al.*, 2012
	Malagabdella sp.	*Gephyromantis luteus*	Rocha *et al.*, 2012
	Malagabdella vagans	*Mantidactylus* sp. aff. *grandidieri*	Rocha *et al.*, 2012
Acari	*Endotrombicula madagascareniensis*	*Aglyptodactylus madagascariensis, Gephyromantis luteus, Mantidactylus brevipalmatus*	Wohltmann *et al.*, 2007
Monogenea	*Kankana manampoka*	*Cophyla pollicaris*	Raharivolololniaina *et al.*, 2011
	Madapolystoma biritika	*Mantella madagascariensis, M. baroni*	Du Preez *et al.*, 2010
	Madapolystoma cryptica	*Guibemantis liber*	Berthier *et al.*, 2014
	Madapolystoma ramilijaonae	*Guibemantis liber*	Berthier *et al.*, 2014
	Metapolystoma brygoonis	*Ptychadena mascareniensis*	Euzet & Combes, 1964
Acanthocephala	*None*		



Figure 1.5: Madagascan polystomes. (**A**) *Uropolystomoides chabaudi.* (**B**) *Metapolystoma brygoonis*. (**C**) *Madapolystoma biritika*. (**D**) *Madapolystoma cryptica*. (**E**) *Madapolystoma ramilijaonae*. (**F**) *Kankana manampoka*.



Figure 1.6: African metapolystomes. (**A**) *Metapolystoma porosissimae* Holotype. (**B**) *Metapolystoma cachani.*

1.3.1 *Madapolystoma*

Representatives of *Madapolystoma* (Fig. 1.5A-C) has the typical gross morphology of most polystomes with a pyriform, dorsoventrally flattened body, a posterior haptor with three symmetrical pairs of suckers, two hamuli between the posterior-most suckers and sixteen marginal hooklets, which are placed as in other polystomes (Fig. 1.3C) (Du Preez *et al.*, 2010). The two posterior-most marginal hooklets of polystomes are generally larger than the rest of the marginal hooklets, however in *Madapolystoma* all marginal hooklets are equal in size as with

Eupolystoma, Kaw 1950 (Du Preez *et al.*, 2010). *Madapolystoma* is one of the most elusive polystome genera. Very little is known about the reproductive strategies of *Madapolystoma* species regarding the mode of transfer from infected to novel uninfected hosts. The life cycle of Polystomatidae has thus far been characterized by a free-swimming ciliated oncomiracidium (Smyth & Wakelin, 1994). One of the unique features of *Madapolystoma* is the unusual reproductive strategy employed by these parasites, with an intra-uterine development of eggs and no free-swimming oncomiracidia (Du Preez *et al.*, 2010). The Polystomatidae characteristic of having free swimming ciliated larvae, had to be amended to accommodate the internal cycle of *Madapolystoma* with no free-swimming oncomiracidium. Intra-uterine development takes place to the extent that larvae develop hamuli and two pairs of suckers (Fig. 1.7) (Du Preez *et al.*, 2010). Although some other polystomes, e.g. *Eupolystoma*, are also known to employ advanced intrauterine development, hamuli have never been observed (Du Preez *et al.*, 2010). Both *Madapolystoma* and *Eupolystoma* have extended uteri. However, in *Madapolystoma* the ovary is positioned in the middle of the body (Du Preez *et al.*, 2010). With regard to the male reproductive system, *Madapolystoma* has a single testis positioned in the posterior half of the body, the genital bulb is small and crowned with five to eight genital spines (Du Preez *et al.*, 2010).

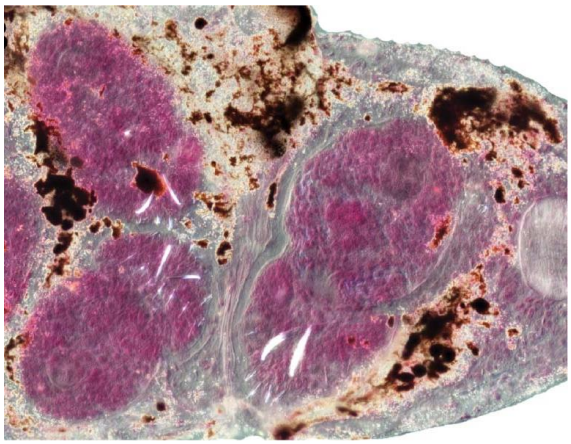


Figure 1.7: Light microscope micrograph of the *in utero* larvae of *Madapolystoma biritika* with hamuli and marginal hooks.

Molecular investigations have revealed *Madapolystoma* to be the sister group to *Eupolystoma (*Verneau *et al., 2009b)*. Morphological studies also highlight similarities between the two genera, as indicated above. Verneau *et al.* (2009a) estimated that *Eupolystoma* and *Madapolystoma* diverged 116 million years ago, with the first crown divergence in *Madapolystoma* 63 million years ago. However, it is still uncertain whether *Madapolystoma* was introduced to Madagascar via Africa or Asia (Verneau *et al.*, 2009a). Several different hypotheses propose either ancestral mantelids, hyloid frogs (Bufonidae Gray, 1825), rachophorids, or microhylids as vectors for *Madapolystoma* (Evans *et al.*, 2008; Van Bocxlaer *et al.*, 2006; Van der Meijden *et al.*, 2007; Vences *et al.*, 2003; Verneau *et al.*, 2009b). However, there is still a lack of information regarding Asian polystomes (Verneau *et al.*, 2009b), which prevent drawing further conclusions on the subject.

Until now, *Madapolystoma* has only been known to infect frogs in the Mantelinae Laurent 1946 subfamily (Family Mantellidae Laurent, 1946) (Du Preez *et al.*, 2010). Molecular studies

revealed the presence of *Madapolystoma* in four of 12 genera in the Mantelidae. These include *Blommersia* Dubois, 1992; *Gephyromantis* Methuen, 1920, *Guibemantis* Dubois, 1992; and *Mantella* Boulenger, 1882 (Verneau *et al.*, 2009b). However, the Mantellidae is one of the families with the greatest radiation of species among Malagasy frogs (Glaw & Vences, 2007). Subsequently, *Madapolystoma* is probably just as diverse as their mantelid hosts (Du Preez *et al.*, 2010).

It is hypothesised that *Madapolystoma* larvae are transmitted, directly or indirectly, to post-metamorphic frogs, hence the absence of free-swimming larvae and the advanced level of intrauterine development (Du Preez *et al.*, 2010). In a direct transmission, physical contact between the two potential hosts, or indirect contact at sites such as egg clutches with the parasite on them, is required (Du Preez *et al.*, 2010). A great majority of the frogs in the Mantelinae subfamily are characterized by derived reproductive modes (Glaw & Vences, 2007). Eggs are laid, for example, outside the water against leaves, twigs, rocks, or mud, allowing tadpoles to drop into the water when they hatch (Glaw & Vences, 2007). Therefore, the lack of an aquatic larval stage in *Madapolystoma* may be related to the terrestrial egg deposition of their hosts (Du Preez *et al.*, 2010). Some of the mantelids display tactile courtship behaviour with prolonged contact, where the male touches the female's dorsum with his femoral glands (Glaw & Vences, 2007). Furthermore, these frogs lack strong amplexus, where the male deposits sperm onto egg clutches next to the female [as with *Guibemantis depressiceps* (Boulenger, 1882)] or on the female's head (Fig. 1.8) where the sperm flows over her back and onto the eggs as with *Guibemantis tornieri* (Ahl, 1928) (Glaw & Vences, 2007). Some species, such as *Mantella laevigata* Methuen and Hewitt, 1913, also display complex parental care (Glaw & Vences, 2007). Parasite transmission then seems plausible in the case of repeated, prolonged contact between hosts.



Figure 1.8: Illustration of *Guibemantis tornieri* in amplexus.

1.3.2 *Metapolystoma*

Metapolystomes has thus far been found to infect *Ptychadena* hosts only, which in general seem to be very suitable hosts for polystomes (Bentz *et al.*, 2001; Du Preez, 2011; Du Preez & Kok, 1992a). The generitype for *Metapolystoma*, *M. cachani*, was originally described as *Polystoma* (*P. cachani*) and later transferred to a new genus *Metapolystoma* (see Combes, 1976). The separation of *Metapolystoma* from *Polystoma* is based on morpho-physiological differences observed in members of these two genera. For example, *Metapolystoma* has a large extended uterus containing many eggs that fills the largest part of the body, and the large ovary is posteriorly positioned (Combes, 1976). In contrast, *Polystoma* has a small uterus that contains only a few eggs, the uterus only fills a small volume of the anterior body, and the ovary is positioned in the anterior half of the body. In general, *Metapolystoma* shows a typical polystome gross morphology, with a dorsoventrally flattened body, a posterior haptor, and marginal hooklets placed as in other polystomes (Fig. 1.3C). Other than in *Madapolystoma*, marginal hooklet pairs one and eight are larger than other hooklets. Furthermore, two large hamuli are present between the posterior-most pair of suckers. The intestine bifurcates directly after the pharynx and converges at the level of the haptor, lacking anastomosis, with medial diverticula located posteriorly to the ovary. Vitellaria are spread throughout the body, except for where female reproductive organs are positioned. The testis is large, follicular and positioned mainly posterior to the ovary, with two lateral processes that sometimes extend anteriorly past the ovary to the level of the mid body.

However, the validity of *Metapolystoma* as a genus has been under dispute ever since its elevation from *Polystoma*, especially since later studies showed that *Metapolystoma* is nested in *Polystoma* at the molecular level (Bentz *et al.*, 2001; Olson & Tkach, 2005; Verneau *et al.*, 2002). Tinsley (1974) considered the morphological differences in the uterine structure as a result of convergence. Bentz *et al.* (2001) ascribed these morphological differences between *Metapolystoma* and *Polystoma*, such as the length of the uterus, to homoplasticity resulting from the host's ecology. Since amphibian polystomes are generally considered host specific, the simultaneous occurrence of both *Metapolystoma porosissimae* Du Preez & Kok, 1992 and *Polystoma sodwanensis* Du Preez & Kok, 1992 in the same host, *Ptychadena porosissima* (Steindachner, 1867), also adds to this dispute (Coetzer, 2017; du Preez & Kok, 1992b), as it might be the case that their morphological differences are the result of reproductive plasticity or convergence. Interestingly, the two parasite species (currently belonging to different genera) employ very different life history strategies. In *M. porosissimae*, many eggs are stored in a large uterus, which is typical of polystomes that occur in hosts from arid environments (Du Preez, 2015; du Preez & Kok, 1992b). In such habitats, water required for breeding is only available to frogs during specific times of the year. Therefore, the parasite needs to release enough eggs during

this timeframe to ensure its survival. *P. sodwanensis,* on the other hand, has a small uterus containing only a few eggs, which is characteristic of parasites inhabiting a more water-dependent host (Du Preez & Kok, 1992a). In humid habitats used by this group of hosts, water is more readily available, and breeding may last over an extended period. These conditions increase both survival and infection rates in frog-infecting parasites, which can rely on a lower number of produced and stored eggs. These two different life strategies of two different genera in the same host make this synoptic occurrence even stranger. Bentz *et al.* (2001) ascribed these morphological differences to ecologically induced morphological plasticity in situations where hosts have undergone geographical relocation or in the instance of climate change (Bourgat & Murith, 1980; du Preez & Kok, 1992b). Consequently, the validity of *Metapolystoma* is still uncertain. It is, however, possible that *Metapolystoma* might be valid in the case of divergence from *Polystoma* (see Coetzer, 2017).

Metapolystoma species have different reproductive strategies (Fig. 1.9). Metapolystomes are partly ovoviviparous where some eggs develop and hatch *in utero* to reinfect the same host as the parent (Murith *et al.*, 1977). Eggs that didn't hatch *in utero* will hatch after being expelled from the host to partake in either a neotenic branchial or a mature branchial to bladder cycle (Murith *et al.*, 1977). A neotenic cycle is followed when an oncomiracidium attaches to the gills of a tadpole in pre-metamorphosis (Combes, 1968; Williams, 1961). In such a case, the oncomiracidium will develop into a neotenic parasite and start producing eggs after two to three weeks until the host reach metamorphosis where the parasite will detach and die (Combes, 1968; Williams, 1961). However, if an oncomiracidium attaches to the gills of a tadpole in pro-metamorphosis, the parasite will develop normally into a urinary bladder destined parasite (Badets & Verneau, 2009; Du Preez & Kok, 1999). Upon host metamorphosis, the young parasite migrates over the host's abdomen to the cloaca, where it enters the urinary bladder to mature and reproduce in synchrony with the host (Badets & Verneau, 2009; Du Preez & Kok, 1999). There are various theories regarding the function and origin of this alternative development (Badets *et al.*, 2013). However, it is most probable that the neotenic parasite serves the function to boost egg production in the beginning of frog breeding seasons (Badets *et al.*, 2013; Badets *et al.*, 2010). During the onset of the breeding seasons, the available population of tadpoles to be infected would be in pre-metamorphosis which will induce the development of neotenic phenotypes (Badets *et al.*, 2010; 2013). Parasites that hatch from eggs produced by the neotenic parasites will then have tadpoles in pro-metamorphosis to infect, which will lead to the development of bladder parasites (Badets *et al.*, 2010; 2013;).

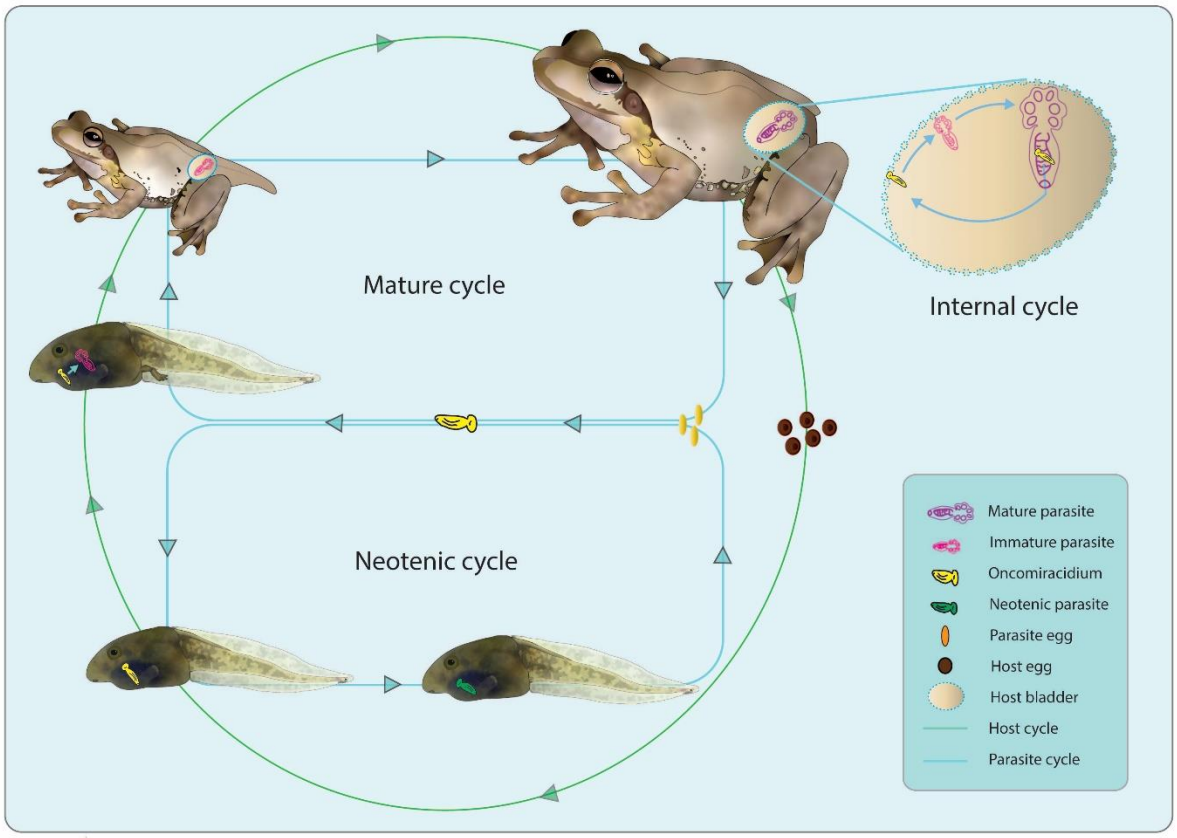


Figure 1.9: Mature, neotenic, and internal life cycles of a *Metapolystom*a species parasitising *Boophis doulioti.*

1.4 General problem statement, aims, and objectives

Madagascar, one of the world biodiversity hotspots, provides habitats to many endemic flora and fauna species (Myers *et al.*, 2000). Although some of these taxa are relatively well-studied, new species, including amphibians, are regularly discovered from Madagascar, and the real extent of the island biodiversity is far from being fully documented (Glaw & Vences, 2006; 2007). For example, many of the local invertebrates, especially small-sized parasites, are at best underexplored (Goodman & Benstead, 2005; Myers *et al.*, 2000). In the last two decades, some attention was given to parasites infecting frogs, chameleons, lemurs, snakes, and stingrays (Clough, 2010; Clough *et al.*, 2010; De Chambrier *et al.*, 2010; Hastriter & Dick, 2009; Junker *et al.*, 2010; Kuchta & Caira, 2010; Lhermitte-Vallarino *et al.*, 2010; Westergren & Siddall, 2004), including the amphibian-infecting polystomes (Berthier *et al.*, 2014). Up to 2009, *Metapolystoma brygoonis* was the only polystome species known from Malagasy frogs. However, an intensive survey carried out in the years 2002–2009 led to several discoveries and new taxa descriptions, including two new genera and several new species (Verneau *et al.*, 2002; Verneau *et al.*, 2009b). During the re-examination of materials collected by Verneau *et al.* (2002; 2009b) two new species

of *Madapolystoma* from Mantellidae frogs and six new species of *Metapolystoma* from Laliostominae Vences & Glaw, 2001 and Boophinae Vences & Glaw, 2001 frogs have been discovered. The aim of the current study was to formally describe these new taxa. The proposed descriptions and diagnoses are based on both molecular analyses and thorough morphological observations using light and scanning electron microscopy as well as marginal hooklet morphometry and standard histological examinations. Twelve more *Madapolystoma* species collected during the 2002–2009 investigations (Table 1.2) in Madagascar await formal description, however, sample sizes are insufficient and additional sampling would be required to formally describe these species.

More concise aims and objectives will be provided within individual chapters.

Table 1.2. *Madapolystoma* species awaiting description due to insufficient sample sizes

Host	**Polystome species**	**Number of specimens available**		
		Adult	**Juvenile**	**Molecular support**
Blommersia blommersae	*Madapolystoma sp.*	1	85	Yes
Blommersia wittei	*Madapolystoma sp.*	0	1	Yes
Gephrynomantis sculpturatus	*Madapolystoma sp.*	0	1	Yes
Mantella aurantiaca	*Madapolystoma sp.*	0	21	No
Mantella betsileo	*Madapolystoma sp.*	0	1	No
Mantella cowani	*Madapolystoma sp.*	0	1	No
Mantella crocea	*Madapolystoma sp.*	0	1	No
Mantella laevigata	*Madapolystoma sp.*	1	0	No
Mantella pulchra	*Madapolystoma sp.*	0	4	No
Mantidactylus luteus	*Madapolystoma sp.*	0	4	No
Mantidactylus tornieri	*Madapolystoma sp.*	3	19	No
Mantidactylus femoralis	*Madapolystoma sp.*	0	1	No

CHAPTER 2:

GENERAL MATERIALS AND METHODS



A mind that is stretched by a new experience can never go back to its old dimensions
-Oliver Wendell Holmes

Materials and Methods that have relevance to only a specific chapter, will be dealt with in that chapter.

2.1 Host and Parasite Sampling

During four research visits to Madagascar (2005–2008) various species of frogs belonging to the Boophinae, Laliostominae, and Mantelinae were collected and examined for the presence of polystomes.

Frogs were collected by hand after nightfall (Ethics clearance number: NWU-00380-16-A5-04), mostly following rainfall events and placed individually in clear plastic bags containing 50 ml water. After 24 hours the water in which the frog was being kept was poured through two plankton sieves with respective mesh sizes of 500 and 100 µm. The 500 µm sieve retained coarse debris and most of the faeces, while the 100 µm sieve retained the parasite eggs and fine debris. The content of the 100 µm sieve was then washed into a Petri dish and inspected for the presence of polystome eggs using a dissecting microscope. Frogs that released polystome eggs were euthanised prior to dissection using 500mg/l ethyl-3-aminobenzoate methanesulfonate (MS222). If no polystomatid eggs were found a representative per species per locality was dissected to check for non-egg-releasing polystomatids. Upon dissection, the kidneys, bladder, accessory bladder (if present) and the Wolffian ducts were removed and placed in a Petri dish with 0.6% amphibian saline. These organs were dissected and inspected with a Nikon SMZ-645 (Nikon, Netherlands) stereo microscope to confirm the presence of polystomes. Polystomatids were retrieved and kept in amphibian saline until fixation. Frogs that were not dissected were released at the site of collection. Dissected frogs were fixed, curated and incorporated into the Zoological Collection of Miguel Vences (ZCMV) or the Herpetological collection at the University of Antananarivo, Madagascar. Some of the eggs, that were collected during the process of frog screening, were kept in dechlorinated tap water to incubate.

Tadpoles were collected by dip net, kept in clear plastic bags. To look for the presence of neotenic polystome parasites on their gills, they were sedated in a 500mg/l MS222 solution and viewed through the semi-transparent gular skin using a portable field stereo microscope. Tadpoles that were infected were dissected to retrieve parasites from the gills. Fine scissors were used to cut open the gular region, starting from the sinistral spiracle. Parasites were removed with a fine camel hair paintbrush and a fine needle and temporarily kept in amphibian saline until fixation or temporary mounting in lactophenol. Uninfected tadpoles were released where collected.

2.2 Temporary mounts using lactophenol or ammonium picrate

Some specimens were temporarily mounted, using lactophenol or ammonium picrate, to reveal sclerotised structures. Hatched oncomiracidia and some juvenile parasites were placed in a drop of saline on a slide from which excess saline was removed. A drop of lactophenol or ammonium picrate was placed on a coverslip, inverted, and lowered on the specimen. To secure the coverslip its edges were sealed to the microscope slide with clear, transparent nail varnish. In the instance of oncomiracidia their position was indicated on the slide using a fine permanent marker.

2.3 Fixing, staining and mounting specimens on permanent mounts

Specimens were heat killed on a microscope slide over a butane lighter (Snyder & Clopton, 2005), over which a coverslip was placed, and the water was replaced with 10% neutral buffered formalin (NBF). This replacement was done by adding a drop of NBF to one side of the coverslip, while extracting the water with a paper towel from the opposite side of the coverslip. Specimens were left to fix for one hour under coverslip pressure. Prior to staining for light microscopy fixed parasites were hydrated for 30 minutes in tap water. The specimens were then stained overnight in a weak acetocarmine solution (Fig. 2.1). After staining, specimens were dehydrated in an ethanol (EtOH) series (30%, 50%, 70%, 80%, 90%, 96%, 100%, 100%) and cleared in xylene (Fig. 2.1). In the event of overstaining a one percent hydrochloric acid in 70% EtOH was used to extract excessive stain. Specimens were mounted on microscope slides using Canada balsam and dried in a drying oven at 50°C for three days.

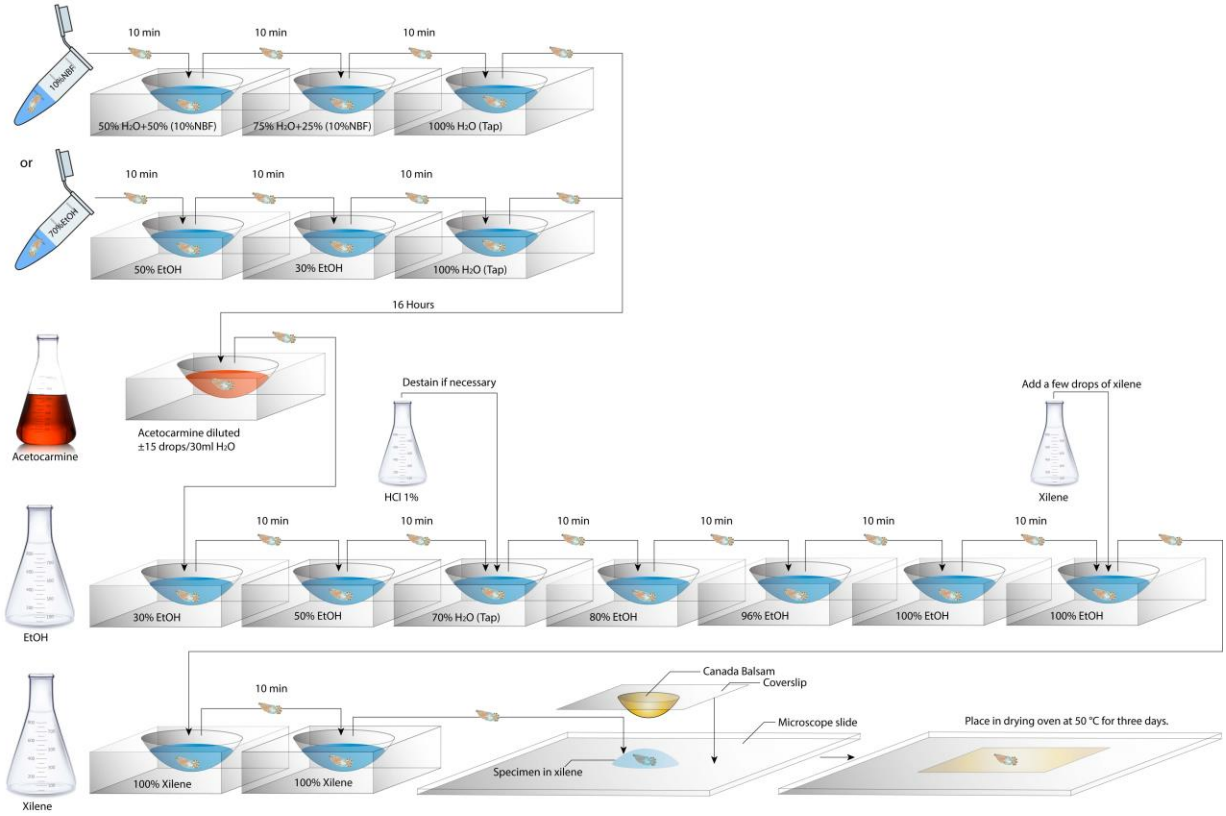


Figure 2.1: Procedures for the staining of polystome parasites with acetocarmine and mounting on a microscope slide in Canada balsam.

2.4 General morphology

Mature specimens were measured as illustrated in figure 2.2 A–D. Specimens were examined and photographed with various light microscopes, as indicated in specific chapters, and taxonomically drawn from compiled micrographs on Adobe Illustrator CC (Adobe, California). All measurements were performed using each microscope-associated software package, as stipulated in the following chapters.

Marginal hooklets were measured, using the method proposed by du Preez and Maritz (2006) (Fig. 2.1C). The total length of the marginal hooklet was multiplied by the guard width. The product of the total hooklet length and the distance between the guard and the tip of the blade was also determined. These results were plotted on a scatterplot together with those of other known species within genera.

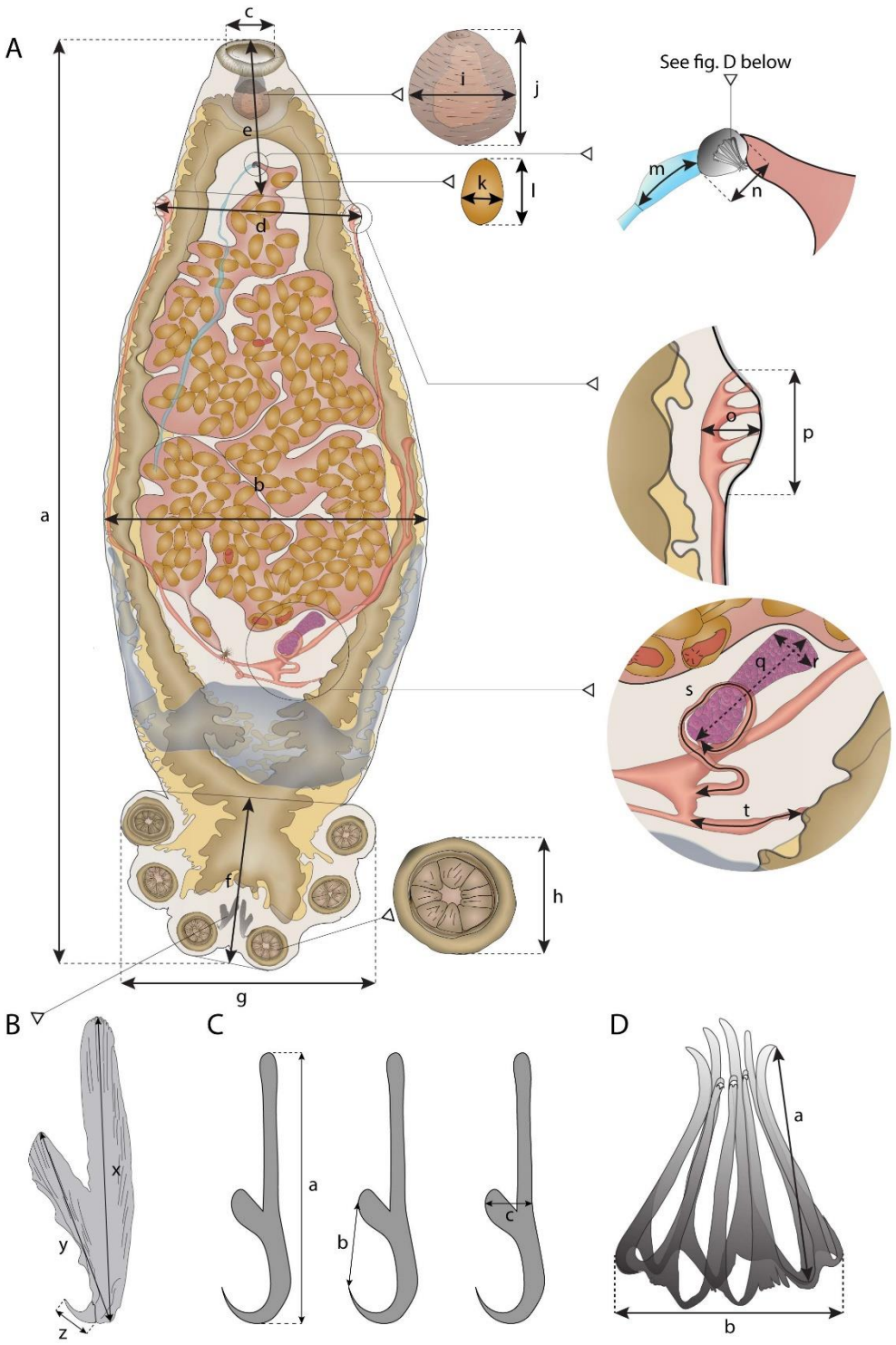


Figure 2.2: Taxonomically important morphometrics of polystomes used in this study. **(A)** a: total length, b: greatest width, c: mouth width, d: width at vagina, e: distance of vagina from anterior end, f: haptor length, g: haptor width, h: haptoral sucker diameter, i: pharynx width, j: pharynx length, k: egg width, l: egg length, m: semen vesicle length, n: genital bulb diameter, o: vagina width, p: vagina length, q: ovary length, r: ovary greatest width, s: oviduct length, and t: genito-intestinal canal; **(B)** x: hamulus handle length, y: hamulus guard length, and z: hamulus hook length; **(C)** Marginal hooklet measurements as stipulated in the protocol of du Preez and Maritz (2006); and **(D)** a: genital spine length, and b: genital crown diameter.

2.5 Molecular analysis

Specimens for molecular analysis were preserved in 100% ethanol. In some instances, published sequences for genetic analysis were downloaded from GenBank, while others were self-obtained.

DNA extractions were conducted in 100-150 ul of 10% Chelex 100 sodium (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) with the proteinase K 1 mg·mL−1 final concentration at 55°C for the duration of 1 to 1,5 hours. Enzymatic reactions were stopped at 100 °C for 15 min and DNA was stored at −20°C until used. Amplifications of 18S, 28S and COI were conducted following the procedure described in Héritier *et al.* (2015), regardless of the gene of interest or primers used. The complete 18S rRNA gene and the partial 28S rRNA gene were amplified in two rounds with the respective combinations of primers F18 / 18Rg and 18F3 / IR5 for 18S and LSU5' / IR16 and IF15 / LSU3' for 28S. Primer sequences are reported in Sinnappah *et al.* (2001), Verneau *et al.* (2009a) and Héritier *et al.* (2015). COI was amplified using the combination of primers L-CO1p / H-Cox1p2 (Littlewood *et al.*, 1997). All PCR products were sent to the Genoscreen Company (Lille, France) for purification and sequencing with their respective forward and reverse PCR primers. Sequences were read and edited with the software Geneious (Saint Joseph, Missouri, USA) to check chromatograms before use for phylogenetic and distance analyses.

All sequences were edited and aligned independently using ClustalW implemented in MEGA version 7 (Kumar *et al.*, 2016). Further phylogenetic analysis and genetic divergence methods are provided in each of the following chapters as they are unique for each chapter.

Results



The real voyage of discovery
consists not in seeking new landscapes,
but in having new eyes
- Marcel Proust

CHAPTER 3:

FIRST RECORD OF VIVIPARITY IN POLYSTOMATID FLATWORMS (MONOGENEA: POLYSTOMATIDAE) WITH THE DESCRIPTION OF TWO NEW SPECIES OF *MADAPOLYSTOMA* FROM THE MADAGASCAN ANURAN HOSTS *BLOMMERSIA DOMERGUEI* AND *MANTELLA EXPECTATA.*



So, Naturalist observe, a flea
Hath smaller fleas that on him prey,
And these have smaller fleas to bite 'em;
And so ad infinitum
(J. Swift, On Poetry)

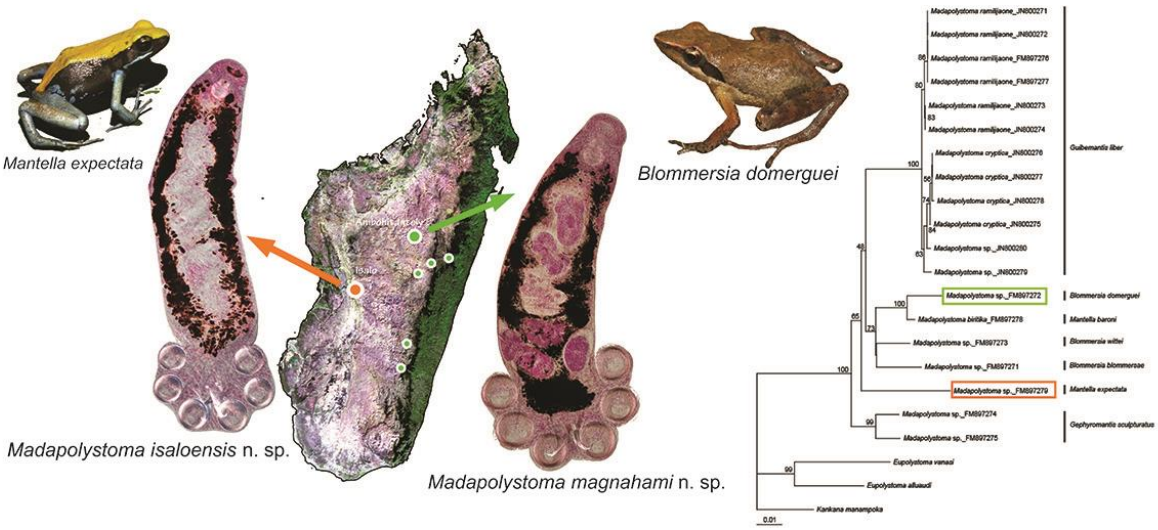
This chapter has been published:

Landman, W., Verneau, O., & Du Preez, L. H. (2018). First record of viviparity in polystomatid flatworms (Monogenea: Polystomatidae) with the description of two new species of *Madapolystom*a from the Madagascan anuran hosts *Blommersia domerguei* and *Mantella expectata. International Journal of Parasitology: Parasites and Wildlife,* 7, 343−354. doi.org/10.1016/j.ijppaw.2018.09.004.

Landman took the lead, prepared the manuscript and all figures.

Abstract

Two frog species, *Blommersia domerguei* and *Mantella expectata*, are reported as hosts for new species of *Madapolystoma*. Phylogenetic analyses and genetic divergences observed in the genus supported the distinction of two morphotypes infecting selectively each host species and morphological investigation combining marginal hooklet morphometrics, genital spine number and measurements further showed that polystomes from the two host species differed from each other and from all other known polystomes. *Madapolystoma magnahami* n. sp. and *Madapolystoma isaloensis* n. sp. are therefore described as two new species. Advanced *in utero* development was illustrated in both polystome species following the observation of well-developed hamuli and two pairs of haptoral suckers in developing embryos. Inside some of these *in utero* embryos a F2 generation embryo was also observed. This is the first report of true viviparity among polystomatid flatworms.



Key words:

Blommersia domerguei, Madagascar, *Madapolystoma isaloensis* n. sp., *Madapolystoma magnahami* n. sp., *Mantella expectata,* Monogenea

3.1 Introduction

As a biodiversity hotspot Madagascar is ranked within the world's top three regions of conservation importance (Myers *et al.*, 2000). The high species diversity and level of endemicity is ascribed to the isolation of the island over an extended period of time (Glaw & Vences, 2007; Goodman & Benstead, 2005; Myers *et al.*, 2000). The separation of Madagascar from other landmass began during the breakup of Gondwana about 156 to 165 Mya (Rabinowitz *et al.*, 1983) and ended when it separated from India about 84-94 Mya (Storey *et al.*, 1995). Globally, Madagascar is ranked as the country with the twelfth highest amphibian species richness (Andreone *et al.*, 2008). While the true species number of anuran species was reckoned to be close to 465 species (Glaw *et al.*, 2010; Glaw & Vences, 2007; Vieites *et al.*, 2009), 345 frog species are currently described from the island (Frost, 2018) with a near 100% endemicity. Only two non-native invasive species have been recorded, namely *Hoplobatrachus tigerinus* Daudin, 1802 and *Duttaphrynus melanostictus* Schneider, 1799 (Glaw *et al.*, 2010; Moore *et al.*, 2015).

In spite of the conservation status of Madagascar, the diversity and endemicity of the less prominent taxa are poorly known (Goodman & Benstead, 2005; Myers *et al.*, 2000). As can be expected, the species richness is not restricted to the herpetofauna alone but also applies to their parasites (Junker *et al.*, 2010; Kuzmin *et al.*, 2013; Rocha *et al.*, 2012; Wohltmann *et al.*, 2007). Four genera of polystomes (Monogenea: Polystomatidae) have been described in Madagascar: (i) *Uropolystomoides* Tinsley and Tinsley, 2016 with a single species *Uropolystomoides chabaudi* (Euzet and Combes, 1965) from the chelonian host *Pelomedusa subrufa (*Lacépède, 1788); (ii) *Metapolystoma* Combes, 1976, with a single species *Metapolystoma brygoonis* from the anuran host *Ptychadena mascareniensis*; (iii) *Madapolystoma* Du Preez, Rahariviololoniaina, Verneau and Vences, 2010 with three species infecting frogs, namely *Madapolystoma biritika* Du Preez, Rahariviololoniaina, Verneau and Vences, 2010 from *Mantella madagascariensis* (Grandidier, 1872), *Madapolystoma cryptica* Berthier, Du Preez, Rahariviololoniaina, Vences and Verneau, 2014 and *Madapolystoma ramilijaonae* Berthier, Du Preez, Rahariviololoniaina, Vences and Verneau, 2014, from the same host *Guibemantis liber* (Peracca, 1893); and (iv) *Kankana* Rahariviololoniaina, Verneau, Berthier, Vences and Du Preez, 2011, with a single species *Kankana manampoka* Rahariviololoniaina, Verneau, Berthier, Vences and Du Preez, 2011 from the frog *Cophyla pollicaris* (Boulenger, 1888).

Because it has been shown that polystomes coevolved with their hosts since their origin in the Palaeozoic age (Héritier *et al.*, 2015; Verneau *et al.*, 2002; Verneau *et al.*, 2009b), investigating their phylogeny can provide relevant insights into the diversification of amphibians over ancient and recent geological periods (Badets *et al.*, 2011). Out of the 345 known anuran species from Madagascar, 86 species from a few selected localities were screened for

polystomes (Verneau *et al.*, 2009a). At least twelve polystome morphotypes were identified from these amphibians, suggesting that a great number of polystome species from Madagascar still await description. It is therefore important to study their systematics and evolution as this particular frog-polystome association may not only provide significant information on the biogeographical origins of Malagasy frogs (Verneau *et al.*, 2009a), but also, ultimately, aid in their conservation as discussed by Berthier *et al.* (2014) for the host *Guibemantis liber* (Petacca, 1893).

In January 2005 during a herpetological survey conducted in Madagascar (Fig. 3.1a), two frog species examined for polystomes were found to be infected with two distinct *Madapolystom*a morphotypes. *Blommersia domerguei* Guibé, 1974 (Fig. 3.1b) was collected from the Ambohitantely Special Reserve (Fig. 3.1a) and *Mantella expectata* Busse and Böhme, 1992 (Fig. 3.1c) was collected in the Isalo region (Fig. 3.1a). Since the discovery of these two groups of parasites, the collection of additional specimens of *B. domerguei* and *M. expectata* has been hampered by administration difficulties for sampling amphibians in Madagascar, and because of the conservation status of the second species. Therefore, despite the low sample size, we now describe the two new species herein since it is unlikely that we will have the opportunity to collect additional material in the foreseeable future.

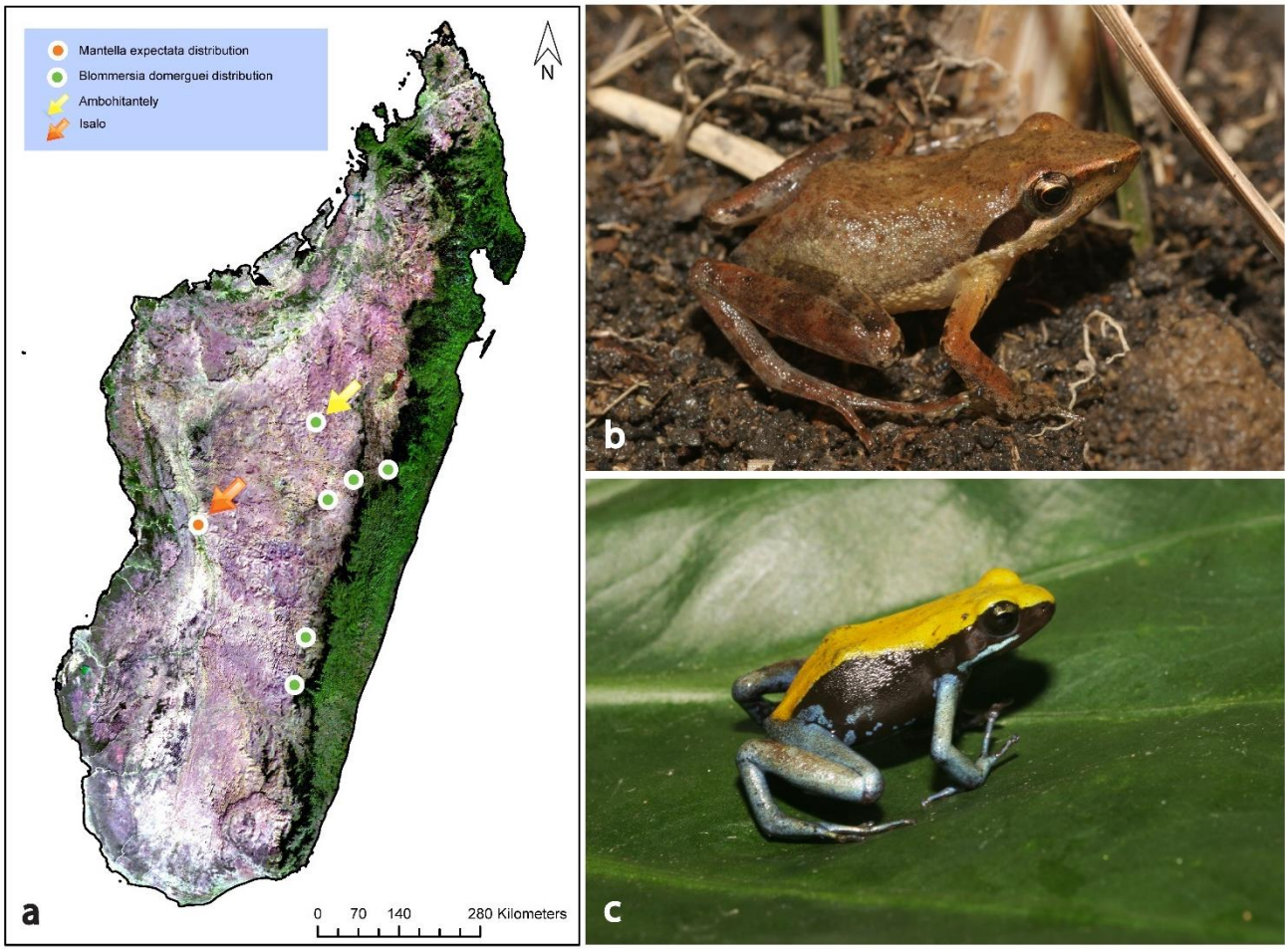


Figure 3.1: (**a**) Map of Madagascar with the distribution areas and sampling localities of the two investigated frogs. (**b**) *Blommersia domerguei*. (**c**) *Mantella expectata*. (Map-Library, 2007).

3.2 Material and Methods

3.2.1 The Host

Blommersia domerguei and *M. expectata* are both small frogs of the family Mantellidae, which is the most diverse amphibian family in Madagascar (Glaw & Vences, 2006). *Blommersia domerguei* is known from six small areas along the east coast of Madagascar. It occurs in swamps at a relatively high altitude (Glaw & Vences, 2007) and its conservation status is considered to be “Least Concern” (IUCN, 2017). Species in this genus lay their eggs against structures overhanging ponds or streams (Glaw & Vences, 2007). In contrast, *M. expectata* is listed as “Endangered” (IUCN, 2017) and is known from only a small geographical area in the dry sandstone massif near Isalo (Glaw & Vences, 2007) (Fig. 3.1b). The majority of *Mantella* species lay their eggs in excavated terrestrial nests. After flooding, tadpoles leave the nest and move to ponds or streams (Glaw & Vences, 2007).

3.2.2 Host and Parasite Sampling

Fifteen adult specimens of *B. domerguei* were collected in the Ambohitantely Special Reserve in Madagascar in January 2005 (Fig. 3.1b). Frogs were collected by hand and temporarily kept in clear plastic bags containing plant material and water, until dissection. The six specimens of *M. expectata* used in this study were not collected in the field but were obtained from an exporter in Antananarivo during the same period. Prior to dissection, frogs were anesthetised and subsequently killed with MS222 (ethyl-4-aminobenzoate). Dissection and internal inspection were performed using a Nikon SMZ-645 dissecting microscope. The urinary bladder and kidneys were removed and inspected for worms in a small glass Petri dish containing 0.6% Ringers solution. Adult parasites were fixed in 10% buffered formalin under coverslip pressure while most of the subadult polystomes were mounted in ammonium picrate glycerine. Some of the juveniles were preserved in absolute ethanol for molecular studies. Adult polystomes were washed free of fixatives in tap water and stained overnight in a weak acetocarmine solution, dehydrated, cleared in xylene and mounted in Canada balsam.

3.2.3 Sequence Analysis

28S rDNA sequences of *Madapolystoma* spp. that were reported in Verneau *et al*. (2009a) and (Berthier *et al.* (2014) were obtained from GenBank (Table 3.1). Sequences of *K. manampoka, Eupolystoma alluaudi* (De Beauchamp, 1913) and *Eupolystoma vanasi* Du Preez, Tinsley and De Sa, 2003, were also selected for rooting the tree according to Raharivololoniaina *et al.* (2011). Sequence alignment was done with the help of ClustalW (Thompson *et al.*, 1994) implemented in the MEGA software version 7 (Kumar *et al.*, 2016) with regard to the 28S ribosomal secondary structure defined for polystome species (Badets *et al.*, 2011; Héritier *et al.*, 2015).

To depict the relationships within *Madapolystoma*, a Minimum Evolution (ME) tree was inferred from the MEGA software, based upon the calculation of the Kimura 2-parameter distance after excluding gaps and partially sequenced regions in the final alignment (complete deletion option). One thousand replications were completed to evaluate the robustness of the nodes. Finally, genetic divergences (uncorrected p-distances) as well as total differences were determined for species delimitation following the complete deletion option in MEGA-7.

3.2.4 Morphology and Morphometry

Specimens were examined using a Nikon NiE compound microscope (Nikon, Netherlands) fitted with a Nikon DS-Ri1 digital camera. Morphological structures and organs were measured in micrometres using a Nikon NIS elements D software program. Marginal hooklets were measured

and plotted according to the procedure of Du Preez and Maritz (Du Preez & Maritz, 2006), in order to discriminate distinct species.

3.3 Results

3.3.1 Phylogenetic Relationships and Genetic Divergence

Regarding the ME tree (Fig. 3.2), a sister species relationship was unambiguously evidenced between *M. biritika* and the undescribed species of *Madapolystoma* from *B. domerguei*, with bootstrap support of 100%. Considering the 1.2 % genetic divergence that was calculated between the two polystomes (Table 3.2), we consider that they are separate species according to the 28S species-level threshold defined by Du Preez *et al.* (2007) for amphibian polystomes, which was estimated to about 0.07%. Furthermore, 22 substitutions were observed between these two polystomes following pairwise sequence comparisons, among which 11 corresponded to individual changes in the undescribed species of *Madapolystoma* from *B. domerguei*, suggesting it is a distinct species.

The undescribed species of *Madapolystoma* from *M. expectata* occupied a more basal position within *Madapolystoma*, being in an intermediate position between two undescribed polystomes from *Gephyromantis sculpturatus* (Ahl, 1929) and all other polystome species, however with low bootstrap support (Fig. 3.2). Because the genetic divergences between this polystome and all other polystomes ranged from 5.46 to 6.32% (Table 3.2), it is likely that this polystome is also a separate species according to the 28S species-level threshold defined by Du Preez *et al.* (2007). Similarly, 70 to 81 substitutions were observed between this polystome and all others, among which 25 corresponded to unique changes, thus supporting our conclusion regarding the systematic status of this polystome.

Table 3.1. Polystome species investigated, host species, geographical locations and 28S Genbank accession numbers

Polystome species	Host species	Location	Genbank Accession Number
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: Andasibe	JN800271
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: An'Ala	JN800272
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: Ranomafana	JN800273
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: Ranomanafakely	JN800274
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: An'Ala	FM897276
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: Andasibe	FM897277
Madapolystoma cryptica	*Guibemantis liber*	Madagascar: Tsaratanana	JN800275
Madapolystoma cryptica	*Guibemantis liber*	Madagascar: Andranomapanga	JN800276
Madapolystoma cryptica	*Guibemantis liber*	Madagascar: Ambohitantely	JN800277
Madapolystoma cryptica	*Guibemantis liber*	Madagascar: Makira	JN800278
Madapolystoma sp.	*Guibemantis liber*	Madagascar: Andrakata	JN800279
Madapolystoma sp.	*Guibemantis liber*	Madagascar: Montagne d'Ambre	JN800280
Madapolystoma biritika	*Mantella baroni*	Madagascar: Unknown locality	FM897278
Madapolystoma sp.	*Blommersia wittei*	Madagascar: Isalo	FM897273
Madapolystoma sp.	*Gephyromantis sculpturatus*	Madagascar: An'Ala	FM897274
Madapolystoma sp.	*Gephyromantis sculpturatus*	Madagascar: An'Ala	FM897275
Madapolystoma sp.	*Blommersia blommersae*	Madagascar: An'Ala	FM897271
Madapolystoma sp.	*Blommersia domerguei*	Madafascar: ambohitantely	FM897272
Madapolystoma sp.	*Mantella expectata*	Madagascar: Isalo	FM897279
Kankana manampoka	*Cophyla pollicaris*	Madagascar: Ranomafana	HM854293
Eupolystoma vanasi	*Schismaderma carens*	South Africa	AM157200
Eupolystoma alluaudi	*Bufo* sp.	Togo	AM157199

Table 3.2. Matrix of p-distances (upper right) and total differences (lower left) inferred from pairwise comparisons of 28S sequences of polystome species

	JN800271	JN800272	JN800273	JN800274	FM897276	FM897277	JN800275	JN800276	JN800277	JN800278	JN800279
*Madapolystoma ramilijaonae*_JN800271		0,0000	0,0016	0,0016	0,0000	0,0000	0,0055	0,0062	0,0062	0,0070	0,0062
*Madapolystoma ramilijaonae*_JN800272	0		0,0016	0,0016	0,0000	0,0000	0,0055	0,0062	0,0062	0,0070	0,0062
*Madapolystoma ramilijaonae*_JN800273	2	2		0,0000	0,0016	0,0016	0,0055	0,0062	0,0062	0,0070	0,0047
*Madapolystoma ramilijaonae*_JN800274	2	2	0		0,0016	0,0016	0,0055	0,0062	0,0062	0,0070	0,0047
*Madapolystoma ramilijaonae*_FM897276	0	0	2	2		0,0000	0,0055	0,0062	0,0062	0,0070	0,0062
*Madapolystoma ramilijaonae*_FM897277	0	0	2	2	0		0,0055	0,0062	0,0062	0,0070	0,0062
*Madapolystoma cryptica*_JN800275	7	7	7	7	7	7		0,0008	0,0008	0,0016	0,0055
*Madapolystoma cryptica*_JN800276	8	8	8	8	8	8	1		0,0000	0,0008	0,0062
*Madapolystoma cryptica*_JN800277	8	8	8	8	8	8	1	0		0,0008	0,0062
*Madapolystoma cryptica*_JN800278	9	9	9	9	9	9	2	1	1		0,0070
Madapolystoma sp._JN800279	8	8	6	6	8	8	7	8	8	9	
Madapolystoma sp._JN800280	7	7	7	7	7	7	4	5	5	4	7
*Madapolystoma biritika*_FM897278	55	55	53	53	55	55	55	56	56	57	56
Madapolystoma sp._FM897273	52	52	52	52	52	52	52	53	53	54	55
Madapolystoma sp._FM897274	61	61	61	61	61	61	61	62	62	63	62
Madapolystoma sp._FM897275	58	58	60	60	58	58	60	61	61	62	61
Madapolystoma sp._FM897271	59	59	57	57	59	59	59	60	60	61	60
Madapolystoma sp._FM897272	68	68	67	67	68	68	69	70	70	70	71
Madapolystoma sp._FM897279	76	76	74	74	76	76	77	78	78	78	78
*Kankana manampoka*_HM854293	110	110	108	108	110	110	109	110	110	109	111
*Eupolystoma vanasi*_AM157200	145	145	143	143	145	145	144	145	145	144	146
*Eupolystoma alluaudi*_AM157199	134	134	132	132	134	134	134	135	135	136	134

	JN800280	FM897278	FM897273	FM897274	FM897275	FM897271	FM897272	FM897279	HM854293	AM157200	AM157199
*Madapolystoma ramilijaonae*_JN800271	0,0055	0,0429	0,0406	0,0476	0,0453	0,0461	0,0531	0,0593	0,0859	0,1132	0,1046
*Madapolystoma ramilijaonae*_JN800272	0,0055	0,0429	0,0406	0,0476	0,0453	0,0461	0,0531	0,0593	0,0859	0,1132	0,1046
*Madapolystoma ramilijaonae*_JN800273	0,0055	0,0414	0,0406	0,0476	0,0468	0,0445	0,0523	0,0578	0,0843	0,1116	0,1030
*Madapolystoma ramilijaonae*_JN800274	0,0055	0,0414	0,0406	0,0476	0,0468	0,0445	0,0523	0,0578	0,0843	0,1116	0,1030
*Madapolystoma ramilijaonae*_FM897276	0,0055	0,0429	0,0406	0,0476	0,0453	0,0461	0,0531	0,0593	0,0859	0,1132	0,1046
*Madapolystoma ramilijaonae*_FM897277	0,0055	0,0429	0,0406	0,0476	0,0453	0,0461	0,0531	0,0593	0,0859	0,1132	0,1046
*Madapolystoma cryptica*_JN800275	0,0031	0,0429	0,0406	0,0476	0,0468	0,0461	0,0539	0,0601	0,0851	0,1124	0,1046
*Madapolystoma cryptica*_JN800276	0,0039	0,0437	0,0414	0,0484	0,0476	0,0468	0,0546	0,0609	0,0859	0,1132	0,1054
*Madapolystoma cryptica*_JN800277	0,0039	0,0437	0,0414	0,0484	0,0476	0,0468	0,0546	0,0609	0,0859	0,1132	0,1054

Table 3.2. Continued

	JN800280	FM897278	FM897273	FM897274	FM897275	FM897271	FM897272	FM897279	HM854293	AM157200	AM157199
*Madapolystoma cryptica*_JN800278	0,0031	0,0445	0,0422	0,0492	0,0484	0,0476	0,0546	0,0609	0,0851	0,1124	0,1062
Madapolystoma sp._JN800279	0,0055	0,0437	0,0429	0,0484	0,0476	0,0468	0,0554	0,0609	0,0867	0,1140	0,1046
Madapolystoma sp._JN800280		0,0445	0,0422	0,0492	0,0484	0,0476	0,0546	0,0609	0,0851	0,1132	0,1069
*Madapolystoma biritika*_FM897278	57		0,0289	0,0461	0,0484	0,0312	0,0172	0,0554	0,0835	0,1054	0,0968
Madapolystoma sp._FM897273	54	37		0,0414	0,0453	0,0304	0,0398	0,0546	0,0781	0,1046	0,0952
Madapolystoma sp._FM897274	63	59	53		0,0187	0,0429	0,0570	0,0570	0,0710	0,0991	0,0874
Madapolystoma sp._FM897275	62	62	58	24		0,0476	0,0593	0,0617	0,0788	0,1062	0,0960
Madapolystoma sp._FM897271	61	40	39	55	61		0,0445	0,0546	0,0773	0,1046	0,0898
Madapolystoma sp._FM897272	70	22	51	73	76	57		0,0632	0,0929	0,1124	0,1038
Madapolystoma sp._FM897279	78	71	70	73	79	70	81		0,0937	0,1101	0,1062
*Kankana manampoka*_HM854293	109	107	100	91	101	99	119	120		0,0734	0,0601
*Eupolystoma vanasi*_AM157200	145	135	134	127	136	134	144	141	94		0,0625
*Eupolystoma alluaudi*_AM157199	137	124	122	112	123	115	133	136	77	80	

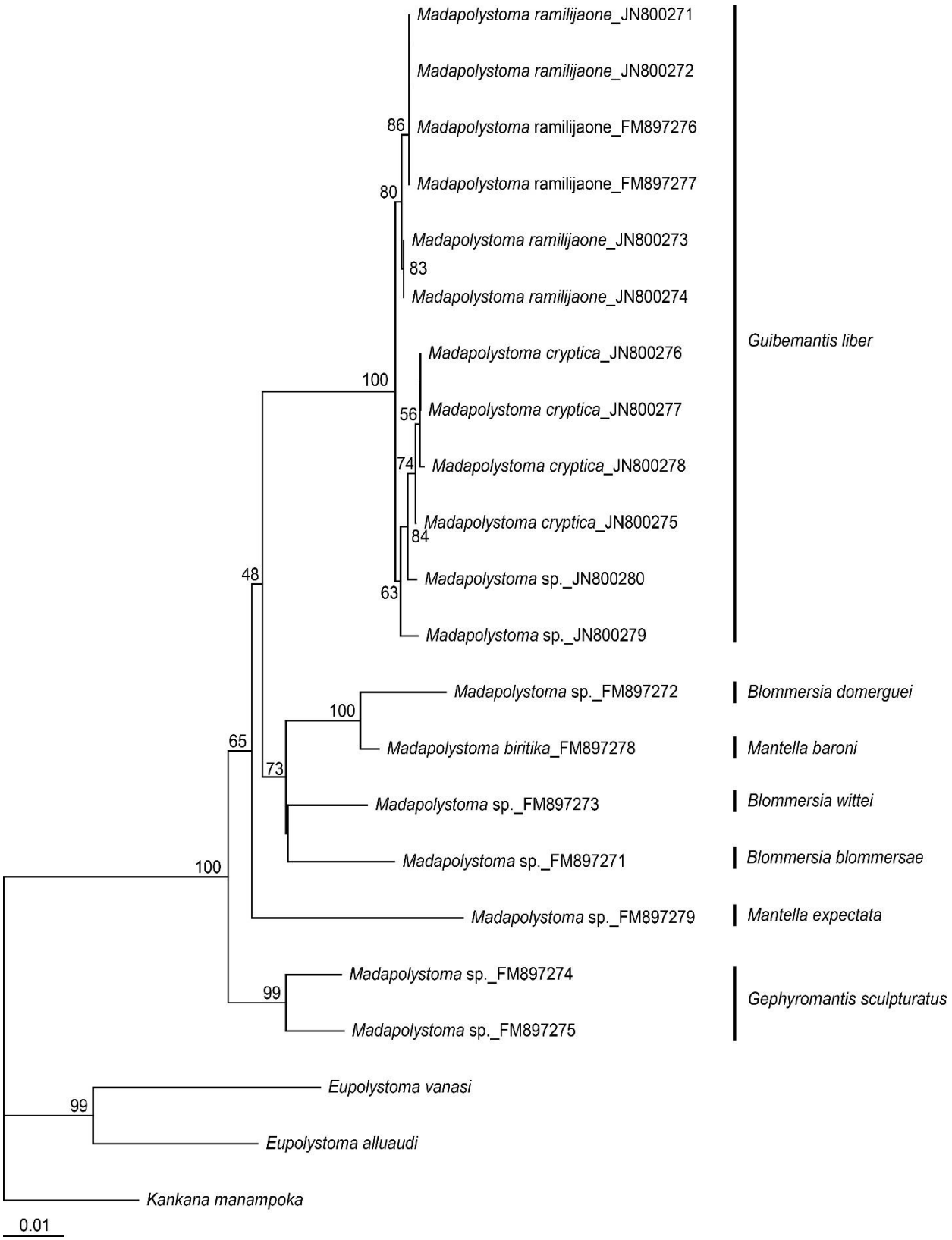


Figure 3.2: Minimum Evolution tree for *Madapolystoma* spp. Numbers on nodes indicate bootstrap support values. *Madapolystoma* sp. from *Blommersia domerguei* refers to *M. magnahami* n. sp. and *Madapolystoma* sp. from *Mantella expectata* refers to *M. isaloensis* n. sp.

3.3.2 Taxonomic summary of *Madapolystoma magnahami* n. sp. (Fig 3.3.; Table 3.3)

3.3.2.1 Classification

Class Monogenea van Beneden, 1858
Order Polystomatidea Lebedev, 1988
Family Polystomatidae Gamble, 1896

Genus *Madapolystoma* Du Preez, Rahariviololoniaina, Verneau and Vences, 2010

3.3.2.2 Type Host

Blommersia domerguei **3.3.2.3 Type Locality**

Ambohitantely Special Reserve, Madagascar (18,166667S; 47,273333E)

3.3.2.4 Site in Host

Mature parasites were found in the urinary bladder while immature stages were found in both urinary and accessory bladders.

3.3.2.5 Level of infection

Of the 15 specimens of *B. domerguei* that were collected, ten frogs were infected by two mature and 27 juvenile parasites, of which nine were found in the accessory bladder (prevalence 67%; mean intensity 2.7).

3.3.2.6 Type Material

Morphological description based on two mature and 27 immature specimens. Two sexually mature specimens (holotype NMBP 474 and paratype NMBP 475) as well as six immature specimens (paratypes NMBP 476 –NMBP 481) from a single locality i.e., Ambohitantely Special Reserve (Fig. 3.1a). Types are deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.3.2.7 Voucher Material

The remaining specimens were deposited in the polystome collection of the North-West University, Potchefstroom, South Africa.

3.3.2.8 Zoobank

The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:4C10D3CF-44C2-4DB4-90B9-648C1F1D0CE1. The LSID for the new name *Madapolystoma magnahami* n. sp. is urn:lsid:zoobank.org:act:EB537C95-A9E2-4BBE-BB0D-E68725F30D10.

3.3.2.9 Etymology

The species epithet *magnahami* is a combination of two Latin words, namely *magna* and *hamus*, meaning respectively great and hook. This refers to the large marginal hooklets of this species that are larger than those of all the other known species of *Madapolystoma*.

3.3.2.10 Description

Measurements in micrometres for mature parasites are given in Table 3.3. Body pyriform with widest point about two-thirds from anterior extremity (Fig. 3.3a, b). Mouth subterminal and surrounded by false oral sucker. Pharynx longer than wide. Intestine bifurcates, converging posteriorly; no prehaptoral anastomoses. Testis position unclear but probably in posterior half of body proper as vas deferens extends into posterior half of body proper; vas deferens widens anteriorly to form seminal vesicle, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal caeca bifurcation; genital atrium muscular, armed with six genital spines. Genital spines of both adult parasites were not measurable but measurable in subadult specimens. Ovary position unclear but based on position of reproductive ducts probably in midbody. Two vaginae, on lateral margins, with marginal opening; vaginal vestibule cup-shape. No distinct vitellaria observed; few small clusters of what appear to be granular vitelline follicles in posterior half of body. Genito-intestinal canal present and prominent; situated behind confluent vitelline duct. Uterus sac-like holds five and eight embryos, respectively. Embryos not ciliated, encapsulated in thin membrane. Four embryos in advanced stage of development with two pairs of suckers and developing hamuli clearly visible (Fig. 3.3a, b). Darker patch of cells observed at midbody in more developed embryos (Fig. 3.3a). Embryos 224–391 long and 152–168 wide. Sucker pair 1 of embryos 39–64 in diameter and sucker pair two 41–47. Haptor of adult parasite with three pairs of suckers. Hamuli well developed; without deep cut between handle and guard (Fig. 3.3c). Marginal hooklet pairs 1 and 2 located along periphery between posterior-most pair of suckers while marginal hooklet pairs 3–5 imbedded in suckers; marginal hooklet pairs 6–8 located anteriorly in haptor between sucker pair 3. Posterior-most marginal hooklet 1 and marginal hooklets 2–8 almost of equal length (Fig. 3.3d).

Table 3.3. Body measurements in micrometres for all known *Madapolystoma* spp.

	***Madapolystoma magnahami* n. sp.**	***Madapolystoma isaloensis* n. sp.**	***Madapolystoma cryptica***	***Madapolystoma biritika***	***Madapolystoma ramilijaonae***
Total length	2179; 2340	2672	1151 (1027–1239)	2041 (1304–3041)	2948 (1493–3,481)
Greatest width	507; 597	871	429 (411–439)	512 (420–597)	705 (602–857)
Haptor length - Body length to ratio	0.29	0.23	0.49	0.33	0.35
Width at vagina	414; 457	683		481 (369–548)	
Haptor length	550; 648	618	387 (337–424)	679 (594–788)	661 (567–771)
Haptor width	769; 934	877	475 (431–550)	882 (707–1046)	922 (765–1013)
Hamulus length X	239 (231–244)	173; 228	227 (215–239)	215 (190–238)	179 (163–195)
Hamulus length Y	204 (193–212)	183; 202	208 (197–219)	199 (175–223)	178 (154–193)
Hamulus hook length	48 (47–49)	46; 49	36 (31–39)	45 (34–50)	39 (31–47)
Oral disk	157; 161	134	71 (58–83)	160 (95–206)	123 (90–156)
Pharynx length	156; 166	149	94	136 (117–164)	175 (164–184)
Pharynx width	140; 153	130	58	119 (105–125)	154 (151–156)
Genital bulb diameter	15	27	31	20 (18–24)	33 (30–38)
Number of genital spines	6	7	7	8	5–8
Genital spine length	17 (16–17)	7.9 (7.4–8.4)	14	10.7 (10.5–10.9)	15 (14–16)
Sucker diameter	209 (193–221)	207 (201–216)	160 (122–186)	200 (160–255)	216 (188–244)
Maximum no of developing eggs or embryos *in utero*	5	0	11	1–32	21
Marginal hooklet 1 length	27.6 (26.6–28.4)	25.5 (24.6–26.6)	23.1 (21.6–24.7)	24.2 (21.4–26.1) 24.6 (20.4–26.9) 23.8 (21.2–26.1)	23.0 (20.6–26.2)
Marginal hooklet 2-8 length	27.0 (26.3–27.4)	25.8 (26.4–26.7)			

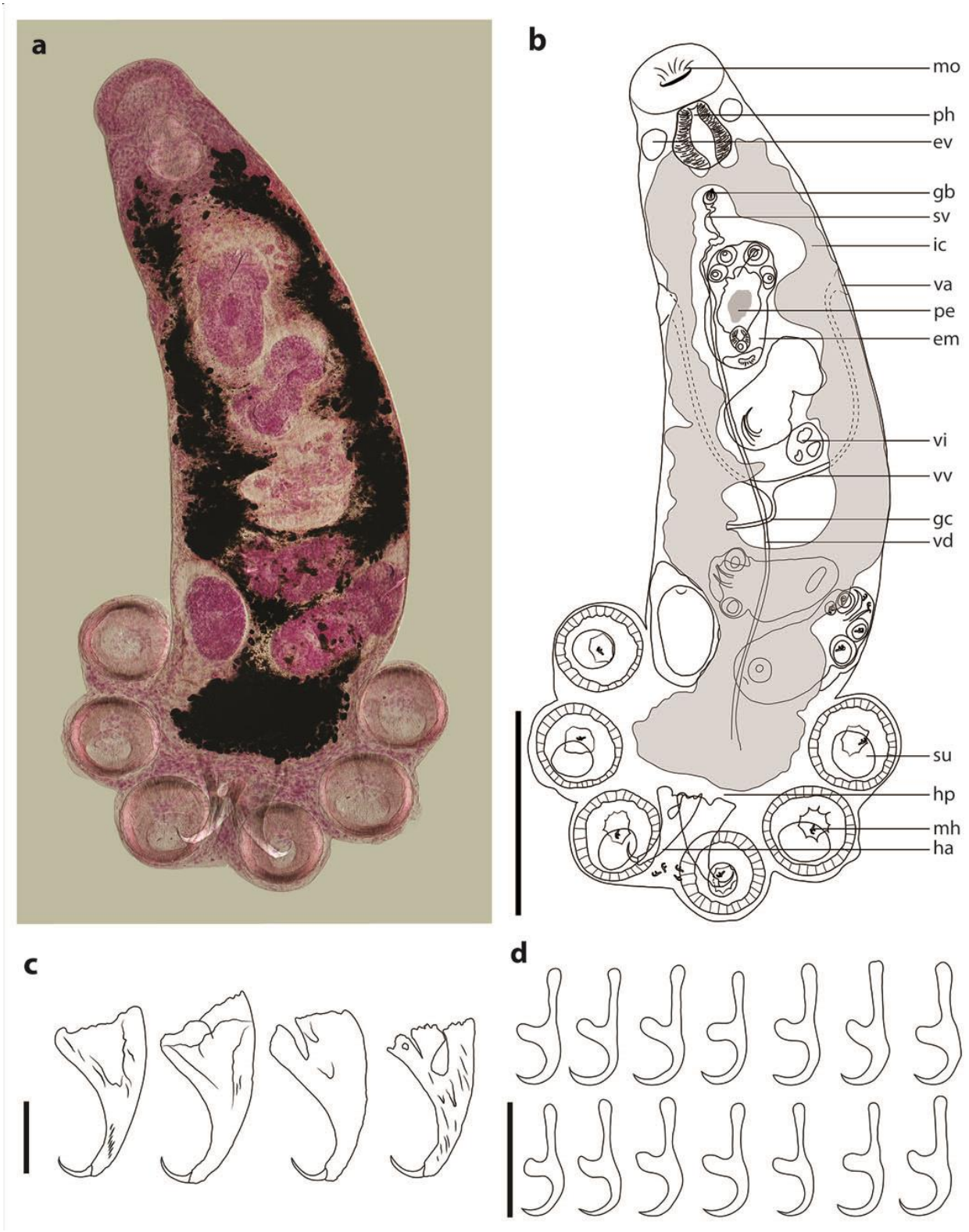


Figure 3.3: (**a-b**) Ventral view of *M. magnahami* n. sp. holotype. (**c**) Hamuli from mature specimens and (**d**) Marginal hooklets 1 (top) and 2-8 (bottom). Scale bars: **B**, 500µm; **C**, 100µm; **D**, 25µm. Abbreviations: em, embryo; ev, excretory vessel; gb, genital bulb; gc, genito-intestinal canal; ha, hamuli. hp, haptor; ic, intestinal cecum; mh, marginal hooklet; mo, mouth; pe, potential embryo; ph, pharynx; su, sucker; sv, seminal vesicle; va, vagina; vd, vas deferens; vi, vitelline follicles; vv, vitelline duct.

3.3.3 Taxonomic summary of *Madapolystoma isaloensis* n. sp. (Fig. 3.4; Table 3.3)

3.3.3.1 Type Host

Mantella expectata (Mantellidae)

3.3.3.2 Type Locality

Isalo, Madagascar (coordinates not known)

3.3.3.3 Site in Host

Mature parasites were found in the urinary bladder while immature stages were found in both urinary and accessory bladders.

3.3.3.4 Level of Infection

All six specimens of *M. expectata* examined were infected by as many as nine subadult parasites. One mature and 24 juvenile polystomes were recovered. This resulted in a prevalence of 100%, with a mean intensity of 5.33.

3.3.3.5 Type Material

Morphological descriptions are based on one mature and 19 immature specimens. The type series comprises one sexually mature specimen (holotype NMBP 482) and six immatures (paratype NMBP 483–488) from a single locality, Isalo (Fig. 3.1a). Types are deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.3.3.6 Voucher Material

The remaining specimens were deposited in the polystome collection of North-West University, Potchefstroom, South Africa.

3.3.3.7 Zoobank

The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:4C10D3CF-44C2-4DB4-90B9-648C1F1D0CE1. The LSID for the new name *Madapolystoma isaloensis* n. sp. is urn:lsid:zoobank.org:act:744DAD34-F102-4946-9135-813DD528118A.

3.3.3.8 Etymology

The species epithet refers to the type locality, Isalo.

3.3.3.9 Description

Measurements in micrometres for mature parasites are given in Table 3.3. Body elongate with widest point just anterior to the haptor (Fig. 3.4a, b); anterior mouth and posterior haptor with three pairs of suckers and pair of hamuli posteriorly between posterior-most sucker pair. Mouth subterminal surrounded by false oral sucker. Pharynx longer than wide. Intestine bifurcates, converging posteriorly; no prehaptoral anastomoses. Testis position unclear but probably posterior in body proper as vas deferens extends into posterior half of body proper; vas deferens widens anteriorly to form seminal vesicle, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal caeca bifurcation; genital atrium muscular, armed with seven genital spines. No distinct vitellaria observed; a few small clusters of what appears to be granular vitelline follicles in posterior half of body. Two vaginae, on lateral margins, with marginal opening; vaginal vestibule cup-shaped. Genito-intestinal canal present, prominent; situated behind confluent vitelline duct. Ovary position unclear but based on the position of reproductive ducts probably in midbody. Uterus sacciform, extending from genital bulb backwards full length of body proper. Haptor with three pairs of suckers. Two hamuli well developed; without cut between handle and guard (Fig. 3.4c). It was not possible to distinguish between marginal hooklets one and two on holotype, but these were measured on juvenile paratypes (Fig. 3.4d). Marginal hooklet pairs 1 and 2 located along periphery between posterior-most pair of suckers; marginal hooklet pairs 3–5 imbedded in the suckers obscured and not measurable; marginal hooklet pairs 6–8 located anteriorly in haptor between sucker pair 3.

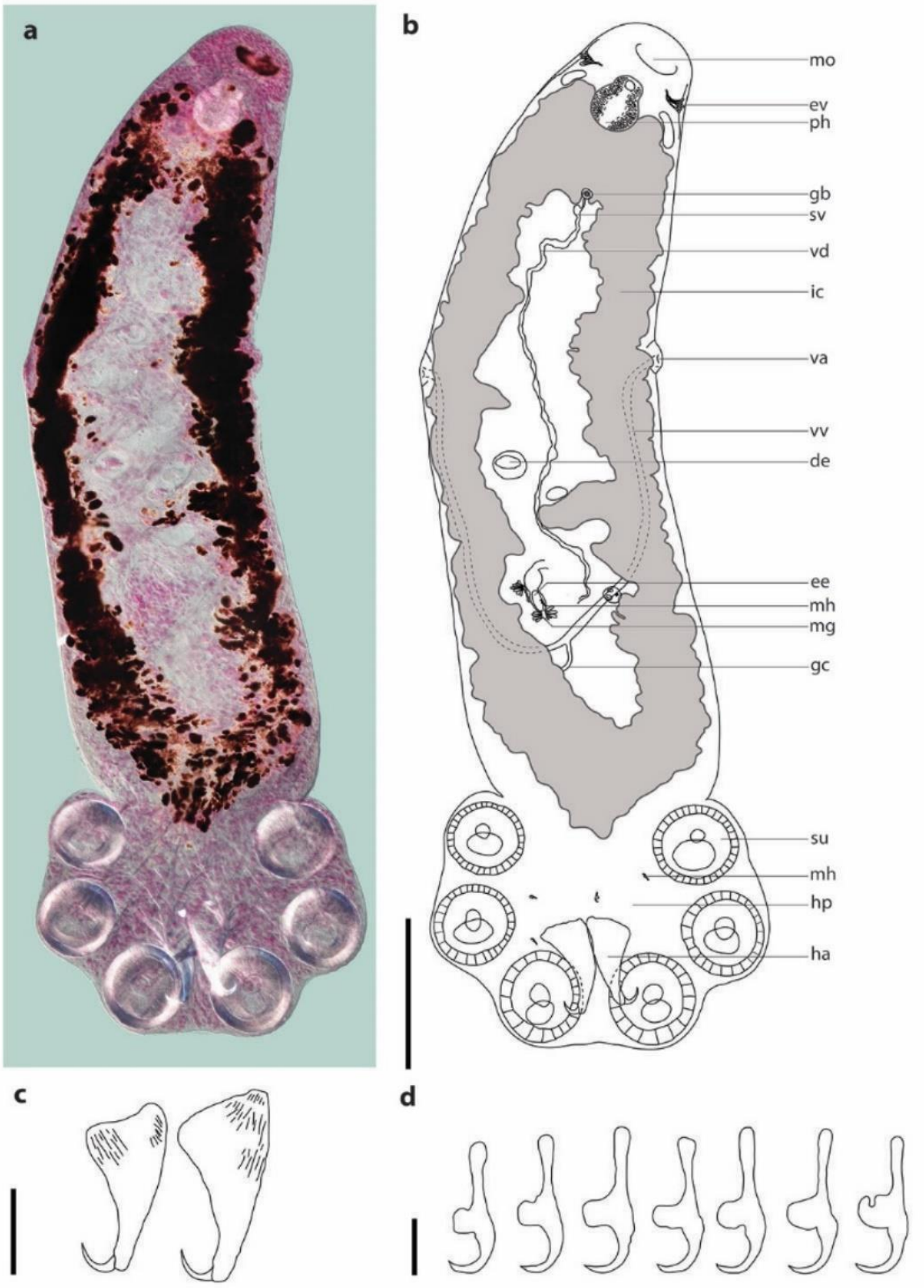


Figure 3.4: (**a-b**) Ventral view of *M. isaloensis* n. sp. holotype. (**c**) Hamuli from mature specimens. (**d**) Marginal hooklets 1−8. Scale bars: **B**, 200µm; **C**, 100µm; **D**, 20µm. Abbreviations: de, developing embryo; ee, early embryo; ev, excretory vessel; gb, genital bulb; gc, genito-intestinal canal; ha, hamuli. hp, haptor; ic, intestinal cecum; mh, marginal hooklet; mo, mouth; ph, pharynx; su, sucker; sv, seminal vesicle; va, vagina; vd, vas deferens; vv, vitelline duct.

3.3.3.10 Marginal Hooklet Morphometrics

Marginal hooklet morphometric measurements separated *M. magnahami* n. sp. of *B. domerguei* from all the other known species of *Madapolystoma*, including *M. isaloensis* n. sp. of *M. expectata*, as no overlaps were evidenced in the scatterplot, produced in Microsoft Excell ® (Fig. 3.5). However, whereas marginal hooklet morphometric measurements separated *M. isaloensis* n. sp. from *M. ramilijaonae*, they did not clearly separate it from *M. biritika* and *M. cryptica* (Fig. 3.5).

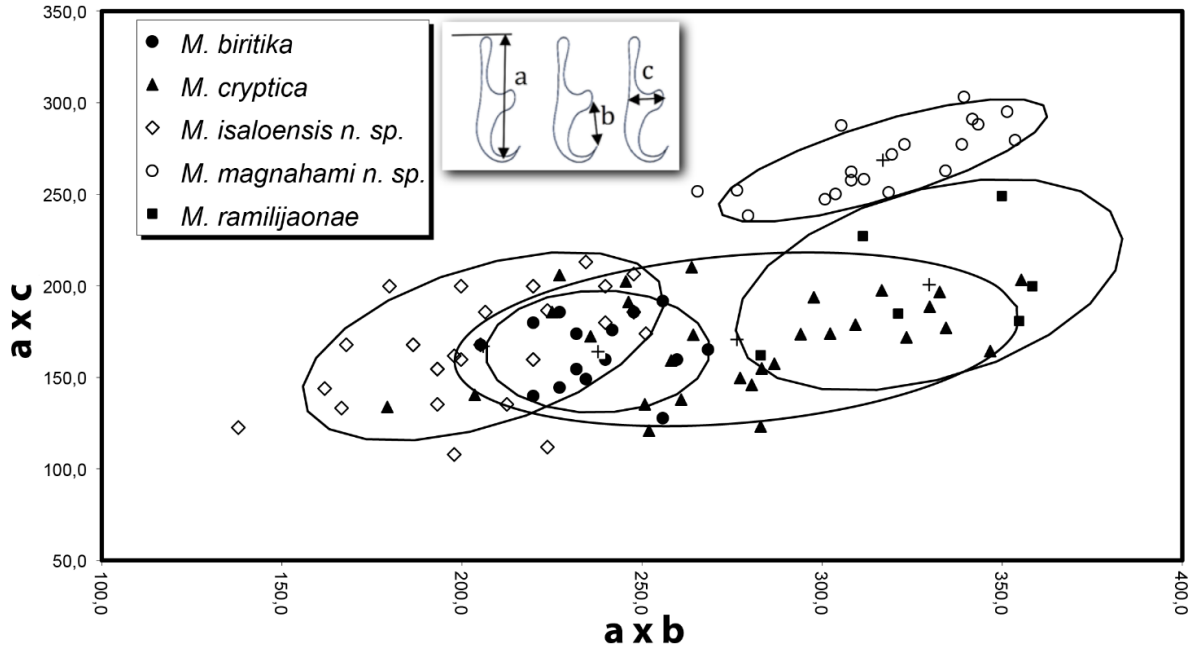


Figure 3.5: Scatter diagram of a × c plotted against b × c for all known *Madapolystoma* spp., *M. magnahami* n. sp. and *M. isaloensis* n. sp. The ellipses represent 95% of the confidence interval about the mean.

3.3.4 Remarks

The phylogenetic position of the two undescribed species of polystomes found among Madagascan frogs clearly indicated they could be both assigned to the genus *Madapolystoma* of the family Polystomatidae. Regarding the genetic divergences estimated within *Madapolystoma* (Table 3.2) and private changes observed within each molecular lineage, i.e. *Madapolystoma* sp. of *B. domerguei* and *Madapolystoma* sp. of *M. expectata*, molecular results thus supported the morphological description of two new species, i.e. *M. magnahami* n. sp. from *B. domerguei* and *M. isaloensis* n. sp. from *M. expectata*. The most significant morphological characteristics that distinguish *M. magnahami* n. sp. and *M. isaloensis* n. sp. from each other and from the three other known *Madapolystoma* spp. (*M. biritika*, *M. madagascariensis* and *M. cryptica*) are the size and shape of marginal hooklets and the number and size of genital spines (Table 3.3).

3.4 Discussion

In species of *Madapolystoma* marginal hooklets C1–C8 were found to be of equal length. This phenomenon has also been reported for species of *Eupolystoma* and *Kankana*. This is in contrast with the usual situation encountered in species of *Polystoma* and most other polystomes where the posterior-most hooklet pair is significantly larger than the rest (see for instance Aisien *et al.*, 2011; Du Preez, 2011; 2013; Du Preez & Kok, 1993; 1995; Du Preez *et al.*, 2002; Tinsley, 1973; Tinsley, 1974). Therefore, this measure may be a good character for species delimitation in *Madapolystoma*. Marginal hooklet morphometrics (Fig. 3.5) were thus useful in separating *M. magnahami* n. sp. from *M. isaloensis* n. sp. and from all the other known *Madapolystoma* species with 95% confidence. *Madapolystoma magnahami* n. sp. currently has the largest marginal hooklets of all known species in the genus. However, the measurements of marginal hooklets were not able to isolate *M.* isaloensis n. sp. from *M. cryptica* nor *M. biritika*. They did however separate *M. isaloensis* n. sp. from *M. magnahami* n. sp. and *M. ramilijaonae.* Regarding the number of genital spines in *M. magnahami* n. sp., although it overlapped with that reported in *M. ramilijaonae*, the length of genital spines was larger on average than for *M. ramilijaonae* (Berthier *et al.*, 2014). Though the number of genital spines recorded in *M. isaloensis* n. sp. overlapped with that reported in *M. cryptica*, length of genital spines was larger than that of *M. cryptica* (Berthier *et al.*, 2014).

The value of sclerotised structures in the description of soft-bodied parasites such as polystomes has been emphasised (Du Preez & Maritz, 2006) and although some taxonomists advocate that polystomes should not be flat fixed (Platt *et al.*, 2011), it is of utmost importance to observe sclerotised structures in flat orientation. Fixing specimens under cover slip pressure does not affect the measurement of sclerites or smaller rigid structures such as the oral sucker, genital bulb or even, in some taxa, the haptoral suckers (Platt *et al.*, 2011). When sufficient material is available we recommend that (1) a specimen be fixed in high quality ethanol or a fixative such as RNAlater for DNA extraction; (2) some of the specimens be heat-killed by placing them in a drop of water on a microscope slide that is then heated from below with a butane lighter until the parasite stops moving followed by fixation in 10% buffered formalin and the remainder of the specimens to be fixed in 10 % buffered formalin under coverslip pressure. Body measurements and placement of organs should be studied from the unflattened specimens while sclerites should be measured in flattened specimens. However, in instances where a limited number of specimens are available, such as here we do recommend fixing specimens flat under coverslip pressure.

Species of *Diplorchis* Ozaki, 1931, *Eupolystoma*, *Kankana*, *Neodiplorchis*, *Parapolystoma*, *Pseudodiplorchis* Yamaguti, 1963 and *Sundapolystoma* Lim & Du Preez, 2001 all have extended uteri (Du Preez *et al.*, 2003; Rahariviololoniaina *et al.*, 2011) allowing for the

accumulation of large numbers of eggs and/or *in utero* development. Therefore, repeated re-infection of a single host may occur either during breeding events, after releasing larvae, or following an internal life cycle inside the host. In the latter case, the oncomiracidium does not leave the host but attaches to the bladder wall alongside its parent inside the urinary bladder. In *Eupolystoma* it has been shown that both ciliated and unciliated oncomiracidia are produced (Combes *et al.*, 1973; Fournier & Combes, 1979); ciliated oncomiracidia are destined to leave the host to swim and find another host, while unciliated oncomiracidia are destined for an internal cycle. In *Madapolystoma* the *in utero* development is taken a step further in that no ciliated oncomiracidia are produced. Embryos develop gradually into juvenile parasites with hamuli and two pairs of suckers (Fig. 3.3a, b). In the most advanced *in utero* developing juveniles, a darker cluster of cells is visible in the middle of the parasites (Fig. 3.3a). We hypothesise that this cluster of darker cells is a developing F2 embryo, implying true vivipary. To confirm whether *Madapolystoma* is viviparous, histological serial sectioning would be necessary; however, no specimens were available for histology. True viviparity has been well documented for the teleost monogenean *Gyrodactylus* (see: Bakke, 2002; Cable & Harris, 2002; Harris, 1983; Harris, 1985; Tinsley, 1983) and reported for the anuran monogenean *Gyrdicotylus* (Harris & Tinsley, 1987; Jackson & Tinsley, 1994). Du Preez *et al.* (2010) and Berthier *et al.* (2014) reported advanced development of embryos with the presence of developing hamuli and suckers in the embryos but did not mention the development of F2 developing embryos within the F1 generation.

The presence of only a small number of developing embryos in species belonging to *Madapolystoma* indicates a unique reproductive strategy. All the known hosts for *Madapolystoma,* namely species of *Blommersia*, *Guibemantis* and *Mantella,* deposit their egg clutches terrestrially or semi-terrestrially. While species of *Blommersia* and *Guibemantis* attach their eggs to vegetation or other objects close to water, *Mantella* spp. deposits their eggs in hidden cavities on the ground (Glaw & Vences, 2007). During a field trip to Madagascar in February 2006, a frog egg mass overhanging a pool was collected and inspected under a stereo microscope. A small polystome embryo was observed on the egg mass. It has been documented that frogs laying eggs outside the water may return, at regular intervals, to urinate on the eggs to keep them moist. We therefore hypothesise that the developing embryo of *Madapolystoma* spp. may leave the host during such an event and stay on the egg mass until another frog visits the egg clutch, when it then enters the cloaca and migrates to the accessory bladder from where it migrates to the urinary bladder.

During stock piling of offspring *in utero,* the reproductive capacity of polystomatids is probably determined by body size (Tinsley, 1990). While the total annual egg production of *Polystoma integerrimum,* with a length of 10 mm, may be as many as 4000 eggs produced in only

a few days (Combes, 1972), in *Pseudodiplorchis americanus* with a similar body length it rarely exceeds 300 (Tinsley, 1990). The maximum reported number of eggs and developing embryos in a single individual of *Madapolystoma* is 32 (Du Preez *et al.*, 2010). In the instance of *M. magnahami* n. sp. and *M. isaloensis* n. sp., with their very small body size of less than 2.5 mm and *in utero* development to a very advanced stage, the annual offspring production is probably very limited.

Well-defined testis tissue and ovaries could not be located in both *M. magnahami* n. sp. and *M. isaloensis* n. sp., in spite of careful examination with a high-end compound microscope. For species of *Gyrodactylus* it has been reported that the testis develops only after the first embryo is produced and that the female reproductive system develops after the male reproductive system (Bakke *et al.*, 2007). Most polystomatids produce chitinous yellow eggs that develop in the water body after being released from the host. In species where *in utero* development is the norm (i.e. *Eupolystoma*, *Kankana*, *Pseudodiplorchis* and *Wetapolystoma* (see: Gray, 1993; Rahariviololoniaina *et al.*, 2011; Tinsley, 1990) eggs are not encapsulated in a yellow rigid shell, but rather a semi-transparent flexible membrane. This allows for direct maintenance of developing larvae through parental nutrients. Whereas vitellaria are distributed throughout most of the body proper in most polystomatids, it is significantly reduced and restricted to lateral fields in species displaying extensive *in utero* development of eggs. For some species of *Eupolystoma* and for *K. manampoka*, the closest relatives to species belonging to *Madapolystoma* (see Rahariviololoniaina *et al.*, 2011), the vitellaria are restricted to two narrow lateral streaks posteriorly in the body proper (Du Preez *et al.*, 2003). The advanced *in utero* development as observed in *Madapolystoma* would involve direct maintenance of offspring by parental nutrients which explain the lack of vitellaria fields. According to Bakke *et al.* (2007), in viviparous forms the vitellaria never fully develop and never produce egg-shell precursor proteins. Vitelline cells in viviparous species appear to be reduced to patches of granular syncytia in the posterior part of the body (Cable *et al.*, 1996). This is in accordance with what we observed for *M. magnahami* n. sp. and *M. isaloensis* n. sp.

3.5 Acknowledgements

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CHAPTER 4:

FIRST RECORD OF *METAPOLYSTOMA* (MONOGENEA: POLYSTOMATIDAE) FROM *BOOPHIS* TREE FROGS IN MADAGASCAR, WITH THE DESCRIPTION OF FIVE NEW SPECIES.



Cheers to the animal that resides within us all.
Machiavellian, insidious
Claws the perfidious.
Herd would be tamed by it
Proclaimed the vulnerability.
I'm no clairvoyant but
Sturdy to acquiesced it.
As it is parasite
We are the host.
-Sheena

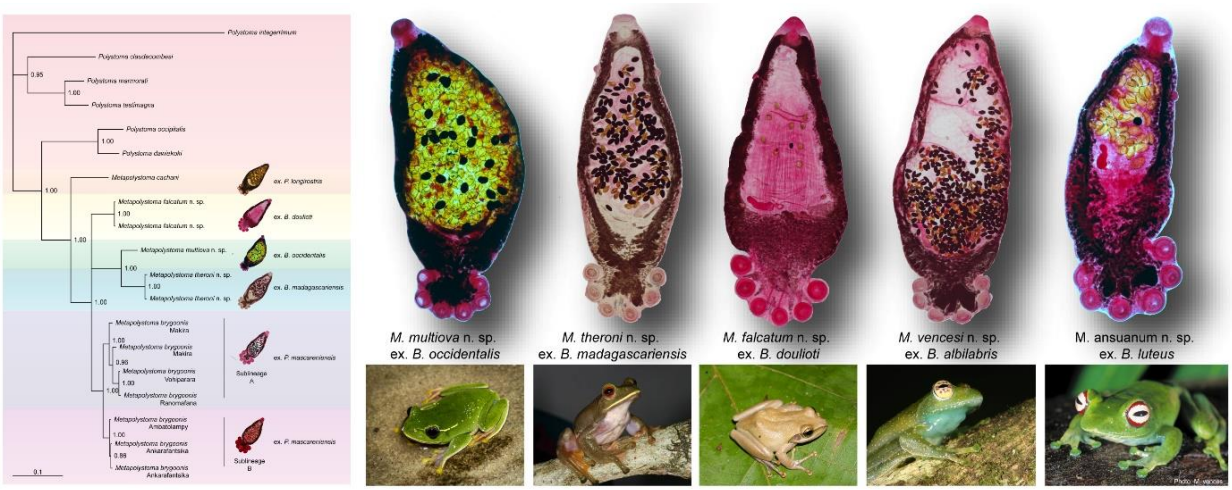
This chapter has been published:
Landman, W.J., Verneau, O., Raharivololoniaina, L., du Preez, L.H. (2021). First record of *Metapolystoma* (Monogenea: polystomatidae) from *Boophis* tree frogs in Madagascar, with the description of five new species. *International Journal for Parasitology: Parasites and Wildlife*. 14, 161−178.

Landman took the lead, prepared the manuscript and all figures.

Abstract

Although Madagascar has more than 350 frog species of which all but two are endemic to the island, the known polystome (Monogenea: Polystomatidae) diversity parasitising Malagasy frogs is low, encompassing five species of *Madapolystoma*, one species of *Kankana* and one *Metapolystoma*. Investigating the parasite diversity of frog parasites at selected Malagasy localities led to the discovery of undescribed polystomes. Five treefrogs, *Boophis albilabris*, *Boophis doulioti*, *Boophis luteus*, *Boophis madagascariensis* and *Boophis occidentalis* were found to be infected and are reported here as hosts for new *Metapolystoma* species. Morphological investigation, combining examination of body length, haptor length, genital bulb width, genital crown diameter, genital spine number, genital spine length, ovary length, egg length, hamulus length, hamulus guard length and hamulus hook length, revealed five distinct morphotypes. Phylogenetic analysis and genetic divergences obtained for three of the five morphotypes, support the distinction of new species. *Metapolystoma ansuanum* n. sp. is described from *B. luteus*, *Metapolystoma falcatum* n. sp. from *B. doulioti*, *Metapolystoma multiova* n. sp. from *B. occidentalis*, *Metapolystoma theroni* n. sp. from *B. madagascareniensis* and *Metapolystoma vencesi* n. sp. from *B. albilabris*. Finally, although the validity of *Metapolystoma* as a taxon is not fully resolved yet, the phylogenetic position of the newly described species and their morphology provide clear evidence for *Metapolystoma* as a valid taxon.

Keywords: *Boophis*, Madagascar, *Metapolystoma*, Monogenea, polystomes



Madagascar is well known for its high species diversity and endemicity, particularly in the cases of plants and vertebrates (Myers *et al.*, 2000). When it comes to amphibian diversity, Madagascar is globally ranked in the top twelve (Andreone *et al.*, 2008). Its unique herpetofauna serve as hosts for an equally unique and diverse assemblage of parasites (Junker *et al.*, 2010; Kuzmin *et al.*, 2013; Landman *et al.*, 2018; Rocha *et al.*, 2012; Wohltmann *et al.*, 2007). However, little is known about Madagascar's anuran polystome flatworm diversity, since only 86 (Verneau *et al.*, 2009a) of the 356 known species (Frost, 2020) have been screened for polystomes at a few accessible localities in the past. Malagasy polystomes are currently represented by four genera encompassing a single chelonian and seven anuran species, all of these were found in the urinary bladder of their host. *Uropolystomoides chabaudi* (Euzet and Combes, 1965) from *Pelomedusa subrufa* (Bonnaterre, 1789) is the only polystome known from turtles. *Metapolystoma brygoonis* (Euzet and Combes, 1964) was the first polystome found within a Malagasy anuran host, as described from *Ptychadena mascareniensis*. Besides these two species, *Kankana manampoka* Rahariviololoniaina *et al.* 2011 was described from *Cophyla pollicaris* (Boulenger, 1888). Five other species of *Madapolystoma* were reported from mantellids, namely *Madapolystoma biritika* Du Preez *et al.* 2010 from *Mantella madagascariensis* (Grandidier, 1872), *M. isaloensis* Landman, Verneau and Du Preez, 2018 from *Mantella expectata* Busse and Böhme, 1922 and *M. magnahami* Landman, Verneau and Du Preez, 2018 from *Blommersia domerguei* Guibé, 1974. *Madapolystoma cryptica* Berthier *et al.* 2014 and *M. ramilijaonae* Berthier *et al.* 2014 were conversely described from the same host species *Guibemantis liber* (Peracca, 1893).

Metapolystoma brygoonis was initially described as *Polystoma* and later elevated (Combes, 1976). This separation of *Metapolystoma* and *Polystoma* was based on morphological characters, including the large extended uterus containing many eggs that fills the largest part of the body and the posterior position of the large ovary (Combes, 1976). The validity of *Metapolystoma* as a genus has, however, been under dispute ever since its nested position within *Polystoma* was demonstrated at the molecular level (Bentz *et al.*, 2001; Olson & Tkach, 2005; Verneau *et al.*, 2002). Up to the present, the three known *Metapolystoma* species have been described from grass frogs belonging to *Ptychadena*: *M. brygoonis* from *P. mascareniensis* in Madagascar, *M. cachani* (Gallien, 1956) from *Ptychadena longirostris* (Peters, 1870) in Ivory Coast and *Metapolystoma porosissimae* du Preez and Kok 1992, from *Ptychadena porosissima* (Steindachner, 1867) in South Africa. It was therefore likely that many Madagascan polystomes awaited discovery (Landman *et al.*, 2018).

Among the 27 anuran genera found in Madagascar (Frost, 2020), *Boophis* Tschudi, 1838 is endemic to Madagascar and the Mayotte Island in the Comoros (Frost, 2020; Glaw & Vences,

2007), totalling 79 currently recognised species (Frost, 2020) representing a diverse and species-rich group within the Mantellidae Günter, 1859. During herpetological surveys conducted in Madagascar in 2005, 2006 and 2007, several species of *Boophis* from different localities were examined for polystome parasites. *Boophis albilabris* (Boulenger, 1888), *Boophis doulioti* (Angel, 1934), *Boophis luteus* (Boulenger, 1882), *Boophis madagascariensis* (Peters, 1874) and *Boophis occidentalis* Glaw & Vences, 1994 were found to be infected with five distinct, unknown *Metapolystoma* species. Since the collection of additional materials was constrained by permit restrictions and administrative difficulties in Madagascar, and because preliminary molecular and morphological data converged towards the same result, our objective was to describe new parasites collected from distinct frog species despite their small sample size.

4.2 Material and methods

4.2.1 Host and parasite sampling

Annually during February–March of 2005 2006 and 2007, several *Boophis* species were collected in Madagascar (Table 4.1). Following collection by hand at night, frogs were individually kept overnight in clear plastic bags containing 50 ml of tap water. After 24 hours, the water in which the frogs were kept was poured through two plankton sieves with mesh sizes of 500 and 100 µm respectively. The 500 µm sieve retained course debris and most of the faeces, while the 100 µm sieve retained parasite eggs and fine debris. The content of the 100 µm sieve was then washed into a Petri dish and inspected for the presence of polystome eggs using a dissecting microscope. Infected frogs were euthanized with MS222 (Ethyl-3-aminobenzoate methanesulfonate) and dissected for parasite investigation using a Nikon SMZ-645 dissecting microscope. Where no eggs were observed, two representatives per species per locality were dissected to check for the presence of non-egg producing polystomes. The kidneys, urinary bladder and accessory bladders were removed and inspected in a Petri dish containing 0.6% Ringers solution. For the purpose of molecular studies, some juvenile polystomes were preserved in absolute ethanol. The remainder of the juvenile polystomes were mounted in ammonium picrate glycerine or preserved in 10% NBF. Adult parasites were fixed in 10% NBF under coverslip pressure. Specimens were rinsed in water, stained with acetocarmine, dehydrated, cleared in xylene and mounted in Canada balsam. Five additional, fixed specimens of *B. albilabris* collected in 1972 by Mr. Blane at Andringita Massif, Madagascar, and archived at the Muséum National d'Histoire Naturelle of Paris, were dissected and parasites retrieved and processed as explained above. Host and parasite distribution maps (Fig. 4.1) were drawn in ArcMap 10.5.1 (Esri, California), and shape files for frog distributions were provided by the IUCN (IUCN, 2016a; IUCN, 2016b; 2016c; 2016d; 2016e).

Table 4.1: Frog species under survey with their site of collection in Madagascar.

Frog species	No. of specimens collected	Locality
B. madagascariensis	30	Andasibe (Indri Reserve)
B. albilabris	6	Ambatolahy and Andringita massif
B. doulioti	22 frogs, 10 tadpoles	Ankarafantsika
B. luteus	9	Isalo (Cascade des Nymphes Special Reserve)
B. occidentalis	5	Isalo (Cascade des Nymphes Special Reserve)

4.2.2 DNA extraction and amplification

All polystome DNA samples used in this study were sourced from Verneau *et al.* (2009a) and Berthier (2011). According to these authors, DNA extractions were conducted in 100-150 ul of 10% Chelex 100 sodium (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) with the proteinase K 1 mg·mL−1 final concentration at 55°C for the duration of 1 to 1,5 hours. Enzymatic reactions were stopped at 100 °C for 15 min and DNA were stored at −20°C until use. Amplifications of 18S, 28S and COI were conducted following the procedure described in Héritier *et al.* (2015), regardless of the gene of interest or primers used. The complete 18S rRNA gene and the partial 28S rRNA gene were amplified in two rounds with the respective combinations of primers F18 / 18Rg and 18F3 / IR5 for 18S and LSU5' / IR16 and IF15 / LSU3' for 28S. Primer sequences are reported in Sinnappah *et al.* (2001), Verneau *et al.* (2009a) and Héritier *et al.* (2015). COI was amplified using the combination of primers L-CO1p / H-Cox1p2 (Littlewood *et al.*, 1997). All PCR products were sent to the Genoscreen Company (Lille, France) for purification and sequencing with their respective forward and reverse PCR primers. Sequences were read and edited with the software Geneious (Saint Joseph, Missouri, USA) to check chromatograms before use for phylogenetic and distance analyses.

4.2.3 Phylogenetic and distance analyses

All *Metapolystoma* sequences were edited and aligned independently using ClustalW implemented in MEGA version 7 (Kumar *et al.*, 2016) under default parameters (Thompson *et al.*, 1994). The alignment included five African *Polystoma* species and a single European *Polystoma* species, that is, *Polystoma integerrimum* (Frölich, 1791), which was used as an outgroup according to Bentz *et al.* (2001). They were subsequently concatenated in a single alignment for Bayesian analysis. The two ribosomal genes were treated as two separate partitions, and the COI genes as three distinct partitions according to their codon position. A two substitution rates model with a proportion of invariable characters was selected for the 18S partition, whereas a GTR + I

model was selected for the 28S partition following the Akaike Information Criterion (AIC) implemented in Modeltest 3.06 (Posada & Crandall, 1998). Concerning COI, six types of substitutions and gamma rates each comprising four gamma rate categories were applied to the first and second positions, whereas six types of substitutions and invariable-gamma rates each comprising four gamma rate categories were applied to the third position. Evolutionary parameters were estimated independently for all five partitions. The Bayesian analysis was run using MrBayes 3.04b (Huelsenbeck & Ronquist, 2001), employing four chains running for ten million generations and sampled every 100 cycles. The Bayesian consensus tree was subsequently drawn after removing the first 10 000 trees (10%) as the burn-in phase, and viewed with TreeView version 1.6 (Page, 1996). Corrected pairwise distances were calculated independently for partial 18S, 28S and COI sequences using the Kimura 2-parameter model and 1000 bootstrap replicates in MEGA version 7 (Kimura, 1980)

4.2.4 Morphology and morphometry

Parasites were examined and photographed using a Zeiss Imager Axio10 compound microscope (Zeiss, Germany) fitted with a Zeiss Axio cam 305 camera (Zeiss, Germany). Morphological structures and organs were measured in micrometres using the Zeiss Zen Blue elements (Zeiss, Germany) software program. Hamuli were respectively measured from the apex behind the hook to the tip of the guard (Length X) and to the handle (Length Y), and the hook from tip to base (Length Z). Full-body images of type specimens were taken using a Nikon AZ100M microscope (Nikon, Netherlands) fitted with a low powered 1X objective. Illustrations were done in Adobe Illustrator CC (Adobe, California). Additional taxonomic measurements for *M. porosissimae* were taken from the type series (Table 4.2).

4.3 Results

4.3.1 Taxonomic summary

Class: Monogenea van Beneden, 1858
Order: Polystomatidea Lebedev, 1988
Family: Polystomatidae Gamble, 1896
Genus: *Metapolystoma* Combes, 1976

4.3.1.1 *Metapolystoma vencesi* n. sp. (Figs. 4.2–4.3; Table 4.2)

4.3.1.1.1 Type host:

Boophis albilabris (Mantellidae).

4.3.1.1.2 Type locality

Ambatolahy, Madagascar (Fig. 4.1), (-21.2438667S; 47.4262167E).

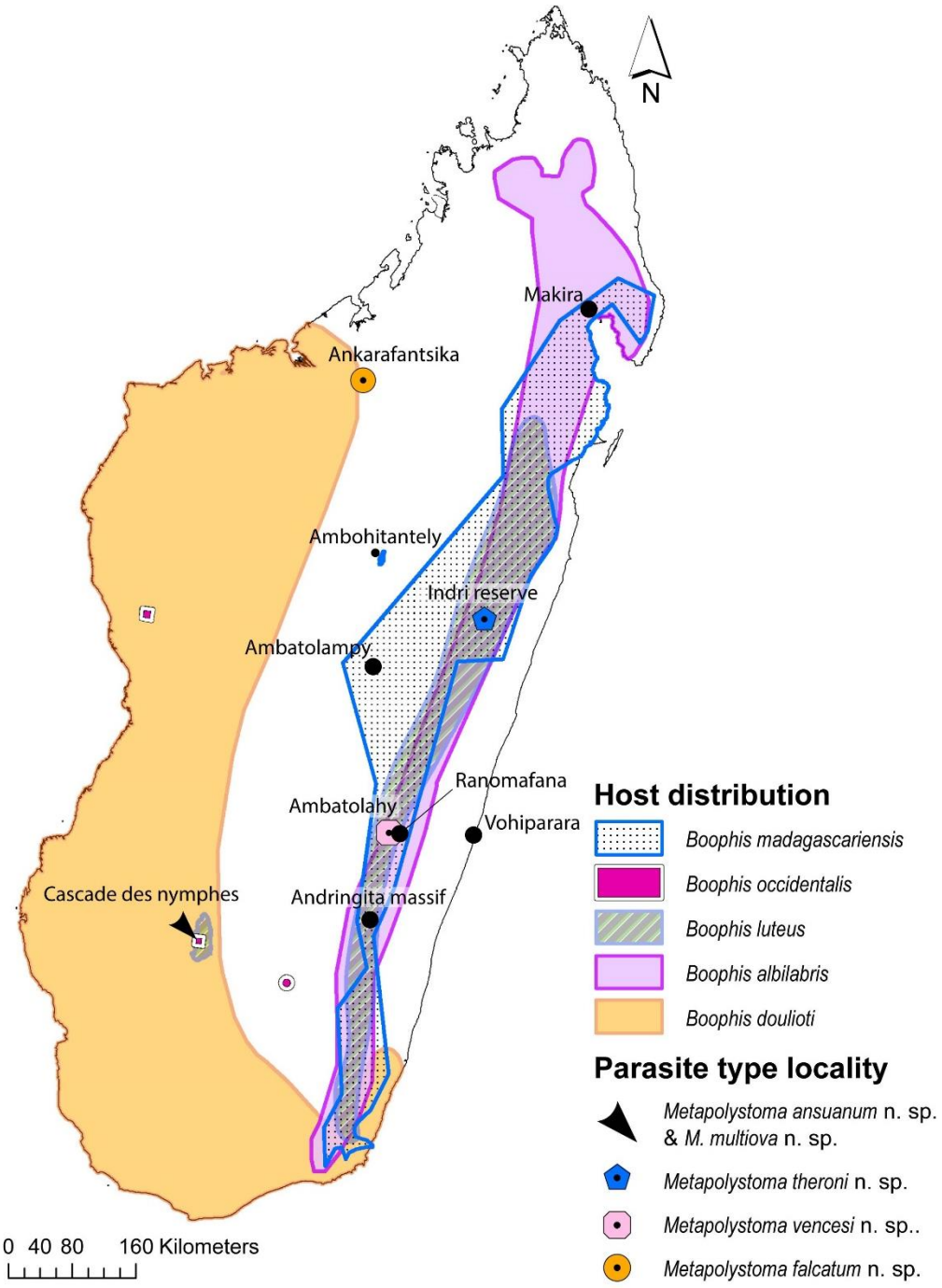


Figure 4.1: Map of Madagascar showing parasite type localities and distribution areas of hosts.

Table 4.2: Body measurements in micrometres for all *Metapolystoma* spp. values.

Measurements	***Metapolystoma vencesi* n. sp.**	***Metapolystoma falcatum* n. sp.**	***Metapolystoma ansuanum* n. sp.**	***Metapolystoma theroni* n. sp.**	***Metapolystoma multiova* n. sp.**
Number of mature specimens	6	1	2	1	2
Total length	9,190	7,871	2,680–3,952	9,086	4,915–5,537
Greatest width	3,496	3,272	1,035–1,436	3,208	2,257–2,419
Width at vagina	2,315	1,783	804–1001	2,011	1,469–2,086
Haptor length – Body length ratio	0.16	0.19	0.23–0.25	0.18	0.18
Haptor length	1,437	1,492	669–912	1,634	916
Haptor width	2,344	2636	1,166–1,385	2,572	1,478
Hamulus length X	269–332 (299 ±32; 3)	393–407	205	392–408	196–248 (215 ±29; 3)
Hamulus length Y	225–302 (270 ±40; 3)	303–314	179	252–257	121–174 (153 ±23; 4)
Hamulus hook length Z	40–52 (47)	66–70	62	50–55	58.2
Oral disk	391	297	307–329	486	248–271
Pharynx length	302–420	327	256–308	401	302–405
Pharynx width	291–390	290	183–222	385	289–320
Genital bulb diameter	115	86	64–73	65	86
Ovary length	861	772	265–338	479	446
Ovary width	194	196	98–144	385	158
Number of genital spines	8	8	10	7	6
Genital spine length	31.5–33.4 (32.5 ±0.6; 9)	24.3–31.6 (27.9 ±1.78; 18)	24.5–28.5 (26.19 ±1.16; 5)	28.9–34.9 (32.9 ±4; 3)	40.4–43.7 (42 ±1.4; 5)
Genital crown diameter	31.8	25.7	29.4–32.8	24.2–27.6 (25.8 ±1; 10)	24.5
Sucker diameter	366–452(424 ±37; 12)	435–498(468 ±29; 6)	241–305(273 ±25; 12)	404–424 (416 ±8; 6)	329–356 (339 ±10; 9)
Maximum number of eggs in utero	367	11	41	176	499+
Egg length	210–230 (219 ±7; 16)	161–185 (171 ±8; 11)	196–217 (205 ±8; 8)	239–265(250 ±7; 12)	198–228 (212 ±9; 23)
Egg width	120–147 (130 ±8; 16)	108–123 (117 ±5; 11)	119–137 (127 ±7; 8)	136–152(143 ±5; 12)	144–165 (154 ±7; 23)
Marginal hooklet 1 length	29.7–34.2 (32.5 ±1.3; 13)	32.6–39.7 (36.9 ±2.3; 13)		34.3–39.4 (936.2 ±1.4; 21)	28.6–38.7 (33.8 ±3.3; 11)
Marginal hooklet 2–7 length	20.8–25 (23.6 ±1; 30)	21.8–27 (23.8 ±1.52; 30)	22.3–24.79 23.5 ±1.17; 4)	22.6–27.2 (24.8 ±1.1; 44)	21.3–26.9 (24.7 ±2; 17)
Marginal hooklet 8 length	27.4–32.4 (30.5 ±1.6; 12)	28.8–33.5 (31.3 ±1.5; 14)		30.5–34.1 (32.2 ±1; 19)	28.4–36.3 (32.4 ±2.8; 5)
Widest section from front	57%	66%	16–65%	51%	50%–57%
Times longer than wide	2.7	2.4	2.6–2.8	2.6	2.2–2.3

Values: (Min – Max (Mean ±Ơ; N)), numbers in bold indicate additional measurements taken from species described prior to the present study

Table 4.2: Continued

Measurement	***Metapolystoma porosissimae*** [a]	***Metapolystoma brygoonis*** [b]	***Metapolystoma cachani*** [c]		
Number of mature specimens	15	10	10	3	57
Total length	4,150–6,710 (5,870 ±0.6; 10)	3,400–4,800 (4,200)	4,000	3,880–5,628 (4,594)	3.680–7.250 (5.070)
Greatest width	1,570–2,360 (2,040 ±0.2; 11)	1,220–1,500 (1,340)	1,500	1,120–2,880 (1,793)	1.160–2.880 (1.980)
Width at vagina	1.120–1.700 (1.460 ±0.2; 11)	650–1,030 (800)			880–1.760 (1.220)
Haptor length – Body length ratio	0.26–0.33 (0.29 ±0.1; 10)	0.22–0.23 (0.22)	0.25	0.17–0.21 (0.19)	0.15–0.32 (0.23)
Haptor length	1.540–1.800 (1.670 ±01; 12)	760–1,070 (900)	1,000	720–1,072 (864)	910–1.500 (1.15)
Haptor width	2.010–2.660 (2.360 ±0.2; 12)	1,220–1,760 (1,530)	1,500	1,248–2,800 (1,858)	1.300–2.350 (1.730)
Hamulus length X	432–472 (447 ±14.3; 26)	330–420 (380)	310	280–450 (348)	330–520 (430)
Hamulus length Y	**332.4–385.7 (361.2 ±18.52; 13)**				
Hamulus hook length Z	**72–86.6 (78.3 ±3.97; 15)**				
Oral disk	461–566 (521)	220–340 (270)			
Pharynx length	267–348 (310 ±31.8; 10)				
Pharynx width	234–298 (262 ±20.4; 10)				
Genital bulb diameter	**93–129.5 (105.7 ±11.91; 7)**	80	25		
Ovary length	546–755 (651 ±89.1; 11)	410–460 (440)			
Ovary width	**266**	90–160 (120)			
Number of genital spines	9–10	8	8		
Genital spine length	31 / **21.83–25.97 (24.144 ±1.46; 6)**	30–32 (31)			
Genital crown diameter	**25.18–26.96 (26.35 ±0.57; 7)**				
Sucker diameter	461–566 (521 ±30; 65)	260–420 (340)	350 – 400	272–580 x 288 –544 (386 x 379)	300–500 (420)
Maximum number of eggs in utero	250	100–140	70 – 100	4	8–390 (170)
Egg length	201–229 (214 ±7.2;30) **192–207 (197 ±5.1; 10)**	160–200 (180)	190	176–208 (200)	122–159 (0.138)
Egg width	113–134 (128 ±6.6; 30) **100–131 (115 ±9.3; 10)**	90–120 (100)	120	96–112 (101)	
Marginal hooklet 1	35				
Marginal hooklet 2 – 7	22				
Marginal hooklet 8	33				
Marginal hooklet 1 – 2		31 – 34 (33)	30		
Marginal hooklet 6 – 8		31			

a: From du Preez and Kok (1992a); b: From Euzet and Combes (1964); c: From Galien (1935) (left), Kulo and Kulo (1981) (middle) and Murith (1981b).

4.3.1.1.3 Site in host

Urinary bladder.

4.3.1.1.4 Level of infection

One of six frogs examined from Ambatolahy was infected with one mature and four juvenile parasites (prevalence 16.6%). One of four frogs examined from Ambohitantely was infected with seven mature parasites (prevalence 25%). The prevalence for the combined sample was 20% and the mean intensity 6.5.

4.3.1.1.5 Type material

The morphological description is based on six mature, ten oncomiracidia and four juvenile parasites. Three specimens were sexually mature (Holotype NMBP 578; Paratypes NMBP 579–580) and two immature (Paratypes NMBP 581–582). The holotype and paratypes NMBP 581 and NMBP 582 originated from Ambatolahy, Madagascar, while paratypes NMBP 579 and NMBP 580 originated from Andringita Massif, Madagascar. Juvenile parasites and oncomiracidia were used for marginal hook measurements and drawings. The type material was deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301, South Africa.

4.3.1.1.6 Voucher material

Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

4.3.1.1.7 Zoobank registration

The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48EB-9EA4-881D43956065. The life science identifier (LSID) of the new name *Metapolystoma vencesi* n. sp. Landman *et al.* is: urn:lsid:zoobank.org:act:680E18A9-64D6-40C9-87D9-1AC5F28A1030

4.3.1.1.8 Etymology

In recognition of Professor Miguel Vences, Technical University of Braunschweig, Germany, for his dedication to the study of Madagascan herpetofauna.

4.3.1.1.9 Description

Measurements reflected in Table 4.2. Body pyriform (Fig. 4.2), dorsoventrally flattened, widest section at 57% of total length from anterior end, body length 2.7 times greater than width.

Mouth sub-ventral, surrounded by false oral sucker. Posterior haptor 16% of body length, bearing three pairs of haptoral suckers of equal size. Marginal hooklets placed as for other polystomes: pairs one and two between hamuli, pairs three to five embedded in suckers, pairs six to eight found between anterior suckers, pairs one and eight larger than pairs two to seven (Fig. 4.3a). Well-developed hamuli between posterior-most haptoral suckers without cut between handle and guard (Fig. 4.3b). Medial pharynx length greater than width, positioned immediately posterior or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx, 8% of total length from most anterior point, converging posteriorly at position of 80% of total length from most anterior point, extending into haptor; no prehaptoral anastomoses. Lateral diverticula length equal to width, found only in last third of intestine. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, positioned in a narrow band posterior to the ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 20–32 (27 ±5; 1) wide, 179 long, 2% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening on left ventral half, posterior to intestinal ceca bifurcation, 10% of total length from most anterior point. Genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing eight genital spines (Fig. 4.3c).

Ovary elongate, not lobed, positioned posterior to midbody, length 4.4 times greater than width, measuring 9% of body length. Oviduct 596 long, 29–53 (30 ±7; 1) wide. Uterus massive and wide, occupying most of body proper, tubiform, convolute, containing 367 ovoid, operculate eggs; some with fully developed oncomiracidia. Hatched intrauterine oncomiracidia present. Mehlis' gland obscure. Two vaginae 180–420 long, 16–31 wide, on lateral margins, bearing multiple marginal openings, vaginal vestibule elongate, positioned at 18% from anterior. Vitellaria extended throughout most of body and haptor, surrounds female reproductive organs. Genito-intestinal canal prominent, 580 long, 40–133 (62 ±28; 11) wide, situated posterior to ovary.

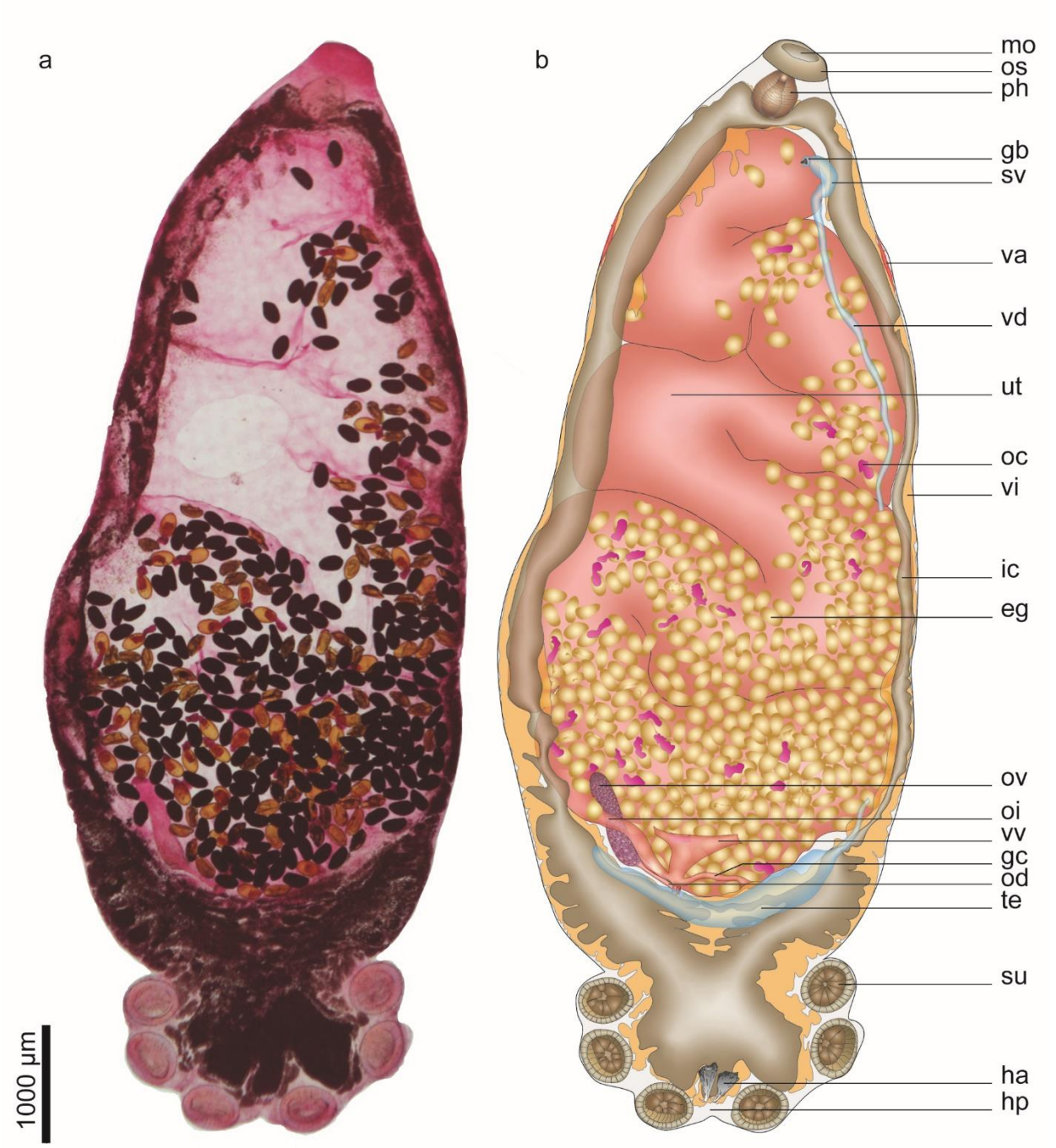


Figure 4.2 a–b: Ventral view of *Metapolystoma vencesi* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vd, vas deferens; vi, vitelline; vv, vitello–vaginal canal.

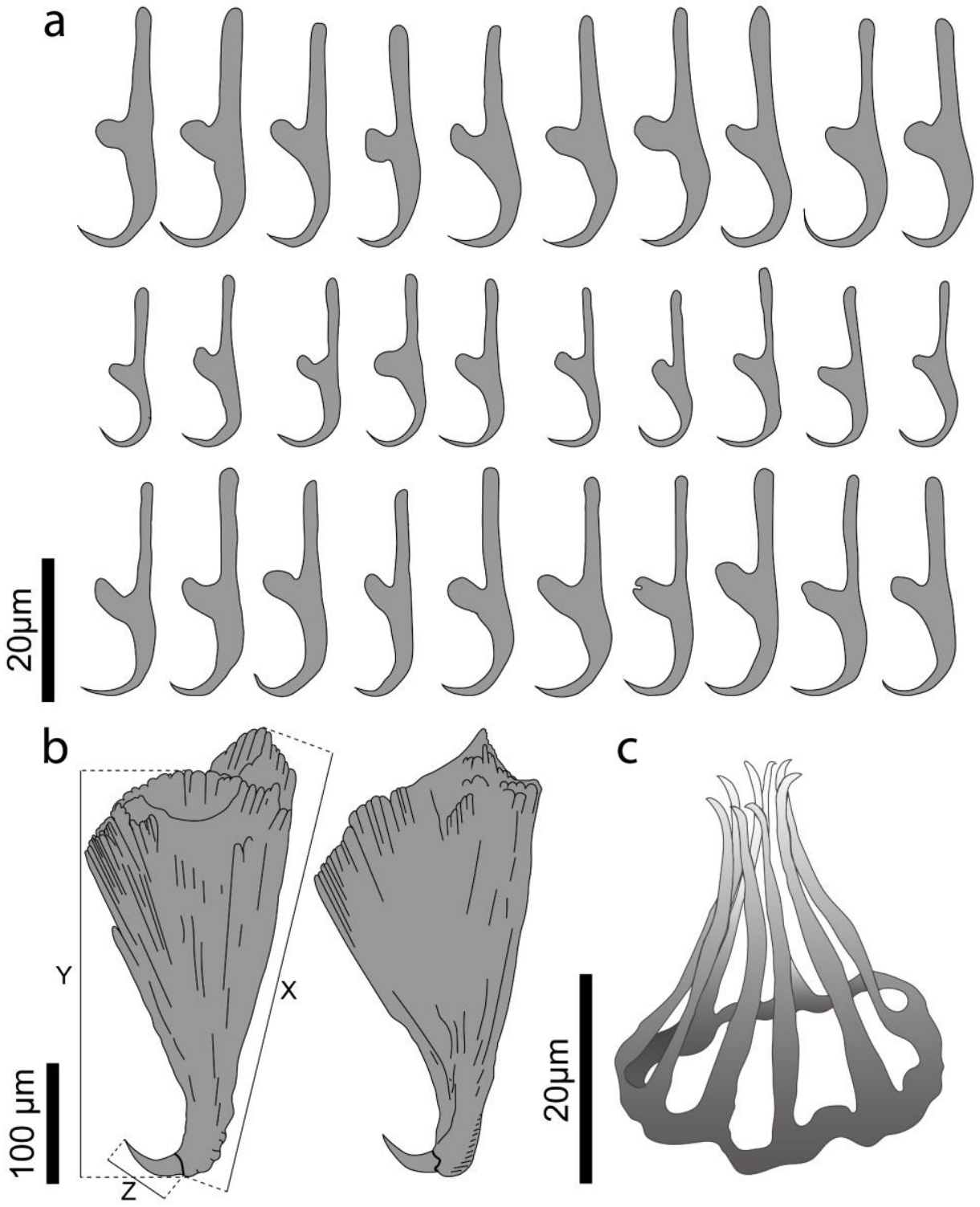


Figure 4.3: *Metapolystoma vencesi* n. sp. from *Boophis doulioti.* **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c**, genital crown from holotype. *Abbreviations*: X, outer length; Y, inner length; Z, hook length.

4.3.1.2. *Metapolystoma falcatum* n. sp. (Figs.4. 4–4.7; Table 4.2)

4.3.1.2.1 Type host

Boophis doulioti (Mantellidae).

4.3.1.2.2 Type locality

Ankarafantsika, Madagascar (Fig. 4.1), (16.115976S;47.095631E).

4.3.1.2.3 Site in host

Gills of tadpoles and urinary bladder of mature frogs.

4.3.1.2.4 Level of infection

Three of 22 frogs collected were infected. One mature and 13 juvenile parasites were recovered, with a maximum of 12 recovered from a single host (prevalence 13.6%. mean intensity 4.7). Seven of 10 tadpoles collected were infected with a total of 13 neotenic parasites, while as many as three parasites were infecting a single host (prevalence 70%; mean intensity 1.85). Though the term neotenic for some polystomes can be confusing (see Badets & Verneau, 2009), it refers here to egg producing parasites that were recovered from the gills of tadpoles.

4.3.1.2.5 Type material

Morphological descriptions are based on one mature parasite, eight juveniles and seven neotenics. One sexually mature specimen (Holotype NMBP 560), four immature bladder parasites (Paratypes NMBP 561–564) and four neotenics (Paratypes NMBP 565–568), all from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

4.3.1.2.6 Voucher material

Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

4.3.1.2.7 Zoobank registration

The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma falcatum* n. sp. Landman *et al.* is: urn:lsid:zoobank.org:act:FB1716E2-5C61-4C53-9A2F-BB364D6782A8

4.3.1.2.8 Etymology

The species epithet refers to the exceptionally long curved tips of marginal hooklets two to seven (*falcatum* = sickle shaped, curved, hooked, armed with scythes).

4.3.1.2.9 Description

Mature parasite (Figs. 4.4–4.5)

Measurements reflected in Table 4.2. Body pyriform (Fig. 4.4), dorsoventrally flat, widest section at 66% of total length from anterior end, body length 2.4 times greater than width. Mouth sub-ventral, surrounded by false oral sucker. Posterior haptor 19% of body length bearing three pairs of haptoral suckers equal in size. Marginal hooklets placed as for other polystomes and as described for *M. vencesi* n. sp., pairs one and eight larger than pairs two to seven (Fig. 4.5a). Well-developed hamuli between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 4.5b). Hamuli development presented in figure 4.5c. Medial pharynx length greater than width, positioned immediately posterior to or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 11% from anterior, converging posteriorly at 82% from anterior, stretching in between haptoral suckers; no prehaptoral anastomoses. Intestine bears lateral diverticula, length equal to width. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, only a small section visible, positioned directly posterior to the ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 23–40 (30 ±6; 1) wide, 301 long, measuring 4% of total length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation at 12% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing eight genital spines (Fig. 4.5d).

Ovary elongated, not lobed, positioned posterior to midbody, length four times greater than width,10% of body length. Oviduct 411 long, 22–48 (30 ±7; 1) wide. Uterus massive and narrow, occupying most of the space between intestinal caeca, tubiform, serpentines between oötype and genital bulb. Eggs operculated. Holotype released 171 eggs with only 11 remaining *in utero*. Some eggs contain fully developed oncomiracidia. Mehlis' gland distinct. Two parallel vaginae 262–235 long, 102 wide, situated on lateral margins, bearing multiple marginal openings. Vaginal vestibule cup-shaped, 21% from anterior. Vitellaria dorsal to intestinal tract, extends throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal canal prominent, 943 long, 10–46 (23 ±9; 1) wide, situated directly posterior to ovary.

Neotenic parasite (Figs. 4.6–4.7)

Measurements obtained from seven egg-producing neotenic parasites. Body pyriform (Fig. 4.6), dorsoventrally flat, ventrally concave, 1.419–3.307 (2.265 ±667; 7) long. Maximum width 904–1.376 (1,121 ±368; 3). Body length 2–5 (3 ±1; 6) times greater than width. Mouth 100–148 (124 ±17; 5) in diameter, sub- ventral, surrounded by false oral sucker. Posterior haptor 313–526 (411 ±89; 7) long, 541–1.166 (936 ±305; 7) wide. Haptor length-body length ratio 0.13–0.24 (0.2 ±0.04; 6), haptor bearing three pairs of haptoral suckers equal in size 103–226 (178 ±32; 47). Hamuli absent. Marginal hooklets placed as for *M. vencesi* n. sp., pairs one 28.2 long (Fig. 4.7a), larger than pairs two to seven 22.6–27.7 (25.5 ±1.5; 15), (Fig. 4.7b), pairs eight 27.5–35 (30.5 ±3.155; 8) long (Fig. 4.7c). Medial pharynx length 199–312 (244 ±45; 5) equal to width 207–312 (245 ±41; 5), positioned immediately posterior or at the margin of false oral sucker. Intestine bifurcates at distance 27–30% (30% ±2%; 3) from anterior, situated posterior to pharynx, converging posteriorly at 77–81% (80% ±2.7%; 3) from anterior, stretching into area between haptoral suckers; no prehaptoral anastomosis. Intestine with lateral diverticula, length greater than width. Diverticula in posterior half longer than anterior. Medial diverticula only posterior to ovary, length greater than width, narrower than lateral diverticula.

Testis follicular, though only a small section was visible, positioned directly posterior to vitello-vaginal canal, ventral to intestine. Vas deferens not visible. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation, at distance 42–51% (46% ±4%; 3) from anterior. Genital bulb diameter 31–51 (41 ±9; 4), muscular, surrounded by glandular cells, armed with genital crown bearing eight to eleven genital spines 7.9–11.8 (9.8 ±1; 10) long.

Ovary elongate, 256–580 (401 ±120; 7) long, 67–117 (91 ±19; 7) wide, not lobed, situated in middle of body, length three-six times greater than width , measuring 16–21% (17 ±2.18%; 6) of body length. Oviduct 15 long, 6–28 (13 ±6; 1) wide. Oötype 171–173 long, ovoid, containing a maximum of one ovoid, operculate egg 171–173 long, 132–142 wide. Uterus absent, eggs laid immediately after production. Mehlis' gland distinct. Vaginae absent. Vitellaria dorsal to intestinal tract, extended throughout most of body, except area occupied by female reproductive organs. Genito-intestinal canal prominent, 257 long, 7–47 (18 ±8; 1) wide, situated posterior to ovary.

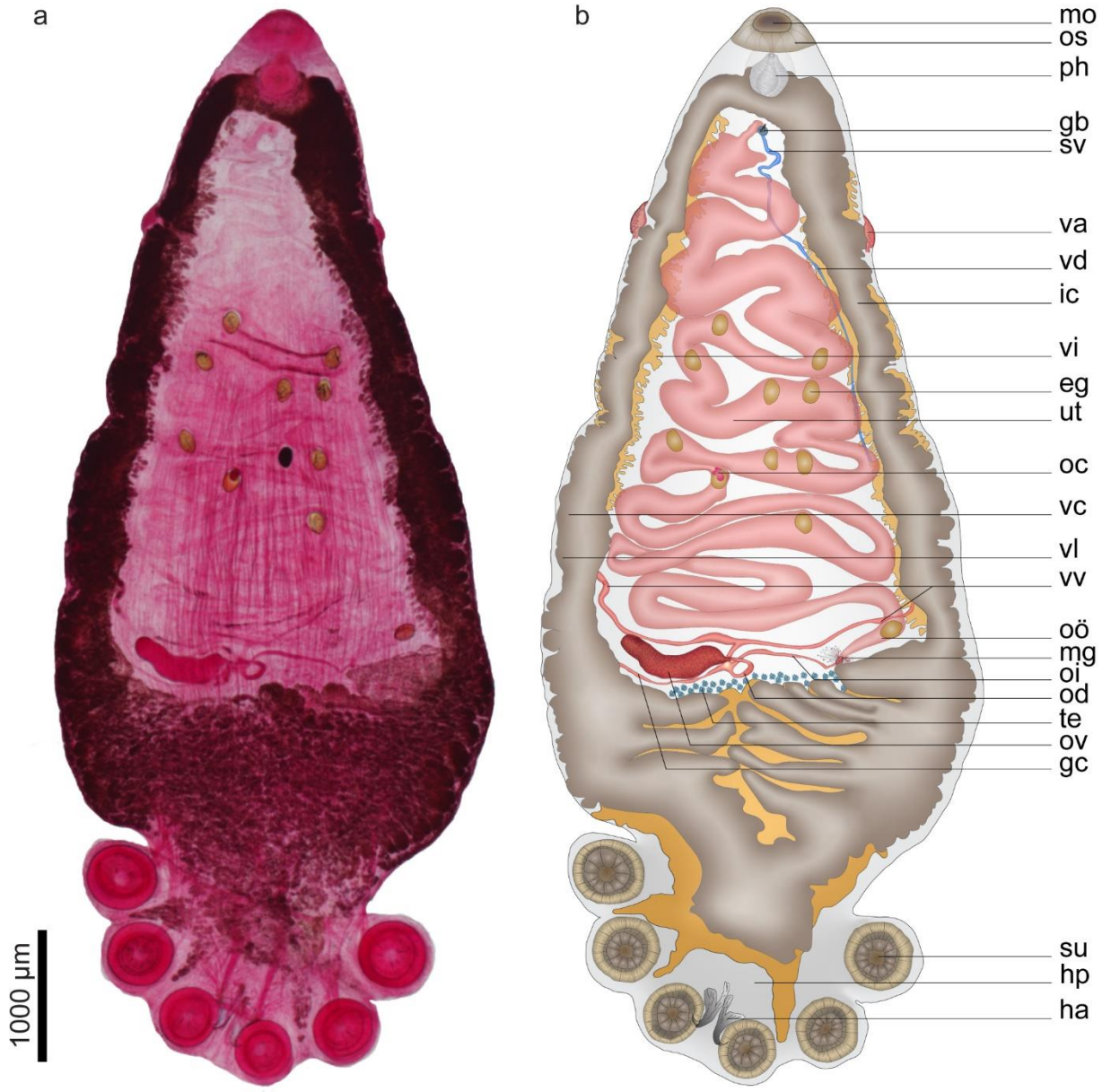


Figure 4.4 a–b: Ventral view of *Metapolystoma falcatum* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vc, vaginal canal; vd, vas deferens; vi, vitelline; vl, vitelline duct; vv, vitello–vaginal canal.

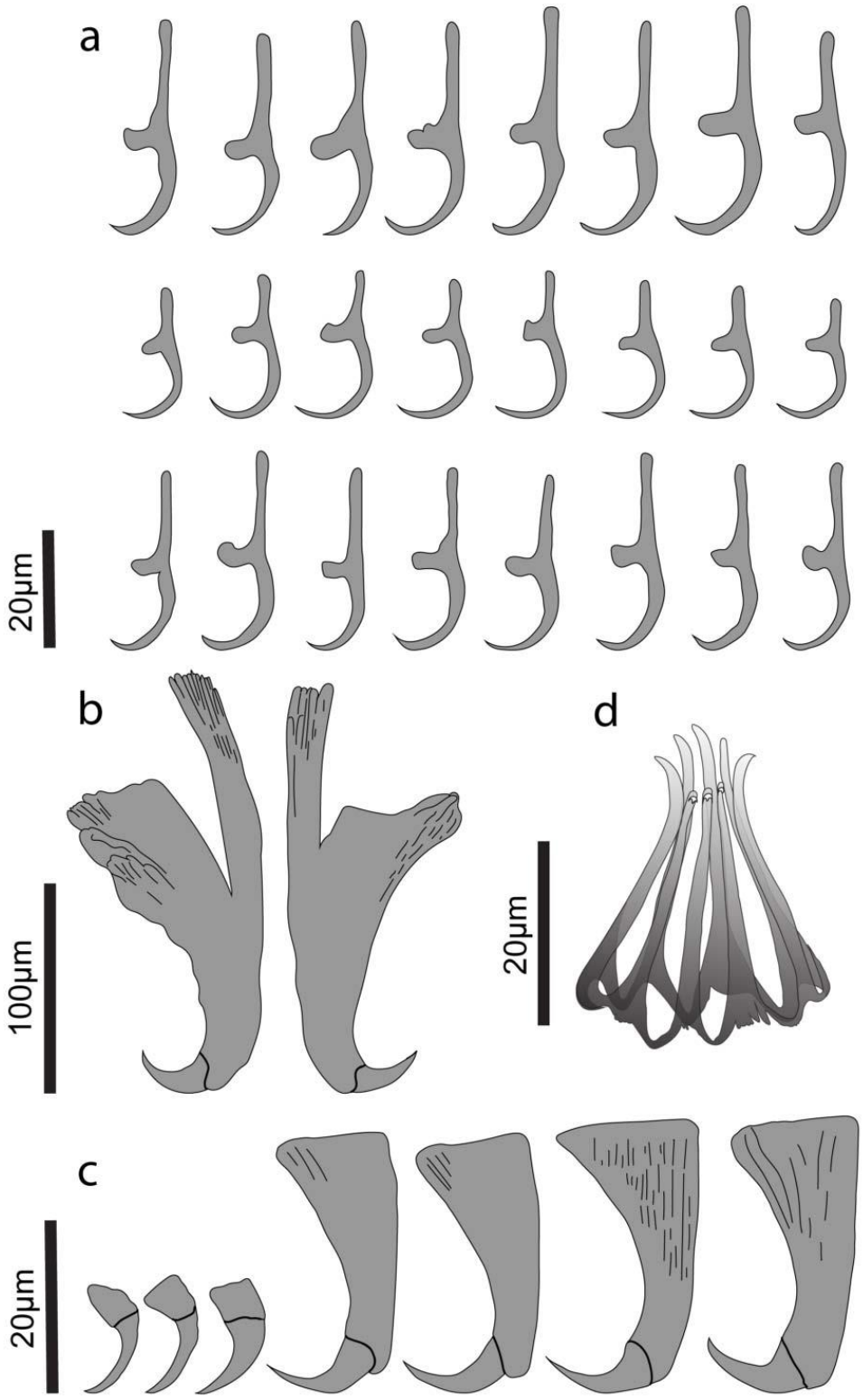


Figure 4.5: *Metapolystoma falcatum* n. sp. from *Boophis doulioti.* **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c**, hamulus development; **d**, genital crown from holotype.

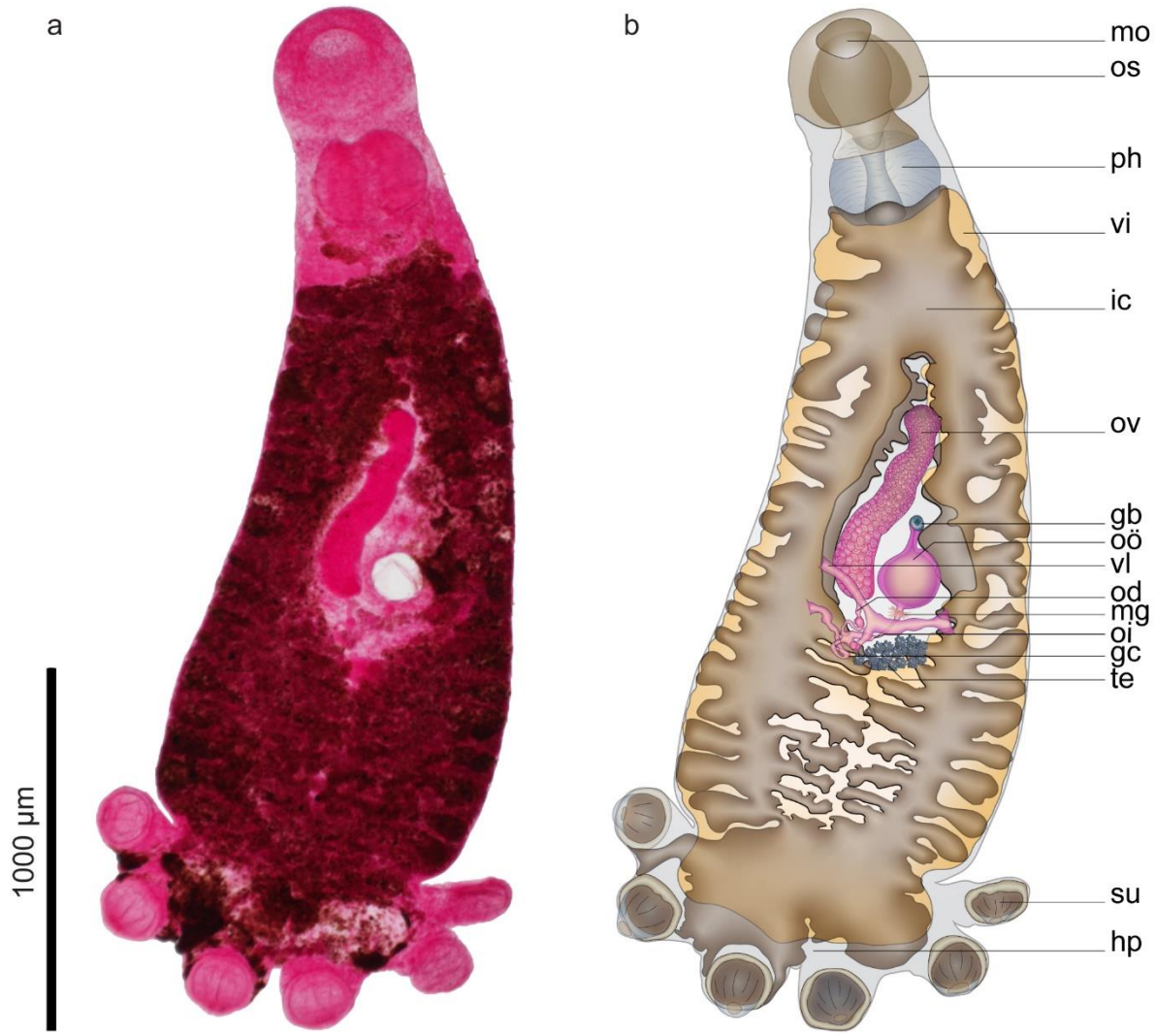


Figure 4.6 a–b: Ventral view of *Metapolystoma falcatum* n. sp. from *Boophis doulioti,* neotenic form. Abbreviations: gb, genital bulb; gc, genito–intestinal canal; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; te, testis; vi, vitelline; vl, vitelline duct.

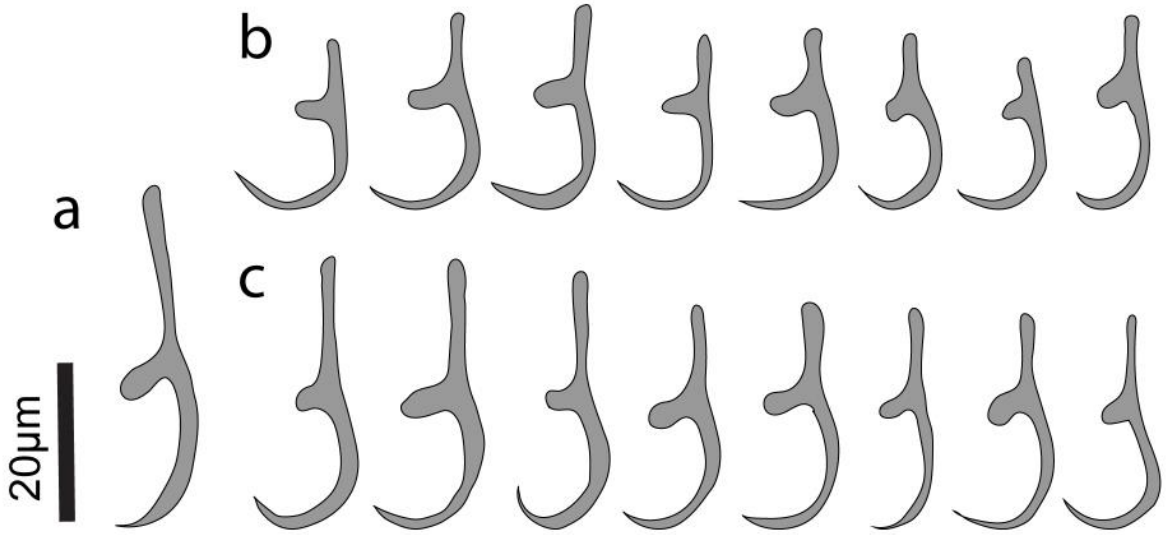


Figure 4.7: *Metapolystoma falcatum* n. sp. from *Boophis doulioti,* neotenic form*.* **a**, marginal hooklets 2–7; **b,** marginal hooklets 8; **c**, marginal hooklet 1.

4.3.1.3 *Metapolystoma ansuanum* n. sp. (Figs. 4.8–4.9; Table 4.2)

4.3.1.3.1 Type host

Boophis luteus (Mantellidae).

4.3.1.3.2 Type locality

Cascade des Nymphes, Isalo National Park, Madagascar (Fig. 4.1), (22.46977S;45.260701E)

4.3.1.3.3 Site in host

Urinary bladder.

4.3.1.3.4 Level of infection

One of nine frogs collected were infected with two mature parasites (prevalence 11.1%).

4.3.1.3.5 Type material

The morphological descriptions are based on two mature parasites (Holotype NMBP 569; Paratype NMBP 570) collected from the same locality in Cascade des Nymphes. The type material was deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301, South Africa.

4.3.1.3.6 Zoobank registration

The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma ansuanum* n. sp. Landman *et al.* is: urn:lsid:zoobank.org:act:4761BF6E-F309-4625-A7D7-CA64F3A3F6F8

4.3.1.3.7 Etymology

This species is named for Mrs Anna-Susan van der Linde, known as Ansu, in acknowledgement of her teaching and inspiration of many secondary school pupils in the field of biology.

4.3.1.3.8 Description

Measurements reflected in Table 4.2. Body pyriform (Fig. 4.8), dorsoventrally flat, ventrally concave, widest section at position 16–65% from anterior end of body. Body length 2.6–2.8 times greater than width. Mouth sub-ventral surrounded by false oral sucker. Posterior haptor measures 24% of total length, bearing three pairs of haptoral suckers of equal size. Well-developed hamuli positioned between posterior-most haptoral suckers with deep cut between handle and guard, hook length Z long relative to Length X (Fig. 4.9a). Marginal hooklets placed as for other polystomes: pairs one and two between hamuli, pairs three to five embedded in haptoral suckers, pairs six to eight positioned between anterior-most haptoral suckers (Fig. 4.9b). Medial pharynx length greater than width, positioned immediately posterior to or at margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 15–18% from anterior, converging posteriorly at 79–81% from anterior; no prehaptoral anastomoses. Lateral intestinal diverticula length equal to width in anterior half, length greater than width in posterior half. Medial diverticula only posterior to ovary.

Testis follicular, large, kidney-shaped, positioned posterior to ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 17–31 (26 ± 4; 1) wide, 422 long, measuring 11% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation at position 16–18% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing 10 genital spines (Fig. 4.9c).

Ovary, elongate, not lobed, positioned posterior to midbody, length 2.3–2.7 times greater than width, measuring 9–10% of body length. Oviduct 345 long, 19–39 (28 ±6; 1) wide. Uterus large, occupying one-third of body proper, tubiform, convolute. Uterus contains 41 ovoid, operculate eggs, some contain fully developed oncomiracidia. Intrauterine oncomiracidia present.

Mehlis' gland distinct. Two parallel vaginae, each 94–132 long (119 ± 18; 4), 66–81 (75 ±8; 4) wide, found on lateral margins, bearing multiple marginal openings; vaginal vestibule cup-shaped at 21–26% from anterior. Vitellaria dorsal to intestinal tract, extending throughout most of body, except area occupied by female reproductive organs. Genito-intestinal canal prominent, 264 long, 30–40 (37 ±4; 1) wide, situated directly posterior to ovary.

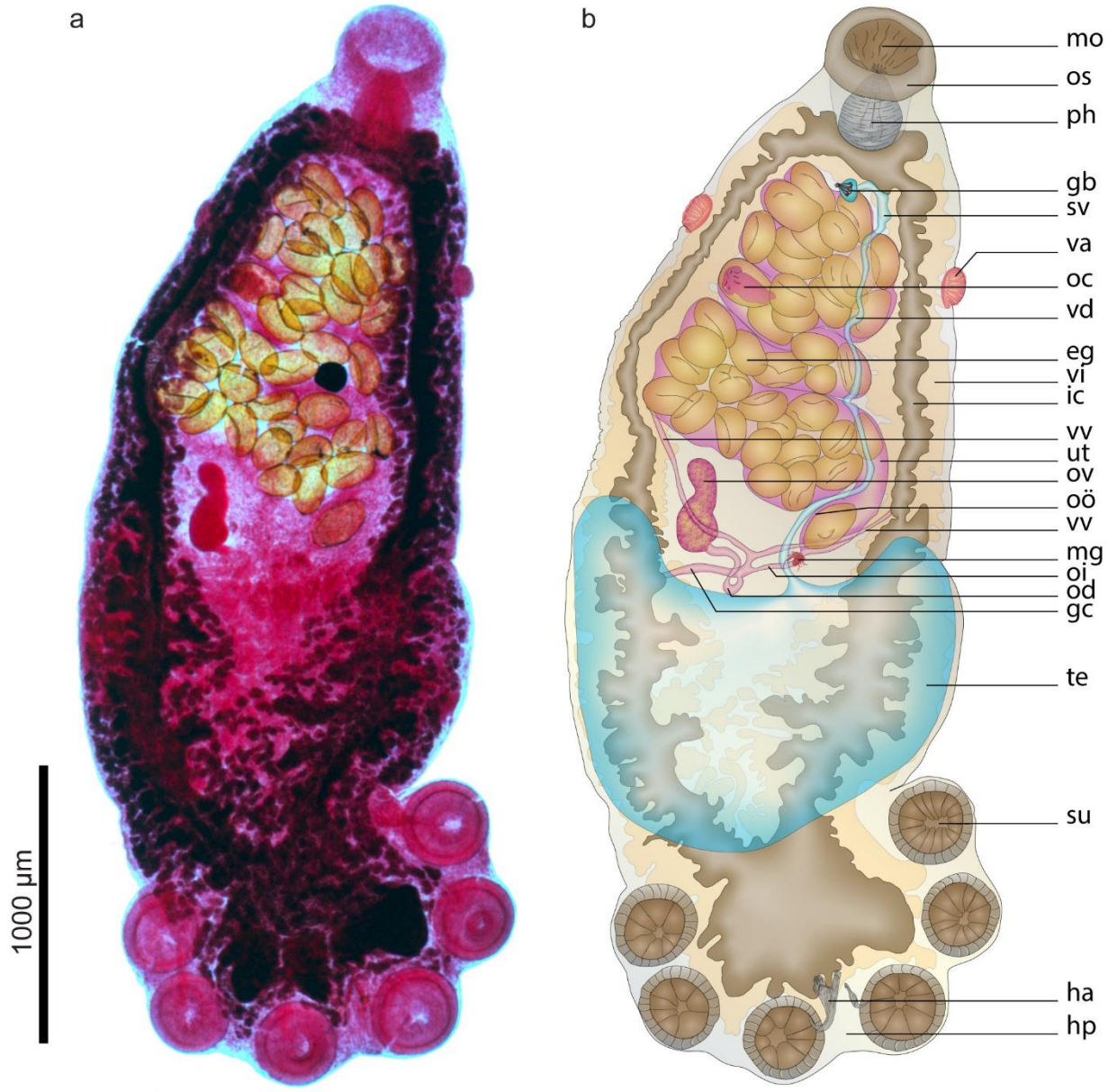


Figure 4.8 a–b: Ventral view of *Metapolystoma ansuanum* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vd, vas deferens; vi, vitelline; vv, vitello–vaginal canal.

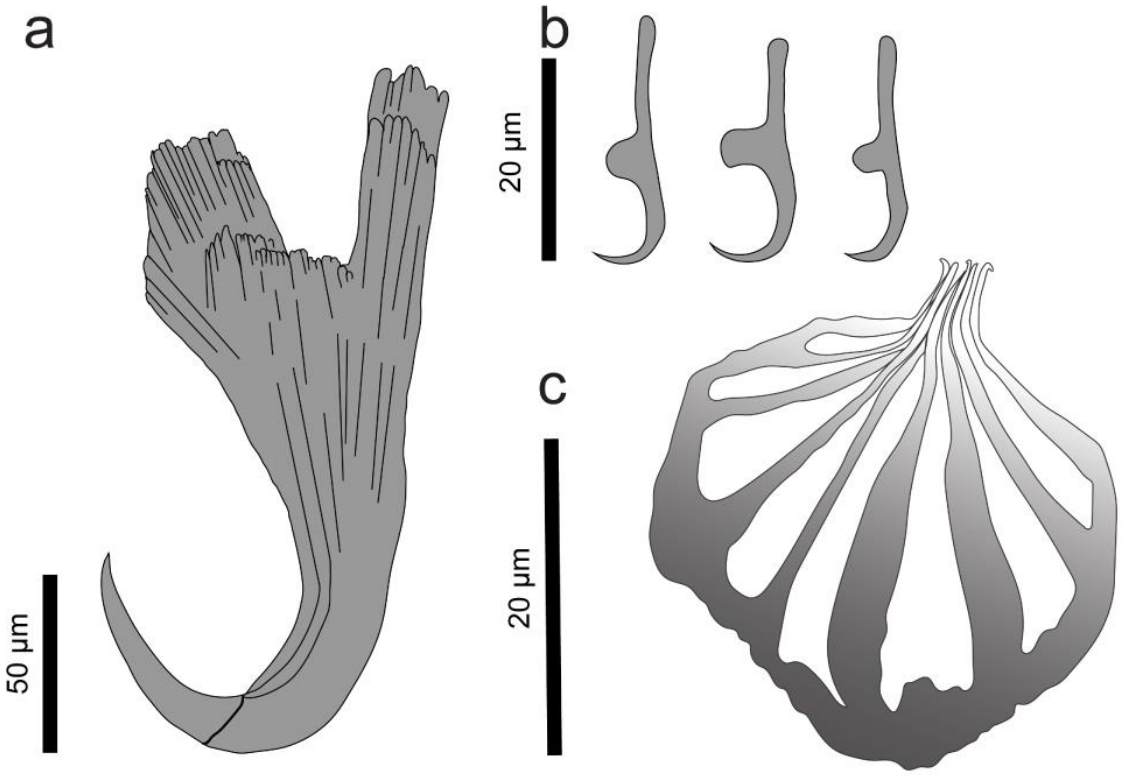


Figure 4.9: *Metapolystoma ansuanum* n. sp. from *Boophis luteus.* **a**, hamulus from holotype; **b**, marginal hooklets 2–7; **c**, genital crown from holotype.

4.3.1.4 *Metapolystoma theroni* n. sp. (Fig. 4.10–4.11; Table 4.2)

4.3.1.4.1 Type host

Boophis madagascariensis (Mantellidae).

4.3.1.4.2 Type locality

Indri Reserve in Andasibe, Madagascar (Fig. 4.1), (18.930856S;48.413611E).

4.3.1.4.3 Site in host

Urinary bladder.

4.3.1.4.4 Level of infection

Three of 30 frogs collected were infected with a total of one mature and 71 juvenile parasites, while as many as 40 parasites were infecting a single host (prevalence of 10%, mean intensity 24).

4.3.1.4.5 Type material

The morphological descriptions are based on one mature and 27 juvenile parasites. One sexually mature specimen (Holotype NMBP 573) and four immature ones (Paratypes NMBP 574–577), all from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

4.3.1.4.6 Voucher material

Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

4.3.1.4.7 Zoobank registration

The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma theroni* n. sp. Landman *et al.* is: urn:lsid:zoobank.org:act:55203AE0-217D-45D0-B3C8-07DDE53FA9A3

4.3.1.4.8 Etymology

This species is named in honour of emeritus Professor Pieter Daniel Theron at the North-West University, South Africa, in recognition of 54 years of inspiring teaching and dedication to the field of zoology.

4.3.1.4.9 Description

Measurements reflected in Table 4.2. Body pyriform (Fig. 4.10) dorsoventrally flat, widest section at 51% from anterior end, body length 2.6 times greater than width, mouth sub-ventral, surrounded by false oral sucker. Posterior haptor occupying 18% of total body length, bearing three pairs of haptoral suckers, equal in size. Marginal hooklets placed as for other polystomes, pairs one and two between hamuli, pairs three to five embedded in suckers, pairs six to eight in area between anterior-most suckers, pairs one and eight larger than pairs two to seven (Fig. 4.11a). Well-developed hamuli positioned between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 4.11b). Hamuli development presented in figure 4.11c. Medial pharynx length greater than width, positioned immediately posterior to or at margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 10% from anterior, converging posteriorly at 80% from anterior; no prehaptoral anastomoses. Lateral intestinal diverticula in first three quarters length equal to width, in last quarter length greater than width. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, u-shaped, mainly positioned posterior to the ovary with two lateral processes extending forward along the lateral line past the ovary up to one-third of the body proper, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 23–65 (46 ±18; 1) wide, 122 long, measuring 1% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation, positioned 13% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing seven genital spines (Fig. 4.11d).

Ovary elongate, not lobed, positioned posterior to midbody, length 1.2 times greater than width, measuring 5% of body length. Oviduct 1,167 long, 20–51 (33 ±9; 1) wide. Uterus massive, occupying 50% of body proper, tubiform, serpentines between posterior connection at oötype and anterior connection at genital bulb, containing 176 ovoid, operculate eggs, some contain fully developed oncomiracidia. Hatched intrauterine oncomiracidia present. Mehlis' gland distinct. Two parallel vaginae 270–304 long, 132–177 wide, on lateral margins, with multiple marginal openings, vaginal vestibule cup-shaped at 18% from anterior. Vitellaria dorsal to intestinal tract, extended throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal canal prominent 457 long, 30–77 (52±16;1) wide, situated posterior to ovary.

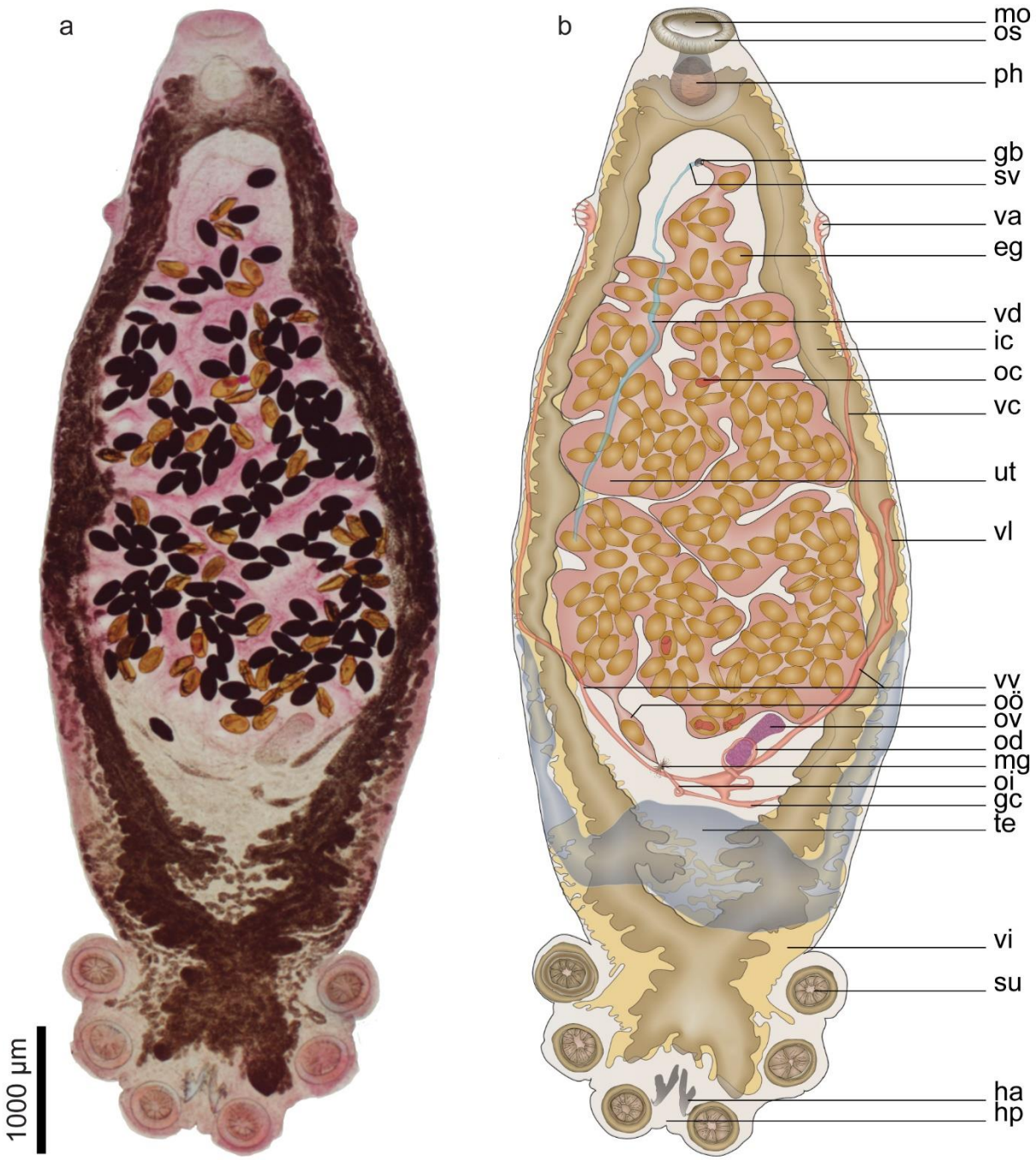


Figure 4.10 a–b: Ventral view of *Metapolystoma theroni* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vc, vaginal canal; vd, vas deferens; vi, vitelline; vl, vitelline duct; vv, vitello–vaginal canal.

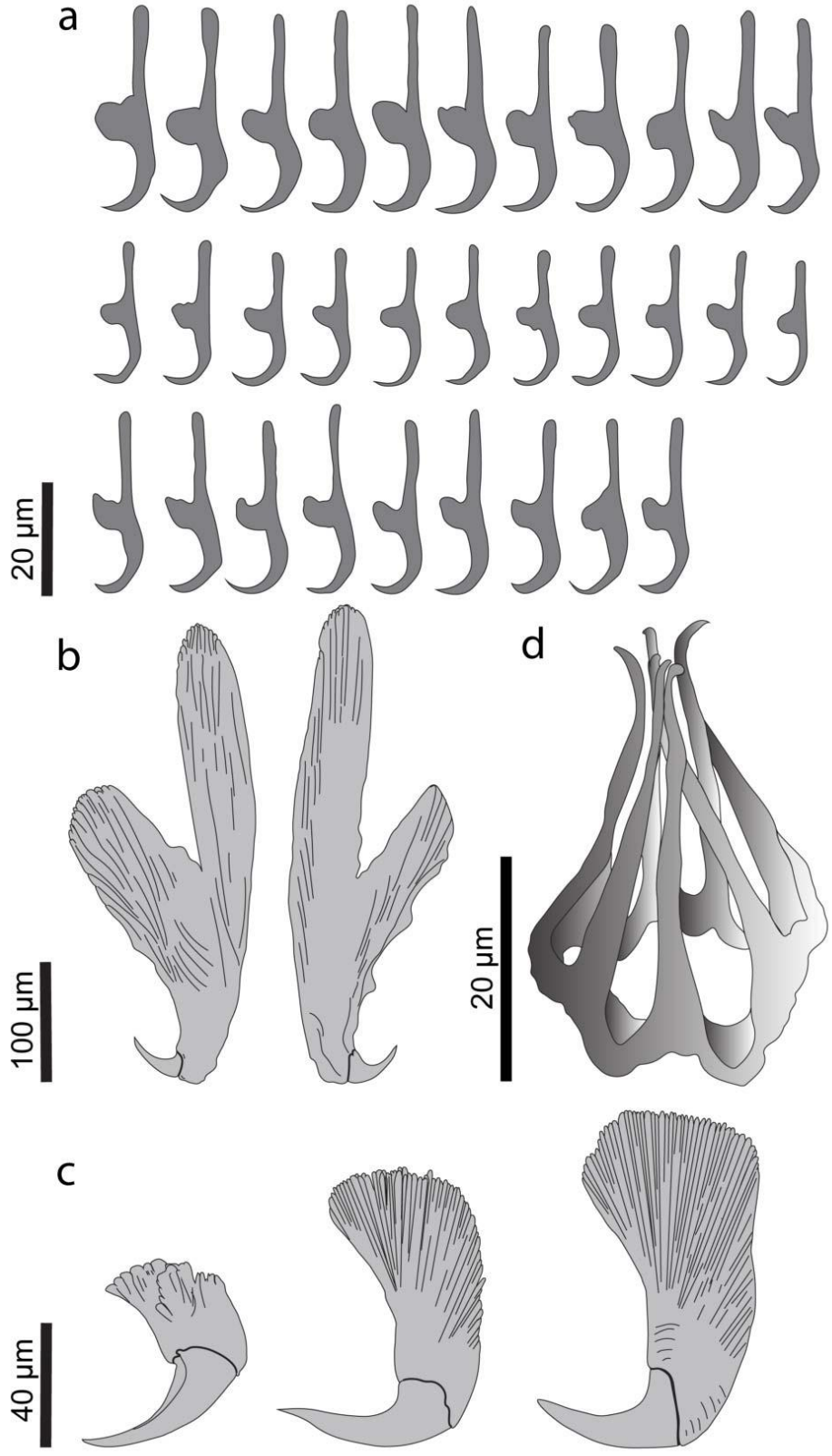


Figure 4.11: *Metapolystoma theroni* n. sp. from *Boophis madagascariensis.* **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c**, hamulus development.; **d**, genital crown from holotype.

4.3.1.5 *Metapolystoma multiova* n. sp. (Figs. 4.12–4.13; Table 4.2)

4.3.1.5.1 Type host

Boophis occidentalis (Mantellidae).

4.3.1.5.2 Type locality

Cascade des Nymphes, Isalo National Park, Madagascar (Fig. 4.1), (22.46977S;45.260701E).

4.3.1.5.3 Site in host

Urinary bladder.

4.3.1.5.4 Level of infection

One of five frogs collected were infected with two adult parasites (prevalence 20%).

4.3.1.5.5 Type material

The morphological descriptions are based on two mature parasites. Two sexually mature specimens (Holotype NMBP 571, Paratype NMBP 572), both from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

4.3.1.5.6 Zoobank registration

The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma multiov*a n. sp. Landman *et al.* is: urn:lsid:zoobank.org:act:6854087D-44B5-492B-9750-3647188F0F26

4.3.1.5.7 Etymology

The species epithet is derived from Latin and related to the vast number of eggs (±500) carried by this species in contrast with other currently known species in the genus (*multi* = many + *ova* = eggs).

4.3.1.5.8 Description

Measurements reflected in Table 4.2. Body pyriform (Fig. 4.12), dorsoventrally flat, ventrally concave, widest section at 50–57% from anterior end of body. Body length 2.2–2.3 times greater than width. Mouth directed sub-ventrally, surrounded by false oral sucker. Posterior haptor

measures 18% of total length bearing three pairs of haptoral suckers of equal size. Marginal hooklet pairs six to eight situated between anterior-most haptoral suckers, pairs one and eight larger than pairs two to seven, pairs one and two between hamuli, pairs three to five embedded in suckers (Fig. 4.13a). Well-developed hamuli situated between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 4.13b). Medial pharynx length greater than width, positioned immediately posterior to or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 13% from anterior, converging posteriorly at 79–85% from anterior; no prehaptoral anastomoses. Lateral intestinal diverticula situated in first third of intestine, where length is equal to width, absent in the second third while length is greater than width in the posterior third. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, sickle-shaped, posterior to ovary, ventral to intestine. Vas deferens obscured. Genital pore opening mid-ventrally situated on anterior margin of intestinal ceca bifurcation at 4% from anterior, genital bulb muscular, armed with genital crown bearing six genital spines (Fig. 4.13c).

Ovary elongate, not lobed, positioned posterior to midbody, length 2.8 times greater than width, measuring 8% of body length. Oviduct 988 long, 20–48 (31±9;1) wide. Uterus contains 336–499 ovoid, operculate eggs, some contain fully developed oncomiracidia, some hatched intrauterine oncomiracidia present. Mehlis' gland obscured. Two parallel vaginae, each 266–311 long, 58–62 wide, situated on lateral margins bearing multiple marginal openings. Vaginal vestibule cup-shaped, situated at 18% from anterior. Vitellaria dorsal to intestinal tract, extended throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal canal prominent 633 long, 7–103 (24 ± 24; 1) wide, situated at level of ovary.

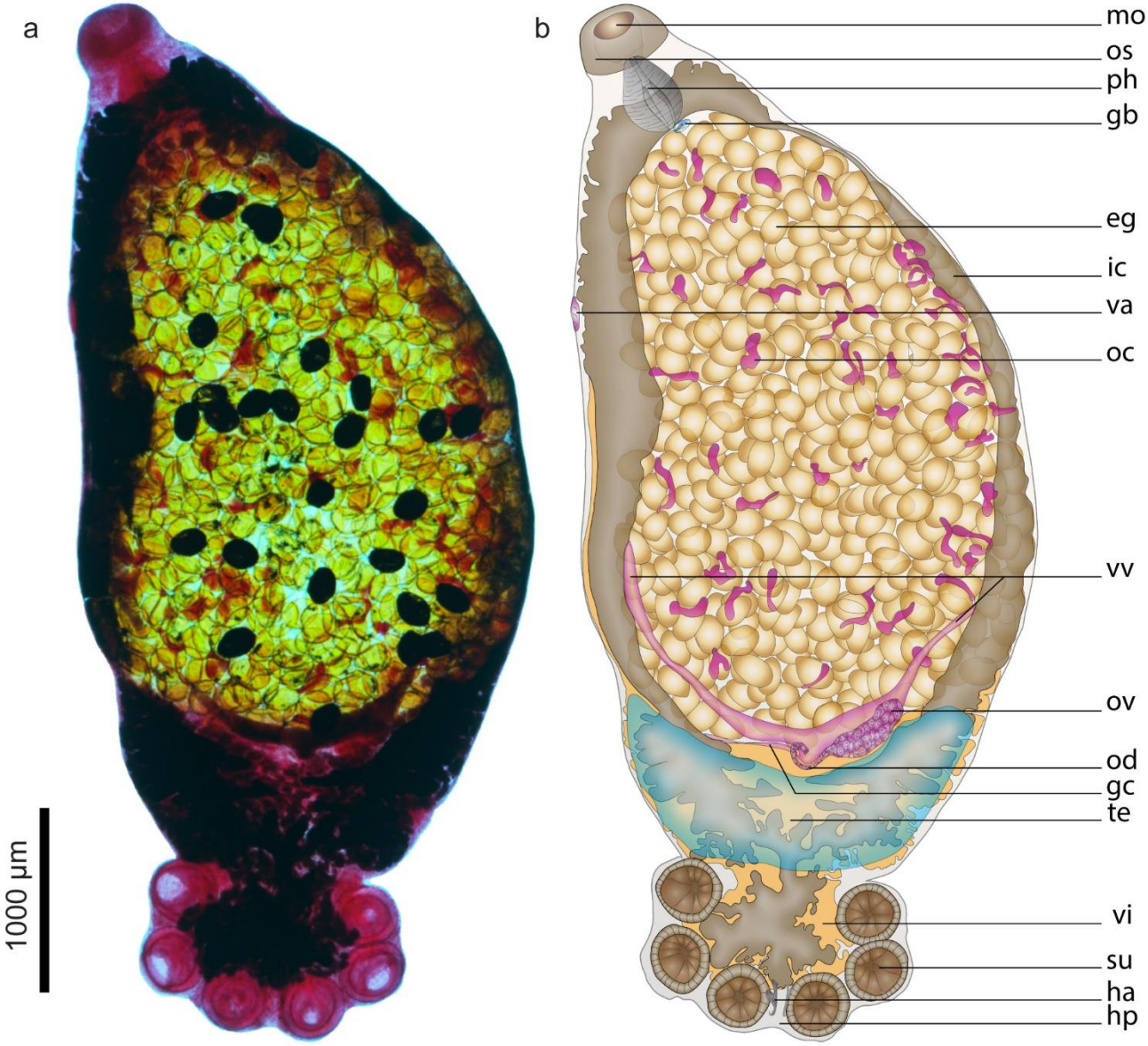


Figure 4.12 a–b: Ventral view of *Metapolystoma multiova* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mo, mouth; oc, oncomiracidium; od, oviduct; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; te, testis; va, vagina; vi, vitelline; vv, vitello–vaginal canal.

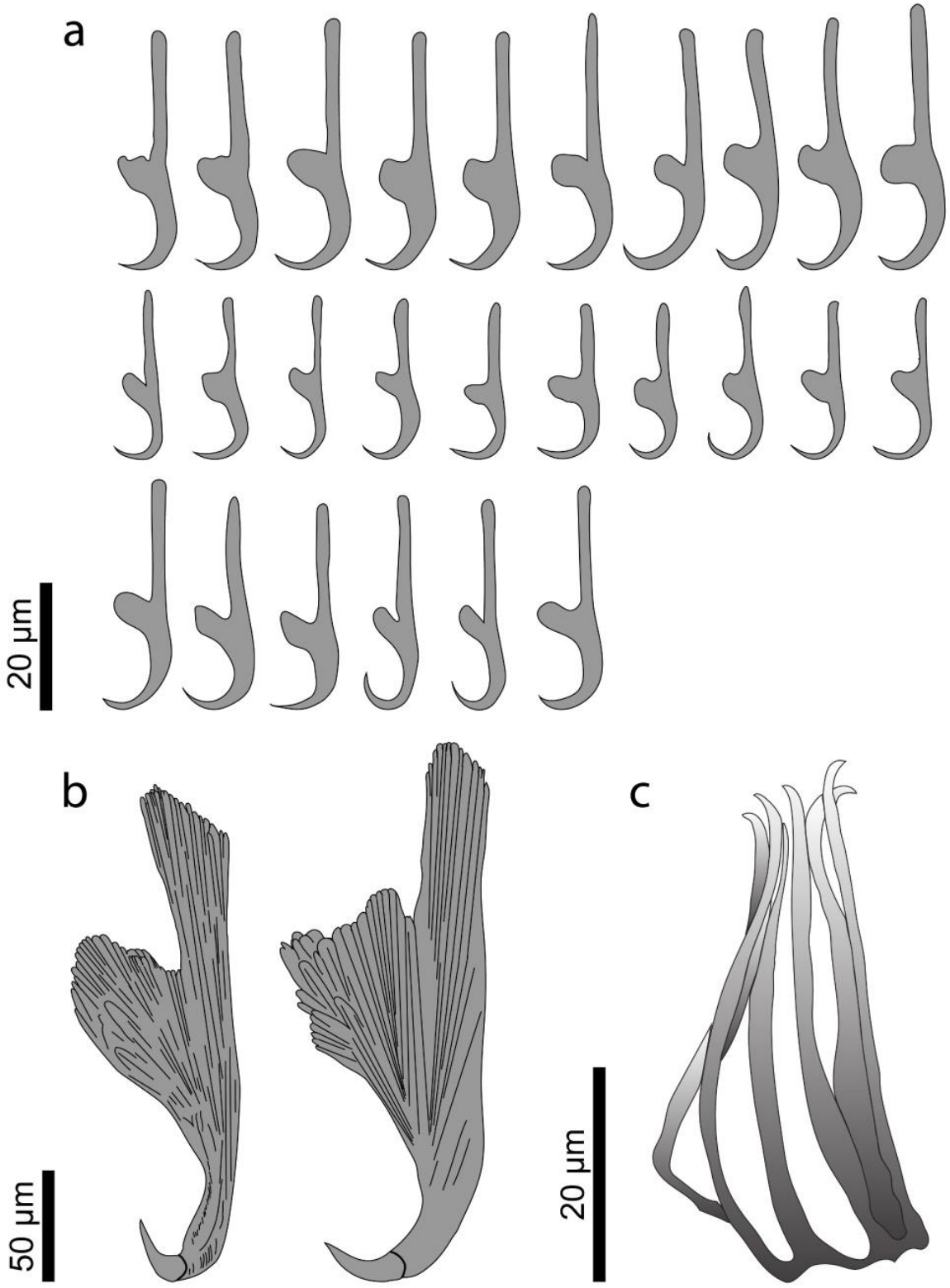


Figure 4.13: *Metapolystoma multiova* n. sp. from *Boophis occidentalis.* **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype (left) and from paratype; **c**, genital crown from holotype.

4.3.2 Genetic divergences between *Metapolystoma* species and parasite phylogeny

Some of the 18S, 28S and COI sequences used in this study were retrieved from GenBank, while the others were obtained and submitted under accession numbers **MW053457**, **MW053458** and **MW054236** to **MW054249** (Table 4.3). The final alignment, which had resulted in 3.977 characters, contained 19 taxa, among which 13 specimens of *Metapolystoma* and six specimens characterizing distinct species of *Polystoma*.

Minimum and maximum distances with standard deviation as estimated among *Metapolystoma* species were as follows. *M. falcatum* n. sp. (18S: 0.051%±0.0003–0.051 ±0.0005%; 28S: 0.42%±0.001–1.42%±0.002; COI: 8.6%±0.016–15.68%±0.023); *M. multiova* n. sp. (18S: 0–0.1%±0.0007); 28S: 0.28%±0.001–1.64%±0.003; COI: 12.54%±0.020–13.78%±0.022); *M. theroni* n. sp. (18S: 0–0.1%±0.0007; 28S: 0.28%±0.001–1.93%±0.003; COI: 11.4%±0.019–15.68%±0.023). With regard to 18S, total differences between species showed no character difference between *M. multiova* n. sp. and *M. theroni* n. sp. while the total 18S differences among all other *Metapolystoma* spp. ranged from 1 to 2 (Table 4.4). Regarding total 28S differences (Table 4.5), values ranged from 4 to 27 among all *Metapolystoma* spp., while the lowest value occurred between *M. multiova* n. sp. and *M. theroni* n. sp. Finally, regarding total COI differences (Table 4.6), values ranged from 25 to 45 between all *Metapolystoma* spp. Though no 18S character difference was detected between *M. multiova* n. sp. and *M. theroni* n. sp., the high levels of genetic divergence between *M. falcatum* n. sp., *M. multiova* n. sp., *M. theroni* n. sp., *M. brygoonis* and *M. cachani* (as estimated on the basis of 28S and COI sequences) led us to consider three new *Metapolystoma* species.

When 18S, 28S and COI sequences were combined for Bayesian analysis (Fig. 4.14), *M. cachani* appears as the most basal species within *Metapolystoma* and Malagasy *Metapolystoma* form a clade. Within this clade, *M. multiova* n. sp. and *M. theroni* n. sp. are sister species; two sublineages are well differentiated within *M. brygoonis.* Sublineage A comprises polystomes found in localities at Makira, Ranomafana and Vohiparara (Fig. 4.1), while sublineage B comprises polystomes of the Ambatolampy and Ankarafantsika areas (Fig. 4.1). Because high levels of 28S and COI genetic divergence (Tables 4.5 and 4.6) were also observed between these two sublineages (28S: 0.14%±0.0009; COI: 5.71%±0.011), it suggests that there could be two genetic entities within *M. brygoonis* infecting the same host species in Madagascar, that is, *Ptychadena mascareniensis.*

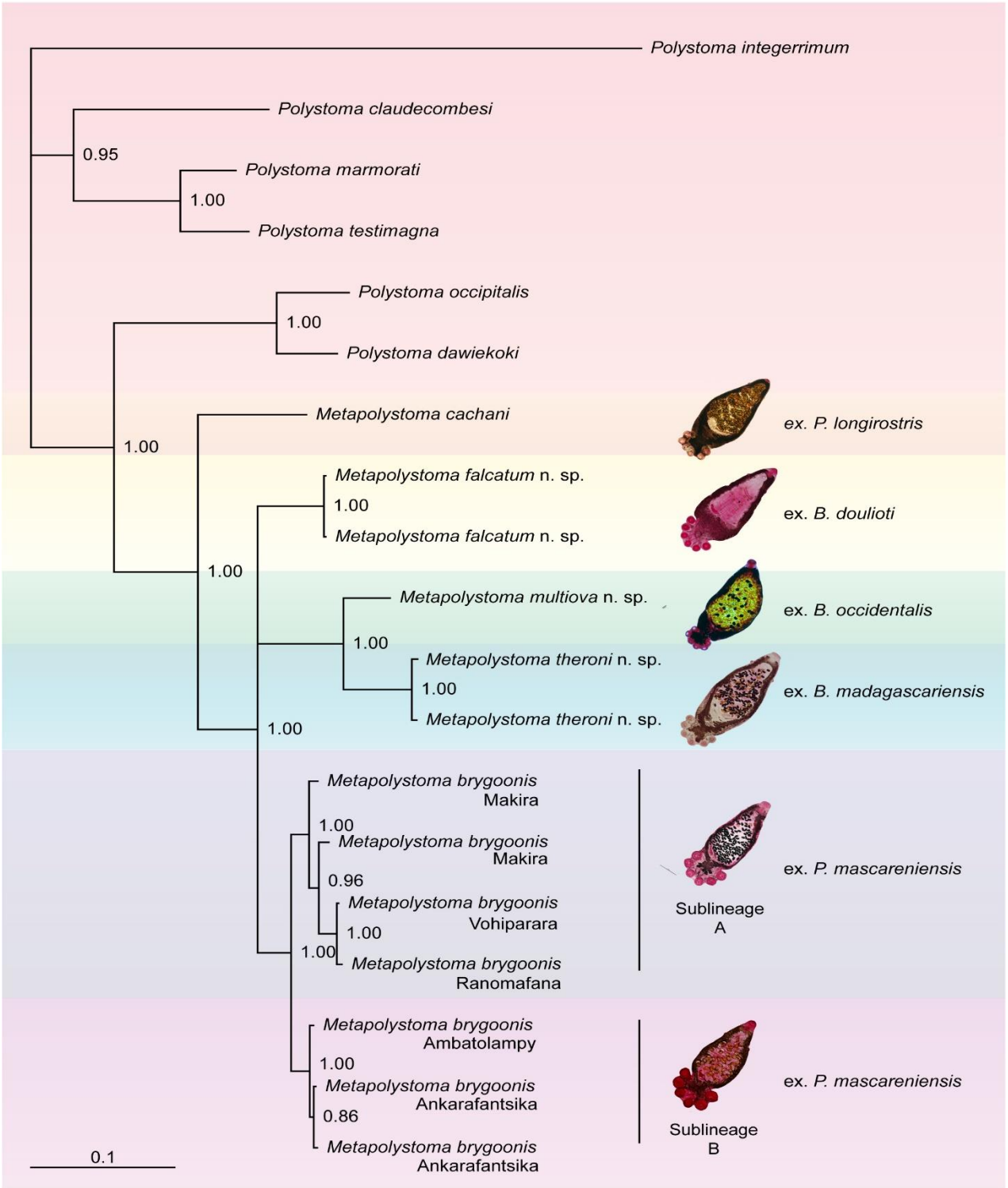


Figure 4.14: Bayesian tree inferred from the analysis of concatenated 18S, 28S and COI gene sequences. Node values indicate Bayesian posterior probabilities.

Table 4.3 Polystome species investigated, host species, locality and Genbank accession numbers for 18S, 28S and COI genes.

Polystome species	Host species	Locality	18S Accession number	28S Accession number	COI Accession number
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ambatolampy	**FM897287**[a]	**FM897270**[a]	**FM897300**[a]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ambatolampy	**MW054243**[g]	**JN800281**[b]	**MW053457**[g]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ankarafantsika	**MW054242**[g]	**MW054236**[g]	**JN800285**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Makira	**MW054246**[g]	**MW054239**[g]	**JN800288**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Makira	**MW054247**[g]	**MW054240**[g]	**JN800289**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ranomafana	**MW054245**[g]	**MW054238**[g]	**JN800286**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Vohiparara	**MW054244**[g]	**MW054237**[g]	**JN800287**[b]
Metapolystoma cachani	*Ptychadena longirostris*	Africa: Nigeria	**FM897280**[a]	**FM897262**[a]	**JN800294**[b]
Metapolystoma falcatum n. sp.	*Boophis doulioti*	Madagascar: Ankarafantsika	**MW054248**[g]	**JN800283**[b]	**JN800291**[b]
Metapolystoma falcatum n. sp.	*Boophis doulioti*	Madagascar: Ankarafantsika	**FM897286**[a]	**FM897269**[a]	**MW053458**[g]
Metapolystoma multiova n. sp.	*Boophis occidentalis*	Madagascar: Isalo	**FM897285**[a]	**FM897268**[a]	**FM897301**[a]
Metapolystoma theroni n. sp.	*Boophis madagascareniensis*	Madagascar: Andasibe	**FM897284**[a]	**FM897267**[a]	**FM897298**[a]
Metapolystoma theroni n. sp.	*Boophis madagascareniensis*	Madagascar: Andasibe	**MW054249**[g]	**MW054241**[g]	**JN800293**[b]
Polystoma claudecombesi	*Amietia delalandii*	South Africa	**FM897281**[a]	**FM897263**[a]	(—)
Polystoma dawiekoki	*Ptychadena anchietae*	South Africa	**AM051069**[c]	**AM157204**[d]	**AM913856**[e]
Polystoma integerrimum	*Rana temporaria*	France	**AM051071**[c]	**AM157206**[d]	**JF699306**[f]
Polystoma marmorati	*Hyperolius marmoratus*	South Africa	**AM051073**[c]	**AM157208**[d]	**AM913859**[e]
Polystoma occipitalis	*Hemisus marmoratus*	Ivory Coast	**AM051075**[c]	**FM897264**[a]	(—)
Polystoma testimagna	*Strongylopus fasciatus*	South Africa	**AM157194**[c]	**AM157217**[d]	**AM913860**[e]

[a] From Verneau *et al.* (2009a); [b] from Berthier and Verneau, unpublished; [c] from Bentz *et al.* (2006); [d] from Badets *et al.* (2011); [e] from Du Preez *et al.* (2007); [f] from Rahariviololoniaina *et al.* (2011); [g] Sequences reported in the present study; (—) Not available.

Table 4.4

Mean genetic distances (below diagonal) and total character differences (above diagonal) between species groups as inferred from comparisons of 18S rDNA sequences (1931 characters)

		1	2	3	4	5	6	7	8	9	10	11
1	*M. brygoonis*		1	2	2	2	11	10	13	10	9	25
2	*M. falcatum* n. sp. (*B. d.*)	0,001		1	1	1	10	9	12	9	8	24
3	*M. multiova* n. sp. (*B. o.*)	0,001	0,001		0	2	11	10	13	10	9	25
4	*M. theroni* n. sp.	0,001	0,001	0,000		2	11	10	13	10	9	25
5	*M. cachani*	0,001	0,001	0,001	0,001		9	8	11	10	9	23
6	*P. occipitalis*	0,006	0,005	0,006	0,006	0,005		6	16	15	14	28
7	*P. dawiekoki*	0,005	0,005	0,005	0,005	0,004	0,003		16	15	14	28
8	*P. claudecombesi*	0,007	0,006	0,007	0,007	0,006	0,008	0,008		11	10	24
9	*P. marmorati*	0,005	0,005	0,005	0,005	0,005	0,008	0,008	0,006		1	25
10	*P. testimagna*	0,005	0,004	0,005	0,005	0,005	0,007	0,007	0,005	0,001		24
11	*P. integerrimum*	0,013	0,013	0,013	0,013	0,012	0,015	0,015	0,013	0,013	0,0126	

Abbreviations: *B. d*. = *Boophis doulioti*; *B. o. = Boophis occidentalis*; *B. m. = Boophis madagascariensis*

Table 4.5

Mean genetic distances (below diagonal) and total character differences (above diagonal) between species groups as inferred from comparisons of 28S rDNA sequences (1418 characters)

		1	2	3	4	5	6	7	8	9	10	11	12
1	*M. brygoonis* (Vieilledent *et al.*)	—	2	8	13	15	20	29	29	37	38	37	58
2	*M. brygoonis* (SlB)	0,001	—	6	11	13	18	27	27	35	36	35	59
3	*M. falcatum* n. sp. (*B. d*.)	0,006	0,004	—	11	13	20	26	26	34	35	34	56
4	*M. multiova* n. sp. (*B. o.*)	0,009	0,008	0,008	—	4	23	32	32	40	39	38	62
5	*M. theroni* n. sp.	0,011	0,009	0,009	0,003	—	27	34	34	40	39	38	62
6	*M. cachani*	0,014	0,013	0,014	0,016	0,019	—	32	33	41	42	41	68
7	*P. occipitalis*	0,020	0,019	0,019	0,023	0,024	0,023	—	7	36	38	35	59
8	*P. dawiekoki*	0,021	0,019	0,019	0,023	0,024	0,024	0,005	—	35	35	32	59
9	*P. claudecombesi*	0,027	0,025	0,024	0,09	0,029	0,030	0,026	0,025	—	22	23	53
10	*P. marmorati*	0,027	0,026	0,025	0,028	0,028	0,030	0,027	0,025	0,016	—	5	53
11	*P. testimagna*	0,027	0,025	0,024	0,027	0,027	0,030	0,025	0,022	0,016	0,004	—	52
12	*P. integerrimum*	0,042	0,043	0,041	0,045	0,045	0,050	0,043	0,043	0,039	0,039	0,038	—

Abbreviations: SlA = sublineage A; SlB = sublineage B; *B. d. = Boophis doulioti*; *B. o. = Boophis occidentalis*; *B. m*. = *Boophis madagascariensis*.

Table 4.6

Mean genetic distances (below diagonal) and total character differences (above diagonal) between specimens of *Metapolystoma* species as inferred from comparisons of COI sequences (323 characters)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*M. brygoonis* (Ankarafantsika)	—	1	2	21	21	13	17	27	27	40	39	41	26	36	35	38	55
2	*M. brygoonis* (Ambatolampy)	0,003	—	3	20	20	12	16	26	26	39	38	40	25	35	34	37	55
3	*M. brygoonis* (Ambatolampy)	0,006	0,009	—	21	21	13	17	26	26	39	39	41	28	36	35	38	56
4	*M. brygoonis* (Vohiparara)}	0,068	0,065	0,068	—	1	13	11	30	30	38	37	39	30	43	40	41	58
5	*M. brygoonis* (Ranomafana)	0,068	0,065	0,068	0,003	—	13	12	30	30	38	36	39	29	43	40	41	57
6	*M. brygoonis* (Makira)	0,041	0,038	0,041	0,042	0,042	—	8	25	25	37	34	36	22	35	32	36	54
7	*M. brygoonis* (Makira)	0,055	0,051	0,055	0,035	0,038	0,025	—	30	30	38	36	38	29	40	37	40	55
8	*M. falcatum* n. sp. (*B. d.*)	0,089	0,086	0,086	0,100	0,099	0,082	0,100	—	0	38	43	45	32	38	33	37	60
9	*M. falcatum* n. sp. (*B. d.*)	0,089	0,086	0,086	0,100	0,099	0,082	0,100	0,000	—	38	43	45	32	38	33	37	60
10	*M. multiova* n. sp. (*B. o.*)	0,137	0,133	0,133	0,129	0,129	0,125	0,129	0,129	0,129	—	40	38	40	49	37	42	61
11	*M. theroni* n. sp.	0,133	0,129	0,133	0,125	0,121	0,114	0,121	0,149	0,149	0,138	—	4	36	48	41	45	64
12	*M. theroni* n. sp.	0,141	0,137	0,141	0,132	0,133	0,121	0,129	0,157	0,157	0,130	0,013	—	40	50	41	46	65
13	*M. cachani*	0,085	0,082	0,092	0,099	0,096	0,071	0,096	0,106	0,106	0,136	0,121	0,136	—	35	31	34	51
14	*P. dawiekoki*	0,121	0,117	0,121	0,147	0,147	0,117	0,135	0,128	0,128	0,170	0,166	0,174	0,117	—	38	42	57
15	*P. marmorati*	0,117	0,114	0,117	0,136	0,135	0,106	0,124	0,110	0,110	0,125	0,139	0,139	0,103	0,129	—	25	54
16	*P. testimagna*	0,128	0,124	0,128	0,139	0,139	0,121	0,135	0,124	0,124	0,143	0,155	0,158	0,113	0,143	0,082	—	53
17	*P. integerrimum*	0,193	0,193	0,198	0,205	0,201	0,189	0,193	0,214	0,214	0,218	0,231	0,235	0,177	0,202	0,189	0,185	—

Abbreviations: *B. d. = Boophis doulioti*; *B. o. = Boophis occidentalis*; *B. m*. = *Boophis madagascariensis*

4.3.3 Remarks

The phylogenetic position of *M. falcatum* n. sp., *M. multiova* n. sp. and *M. theroni* n. sp. within the Polystomatidae (Fig. 4.14) confirms that they are members of *Metapolystoma* (Polystomatidae). Genetic divergence estimates among *Metapolystoma* taxa (Tables 4.4, 4.5 and 4.6) sustain morphological descriptions, for these three species.

The lengths of *M. vencesi* n. sp. (9,190) and *M. theroni* n. sp. (9,086) differ from all other Malagasy metapolystomes, which vary in length from 2,680 to 6,710. *Metapolystoma theroni* n. sp. has a haptor length of 1,634, separating it from all other known Malagasy metapolystomes. Haptor length of *Metapolystoma falcatum* n. sp. (1,492) and *M. vencesi* n. sp. (1,437) overlap but differ substantially from *M. ansuanum* n. sp. (669–912), *M. brygoonis* (760–1,070) and *M. multiova* n. sp. (916). Haptor length-body length ratio of *M. vencesi* n. sp. (0.16) separates it from all other species while, in the overlapping cases of *Metapolystoma falcatum* n. sp. (0.19), *M. multiova* n. sp. (0.18) and *M. theroni* n. sp. (0.18), there is a marked difference from *M. ansuanum* n. sp. (0.23–0.25) and *M. brygoonis* (0.22–0.23), the latter two of which overlap in turn.

With a hamulus length X (Fig. 4.3b) of 269–332, *M. vencesi* n. sp. differs from all other known Malagasy metapolystomes, which range in length between 196 and 420. It is also the single species that has no separation between the hamulus handle and guard. *Metapolystoma brygoonis* (330–420), *M. falcatum* n. sp. (393–407) and *M. theroni* n. sp. (392–408) overlap but differ from *M. ansuanum* n. sp. (205) and *M. multiova* n. sp. (196–248), while the latter two also overlap. Hamulus hook length Z (Fig. 4.3b) separates all newly described metapolystomes from one another, ranging from *M. vencesi* n. sp. (40–52) with the smallest hooks to *M. falcatum* n. sp. (66–70) with the largest. *Metapolystoma ansuanum* n. sp. differs from all other metapolystomes in that it has an exceptional long hamulus hook relative to the rest of the hamulus and *M. falcatum* n. sp. differs from all others in that it has an exceptional long curved marginal hooklet tip on hooks two to seven.

With a genital bulb diameter of 115, *M. vencesi* n. sp. has the largest diameter ranging between 64 and 86. *M. brygoonis* (80), *M. falcatum* n. sp. (86), and *M. multiova* n. sp. (86) overlap but are separated from *M. ansuanum* n. sp. (64–73) and *M. theroni* n. sp. (65), while the latter two overlap. *Metapolystoma falcatum* n. sp. (26), *M. multiova* n. sp. (25), and *M. theroni* n. sp. (24–28) overlap in genital crown diameter but are separated from *M. ansuanum* n. sp. (29–33) and *M. vencesi* n. sp. (32), while the latter two overlap. *Metapolystoma brygoonis*, *M. falcatum* n. sp. and *M. vencesi* n. sp. all bear eight genital spines and differ from *M. ansuanum* n. sp., *M. multiova* n. sp. and *M. theroni* n. sp. that respectively bear ten, six and seven genital spines. With

the greatest genital spine length of 40.4–43.7 *M. multiova* n. sp. is separated from all other known Malagasy metapolystomes, except *M. brygoonis*, which ranges between 24.3 to 34.9.

Metapolystoma brygoonis (410–460), *M. multiova* n. sp. (446) and *M. theroni* n. sp. (479) overlap in ovary length but differ from *M. ansuanum* n. sp. (265–338), *M. falcatum* n. sp. (772) and *M. vencesi* n. sp. (861), the latter which also differ from one another. With the greatest egg length of 239–265, *M. theroni* n. sp. differs from all other Malagasy metapolystomes, which range from 160 to 230. *Metapolystoma ansuanum* (196–217), *M. multiova* n. sp. (198–228), and *M. vencesi* n. sp. (210–230) overlap but differ from *M. brygoonis* (160–200) and *M. falcatum* n. sp. (161–185), the latter two which overlap.

To conclude, due to the small number of parasite specimens that were investigated, we cannot exclude that the morphometric variability may represent intra specific variation. However, worms under investigation show a combination of morphological characters, namely shape of the hamulus handle and guard, shape of the hamulus hook, shape of the marginal hooklet tip on hooks and genital spine number that allow the differentiation of five distinct metapolystome species.

4.4 Discussion

Metapolystoma was until now reported only from the Afrotropical realm anuran hosts belonging to *Ptychadena* (Ptychadenidae). *Ptychadena* is a successful and widespread genus in Africa being a well-suited host for polystomes (Du Preez & Kok, 1992a). Of the 56 known *Ptychadena* species (Frost, 2020), 11 are known to host polystomes, including 14 *Polystoma* and three *Metapolystoma* species (see Du Preez & Kok, 1992a). According to Verneau *et al.*, (2009a), *Metapolystoma* may have originated in Africa within the time window 19.8 – 4.3 million years ago (Mya) from ancestors close to *Polystoma* and further dispersed to Madagascar following natural transoceanic dispersal of the ancestor of *P. mascareniensis* at about 14.2 – 2.3 Mya (Verneau *et al.*, 2009b). Because *M. brygoonis* forms a sister group to all other members of Malagasy *Metapolystoma*, *M. cachani* being basal within *Metapolystoma*, a host switch was suggested from the ancestor of *P. mascareniensis* to ancestral *Boophis* (Mantellidae) (Verneau *et al.*, 2009a), which is confirmed in the present phylogenetic study. Even though *Ptychadena* and *Boophis* are not phylogenetically closely related, they both display pleisiomorphic reproductive modes, favouring the possibility of an ancestral host switch in Madagascar (see Verneau *et al.*, 2009a), and ultimately the diversification of *Metapolystoma* within *Boophis*. While *Boophis* is not the only representative of the family Mantellidae infected by polystomes in Madagascar, mantellid frogs of the two genera *Mantella* and *Blommersia* which both exhibit a derived mode of reproduction, are infected by polystomes of another genus, i.e. *Madapolystoma* (see Du Preez *et al.*, 2010;

Landman *et al.*, 2018; Verneau *et al.*, 2009a). In addition, Verneau *et al.* (2009a) reported another undescribed species of *Metapolystoma* from *Aglyptodactylus madagascariensis* (Duméril, 1853). Therefore, the five new *Metapolystoma* species described along this study clearly indicate that a larger diversity of metapolystomes can be expected. This is especially true since Madagascar is inhabited by 79 *Boophis* species (Frost, 2020) that may serve as hosts for *Metapolystoma* species. Furthermore, *M. brygoonis* can be divided in two separate lineages according to genetic differentiation (Fig. 4.14). These results strengthen the fact that *Metapolystoma* in Madagascar still continue to diversify and call for further sampling and investigation.

The validity of *Metapolystoma* has long been disputed. It was suggested that the uterine structure was achieved convergently within *Metapolystoma* (Tinsley, 1974), through the ability of the parasite to adapt to the ecology of its host (Kok & Seaman, 1987; Murith, 1981b; Tinsley, 1983). Bentz *et al.* (2001) claimed it to be invalid and ascribed its morphological differences from *Polystoma* in terms of homoplastic characters. Nevertheless, our phylogeny supports the monophyly of *Metapolystoma*, which is, however, nested within the paraphyletic *Polystoma*. If the morphological differences between *Metapolystoma* and *Polystoma* were the product of reproductive plasticity, *Metapolystoma* spp. would not have clustered together on a molecular level (see Fig. 4.14). The fact that they do, therefore, suggests that the long uterus may have been inherited by descent. It is, however, intriguing to note that two polystome species *M. porosissimae* and *P. sodwanensis*, which display completely different life-history strategies, can occur simultaneously in the same host species, i.e. *Pt. porosissima* in Africa (du Preez & Kok, 1992b). *Metapolystoma porosissimae* displays a strategy where many eggs are stored in a large uterus, which is typical of polystomes that infects hosts within arid environments (Du Preez, 2015; du Preez & Kok, 1992b). Conversely, *P. sodwanensis* has a small uterus containing only a few eggs, which is in line with a water-dependent host (du Preez & Kok, 1992b). Even though morphological differences between the two species are distinct, the simultaneous occurrence of these two species in the same individuals of *Pt. porosissima* have reinforced the dispute (du Preez & Kok, 1992b). Though the validity of *Metapolystoma* at this stage cannot be ruled out, a more in-depth genetic investigation of the two African polystome species *M. porosissimae* and *P. sodwanensis* should help to conclude.

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CHAPTER 5:

METAPOLYSTOMA SP. (MONOGENEA: POLYSTOMATIDAE) FROM THE MADAGASCAN JUMPING FROG *AGLYPTODACTYLUS MADAGASCARIENSIS* (ANURA: LALIOSTOMINAE).



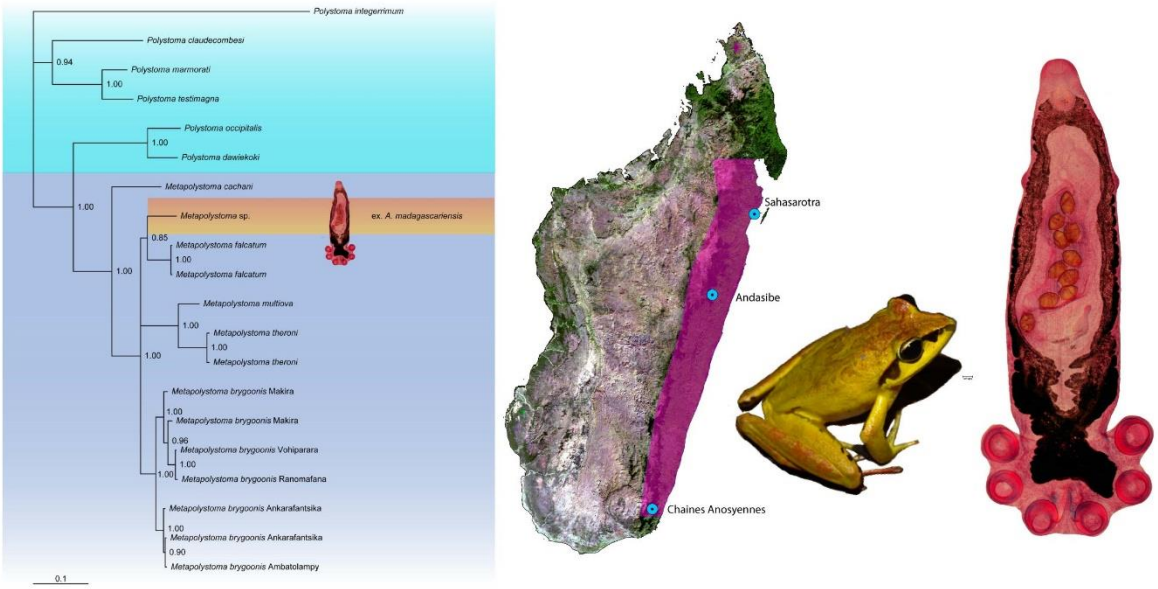
When we try to pick out anything by itself,
we find it hitched to everything else in the universe.
-John Muir

A modified version of this chapter has been submitted to Parasitology International. To comply with international nomenclature protocols, the species name has been replaced with sp.

Abstract

Despite Madagascar's high amphibian diversity of more than 350 species, only a few polystome species are known from the island's frogs. The dissection of preserved frogs from museum collections, together with amphibian and parasite surveys conducted in Madagascar led to the discovery of an undescribed polystome infecting *Aglyptodactylus madagascariensis* (Duméril, 1853). Morphological examination supported by phylogenetic analysis and genetic divergences, revealed distinct differences from all known metapolystomes, supporting the description of a new *Metapolystoma* species. The validity of *Metapolystoma* as a true taxon has been disputed by various authors. Differences were reported between the life cycles of *Metapolystoma* and *Polystoma*, providing additional supporting evidence for the validity of *Metapolystoma* as a taxon. *Metapolystoma* presents true ovoviviparity, while *Polystoma* only presents ovoviviparity in situations of incomplete egg expulsion. The position of *Metapolystoma* on published phylogenetic trees suggests that *Polystoma* might be a complex that requires further investigation. The need for parasite surveys in Madagascar was also emphasized and it was recommended that museum material be examined for polystomes to provide supplementary material and localities for further field investigation.

Keywords: *Aglyptodactylus,* life cycle, Madagascar, *Metapolystoma*, neotenic, polystome



5.1. Introduction

1. Introduction

The Polystomatidae is one of the most diverse families of Monogenea (Badets *et al.*, 2011) with 30 recognised genera and more than 200 species. Polystomes are known to infect amphibians, aquatic chelonians, hippopotami, and the Australian lungfish (Héritier *et al.*, 2015). Seventeen of the 30 polystome genera parasitise anurans and can be found on all continents, excluding only Antarctica and some islands (Du Preez *et al.*, 2008). Polystomes have co-evolved with their hosts and exhibit direct life cycles that led to extensive speciation (Héritier *et al.*, 2015; Hickman *et al.*, 2020; Tinsley & Earle, 1983). This co-evolution is especially evident in the reproductive cycle of polystomes, which is synchronised to that of both the reproductive cycle and behaviour of the host (Armstrong *et al.*, 1997; Cable *et al.*, 1998; Smyth & Smyth, 1980; Tinsley, 1999). *Metapolystoma* species, for instance, have different reproductive strategies. They are partly ovoviviparous where some eggs develop and hatch *in utero* to reinfect the same host alongside the parent (Murith *et al.*, 1977). Eggs that do not hatch *in utero* will hatch after being expelled from the host. The oncomiracidium will infect a host tadpole and partake in either a neotenic branchial or a bladder-destined branchial cycle (Murith *et al.*, 1977). A neotenic cycle follows when an oncomiracidium attaches to the gills of a tadpole in pre-metamorphosis (Combes, 1968; Galien, 1935; Williams, 1961). In such a case, the oncomiracidium will develop into a neotenic parasite and start producing eggs after two to three weeks. When the host metamorphose the parasite will detach and die (Combes, 1968; Galien, 1935; Williams, 1961). If an oncomiracidium attach to the gills of a tadpole in pro-metamorphosis, however, the parasite will develop slowly into a urinary bladder destined parasite (Badets & Verneau, 2009; Du Preez & Kok, 1999). Upon host metamorphosis, the young parasite leaves the branchial chamber and migrates over the host's abdomen to the cloaca, where it enters and migrates to the urinary bladder to mature and reproduce in synchrony with the host (Badets & Verneau, 2009; Du Preez & Kok, 1999).

Until recently, *Metapolystoma* (Monogenea: Polystomatidae) was only known from *Ptychadena* Boulenger, 1917 grass frogs found in Madagascar and the African continent. These include *Metapolystoma cachani* (Gallien, 1956) from *Ptychadena longirostris* (Peters, 1870) in West-Africa, *Metapolystoma porosissimae* Du Preez and Kok, 1992 from *P. porosissima* (Steindachner, 1867) in South Africa and *M. brygoonis* (Euzet and Combes, 1964) from *Ptychadena madagascariensis* (Duméril and Bibron, 1841) in Madagascar. Recently Landman *et al.* (2021) described the first record of metapolystomes from a host genus other than *Ptychadena*, namely, *Boophis* Tschudi, 1838 in the Mantellidae Günter, 1859. These include *M. ansuanum* Landman *et al.* 2021 from *Boophis luteus* (Boulenger, 1882), *Metapolystoma falcatum* Landman,

Verneau *et al.* 2021 from *Boophis doulioti* (Angel, 1934), *Metapolystoma multiova* Landman, Verneau *et al.* 2021 from *Boophis occidentalis* Glaw & Vences, 1994, *Metapolystoma theroni* Landman, Verneau *et al.* 2021 from *Boophis madagascariensis* (Peters, 1874), and *Metapolystoma vencesi* Landman, Verneau *et al.* 2021 from *Boophis albilabris* (Boulenger, 1888) in Madagascar. The validity of *Metapolystoma* as a true taxon has been disputed, by various authors (Bentz *et al.*, 2001; Kok & Seaman, 1987; Murith, 1981b; Tinsley, 1974; 1983), since its elevation from *Polystoma* Zeder, 1800 (see Landman *et al.*, 2021). Morphological separation between these genera are based on *Polystoma* having a small uterus with a low egg holding capacity in contrast to *Metapolystoma* that has a large uterus with a large egg holding capacity (Combes, 1976). Furthermore, the ovary of *Polystoma* is situated in the anterior half of the body, while that of *Metapolystoma* is situated posteriorly (Combes, 1976).

In February 2006, preserved material, provided by the Tsimbazaza Zoo, Madagascar was dissected for parasites. It was found to be infected with a *Metapolystoma* species. In May 2006, more preserved specimens of *Aglyptodactylus madagascariensis* (Duméril, 1853) were dissected for parasites at the Muséum National d' Historie Naturelle in Paris. During a herpetological survey in Madagascar (Fig. 5.1a), February 2008, more specimens of *A. madagascariensis*, (Laliostominae Vences and Glaw, 2001), (Fig. 5.1b) were investigated for *Metapolystoma* species and found to be infected. Genetic analysis by Verneau *et al.* (2009a) confirmed this metapolystome to be a new species. This paper formally describes this new *Metapolystoma* species from *A. madagascariensis*.

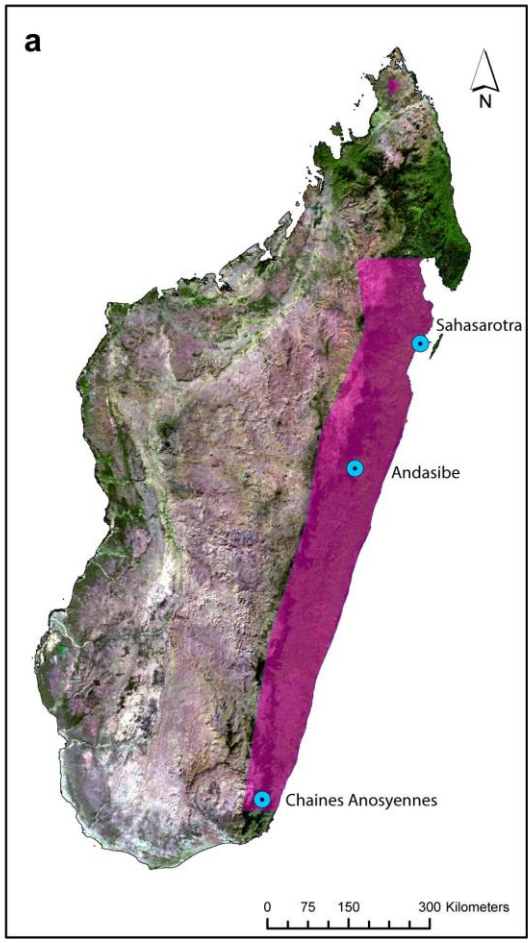




Figure 5.1: a) Map of Madagascar with parasite type localities and host distribution area. b) *Aglyptodactylus madagascariensis.*

5.2. Material and methods

5.2.1. Host and parasite sampling

In February 2006, formalin preserved specimens of *A. madagascariensis* were examined for polystomes at the Tsimbazaza zoo, Antananarivo, Madagascar. They had been collected in November 2005 from three localities, Besarika (five frogs), Mount Mandry Piore, and Sahasarotra (seven). More fixed specimens were examined for polystomes at the Muséum National d' Historie Naturelle in Paris in May 2006. These archived specimens were collected by P. Blane at Chaines Anosyennes, Madagascar (Fig. 5.1a) in November 1971. Thirty-three specimens were dissected and examined for polystomatid parasites. Recovered specimens were not suitable as whole mounts and were used for histological sections. Furthermore, in February 2008, 37 specimens of *A. madagascariensis* were collected by hand at Andasibe, Madagascar (Fig. 5.1a) around a forest pool following a rainfall event. These frogs were screened for the presence of polystome parasite eggs according to the protocol stated in Landman *et al.* (2021). Ethyl-3-aminobenzoate methanesulfonate (MS222) was used to anaesthetize and kill frogs that screened positive for polystome eggs. A Nikon SMZ-645 dissecting microscope (Nikon, Netherlands) was used to inspect the urinary bladder, accessory bladders, Wolffian ducts and kidneys for the presence of polystomatids. Bladders containing polystomatids were removed and transferred to glass cavity blocks containing 0.6% amphibian saline solution.

One specimen for molecular analysis was preserved in 100% ethanol. Adult specimens for morphological examination were fixed and preserved in 10% neutral buffered formalin (NBF) under coverslip pressure. Juvenile specimens for the investigation of marginal hooklets were mounted on microscope slides in ammonium picrate glycerine.

In preparation of whole mounts, specimens were hydrated, stained overnight in a weak acetocarmine solution, dehydrated in an alcohol series and cleared in xylene. Specimens were mounted using Canada balsam.

ArcMap 10.5.1 (Esri, California) was used to compile parasite and host distribution maps, shapefiles for frog distributions were provided by the IUCN (IUCN, 2020).

5.2.2. Phylogenetic and distance analysis

All DNA sequences used in this study were obtained from GenBank. GenBank accession numbers are listed in Landman *et al.* (2021), except for *Metapolystoma* sp. ex. *A. madagascariensis* (18S: FM897283, 28S: FM897266 and COI: FM897299) from Verneau *et al.* (2009a). Default parameters of ClustalW in Mega 7 (Kumar *et al.*, 2016) were used to edit and

align 18S, 28S and COI sequences independently (Thompson *et al.*, 1994). The final alignment comprised 20 taxa, containing 14 *Metapolystoma* and six distinct *Polystoma* species, with *Polystoma integerrimum* (Frölich, 1791) as outgroup. These sequences were concatenated in one alignment for Bayesian analysis. The COI genes were treated as three distinct partitions as determined by codon positions while 18S and 28S genes were treated as two separate partitions. For COI partitions one and two, six different gamma rates and substitutions were applied with four gamma rate categories. The third position was treated similarly, but with invariable-gamma rates. Implemented in Modeltest 3.06 (Posada & Crandall 1998), a two substitution rates model, was selected for 18S and a GTR + I model for 28S in accordance to the Akaike Information Criterion (AIC). Bayesian inference was executed in MrBayes 3.04b (Huelsenbeck & Ronquist, 2001). Four chains were run for ten million generations, sampling every 100 cycles. The first 10 000 trees (10%) were removed at the burn-in phase. Bayesian consensus trees were drawn and viewed with Tree View version 1.6 (Page, 1996). Evolutionary parameters for the five partitions were estimated separately. Corrected pairwise distances were calculated independently for the three genes in MEGA version 7 with the Kimura 2-parameter model and 1 000 bootstrap replicates (Kimura, 1980) and used for species delimitation.

5.2.3. Morphology and morphometry

A Zeiss Imager Axio10 compound microscope (Zeiss, Germany), fitted with a Zeiss Axio cam 305 camera (Zeiss, Germany) was used to investigate and photograph parasites. Taxonomic measurements were all taken in micrometres, using the Zeiss Zen Blue elements (Zeiss, Germany) software program. A Nikon AZ100M stereomicroscope (Nikon, Netherlands) was used to capture full-body micrographs of type specimens. Adobe Illustrator CC (Adobe, California) was used to prepare illustrations from micrographs. Eggs were measured in length (x) and width (y), plotted on a scatterplot, in Microsoft Excell ®, and encircled by a 100% confidence ellipse with the mean $(\bar{x};\bar{y})$ as pivot in order to discriminate between different species groups. Eggs were measured from the type series of *M. ansuanum*, *M. falcatum*, *M. multiova*, *M. porosissimae*, *M. theroni*, and *M. vencesi*. Egg measurements for *M. cachani* were obtained from Kulo and Kulo (1981). Eggs of *M. brygoonis* were measured from parasites in the polystome collection of the North-West University, Potchefstroom, South Africa, collected in September 1988 at Ranomafana, Madagascar. Marginal hooklets were measured according to the protocol of Du Preez and Maritz (2006) in order to discriminate between species groups.

5.2.4 Histology

Specimens retrieved from museum material that had been fixed in 10% neutral buffered formalin (NBF) were used for histological examination. Specimens were washed and dehydrated

in an ethanol series, impregnated with paraffin wax and embedded for sectioning (Fig. 5.2) using a Slee MPS histocene embedding machine (Slee, London, UK). Specimens were sectioned at 6 µm using a Reichert Jung motorised microtome (Reichert-Jung, Nosloch, Germany). Sections were stained with routine eosin and Harris' hematoxylin and mounted using Entellan mounting medium (Fig. 5.3) (Humason & Gretchen, 1962).

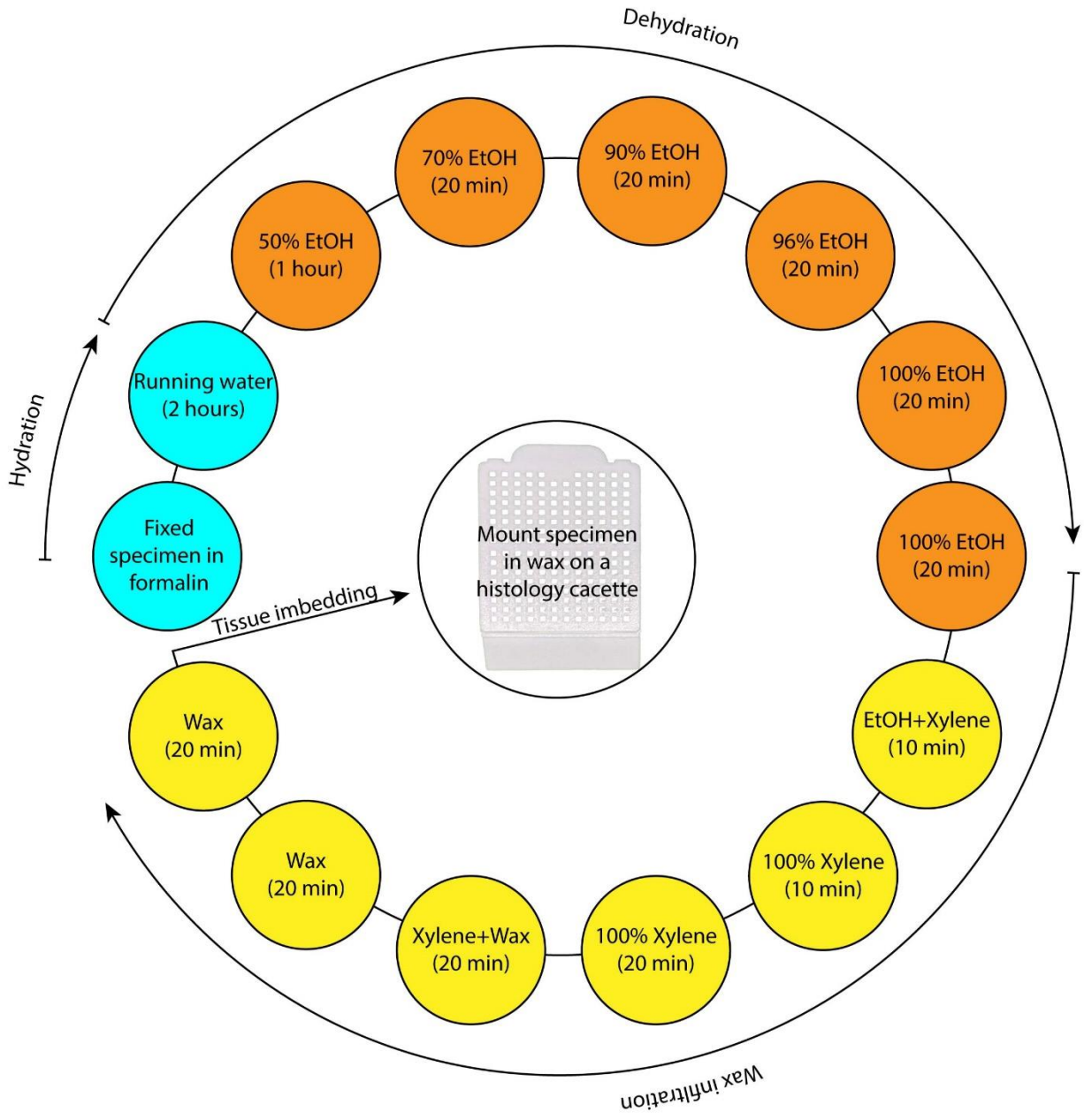


Figure 5.2: Procedure for tissue imbedding in paraffin wax for histology.

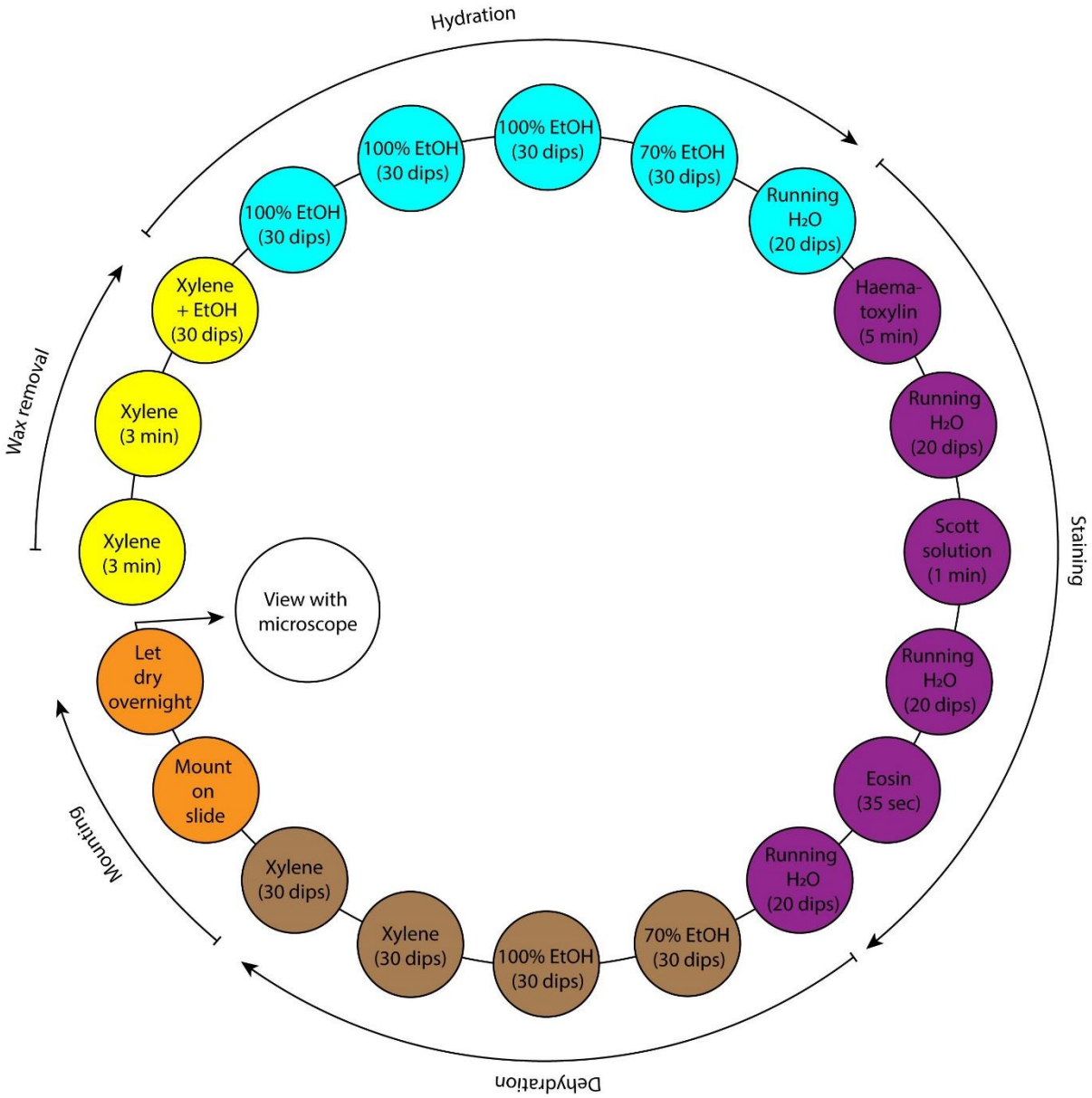


Figure 5.3: Procedure for the staining of histological sections with haematoxylin and eosin.

5.2.5 Sclerite isolation and scanning electron microscopy

Sclerotised hooks were isolated from juvenile parasites using enzyme digestion of soft tissue as described by Harris *et al.* (1999). A dilution of Proteinase K enzyme was used for the digestion. The solution was prepared by adding 160µl of a 2500 g/mg Proteinase K solution to 1840µl of digestion buffer. A 50-ml quantity of digestion buffer was made up with 0.454g (hydroxymethyl)-aminomethane hydrochloride (Tris-HCL), 0.186g ethylenediaminetetraacetic acid (EDTA) and 2.5g sodium dodecyl sulfate (SDS) dissolved in ultra-distilled water.

Haptors were removed from specimens using a scalpel blade and cut into smaller fragments. These fragments were rehydrated in distilled water and placed on a Millipore filter (pore size 0.5 µm). Specimens were then covered with the enzyme solution and incubated at 50ºC for 10-15 minutes. Subsequently, distilled water was forced through the filter with a syringe in order to rehydrate the specimen and remove excess salts and debris. Digestion and rehydration steps were repeated seven to eight times until all soft tissue was removed from the hooks. Filter papers with sclerite hooks were dried overnight in a desiccator prior to the SEM analysis.

Dried material was mounted on aluminium SEM stubs with carbon tape and sputter-coated for 90 seconds with gold palladium using a SPI-Module Sputter Coater (Spi Supplies: Westchester, PA, USA). SEM images were captured using a Phenom Pro Desktop SEM (Phenom-World BV., Eindhoven, Netherlands).

5.3. Results

5.3.1 Level of infection

Two of the seven frogs examined from Sahasarotra (Fig. 5.1) were infected with a total of sixteen mature and eight juvenile parasites, one of these frogs was infected with one mature, and eight juvenile parasites, and the other with 15 mature parasites. One of 33 frogs examined from Chaines Anosyennes was infected with nine adult parasites. Frogs collected from other localities were uninfected. Four of 37 frogs examined from Andasibe (Fig. 5.1) were infected with a total of four mature and 17 juvenile parasites (prevalence 10.8%, mean intensity 5.3). These frogs were all infected with parasites of two to three different sizes.

5.3.2 Phylogenetic relationship and genetic divergence

A concatenated Bayesian inferred phylogeny (Fig. 5.4) of 18S, 28S and COI sequences placed *Metapolystoma* sp. ex. *A. madagascariensis* in the *Metapolystoma* clade. Within this clade, *Metapolystoma* sp. ex. *A. madagascariensis* and *M. falcatum* are sister species. A high Bayesian posterior probability (Fig. 5.4), as well as exceeded 28S and COI species thresholds between *Metapolystoma* sp. ex. and other *Metapolystoma* spp., sustain its morphological description as a new species.

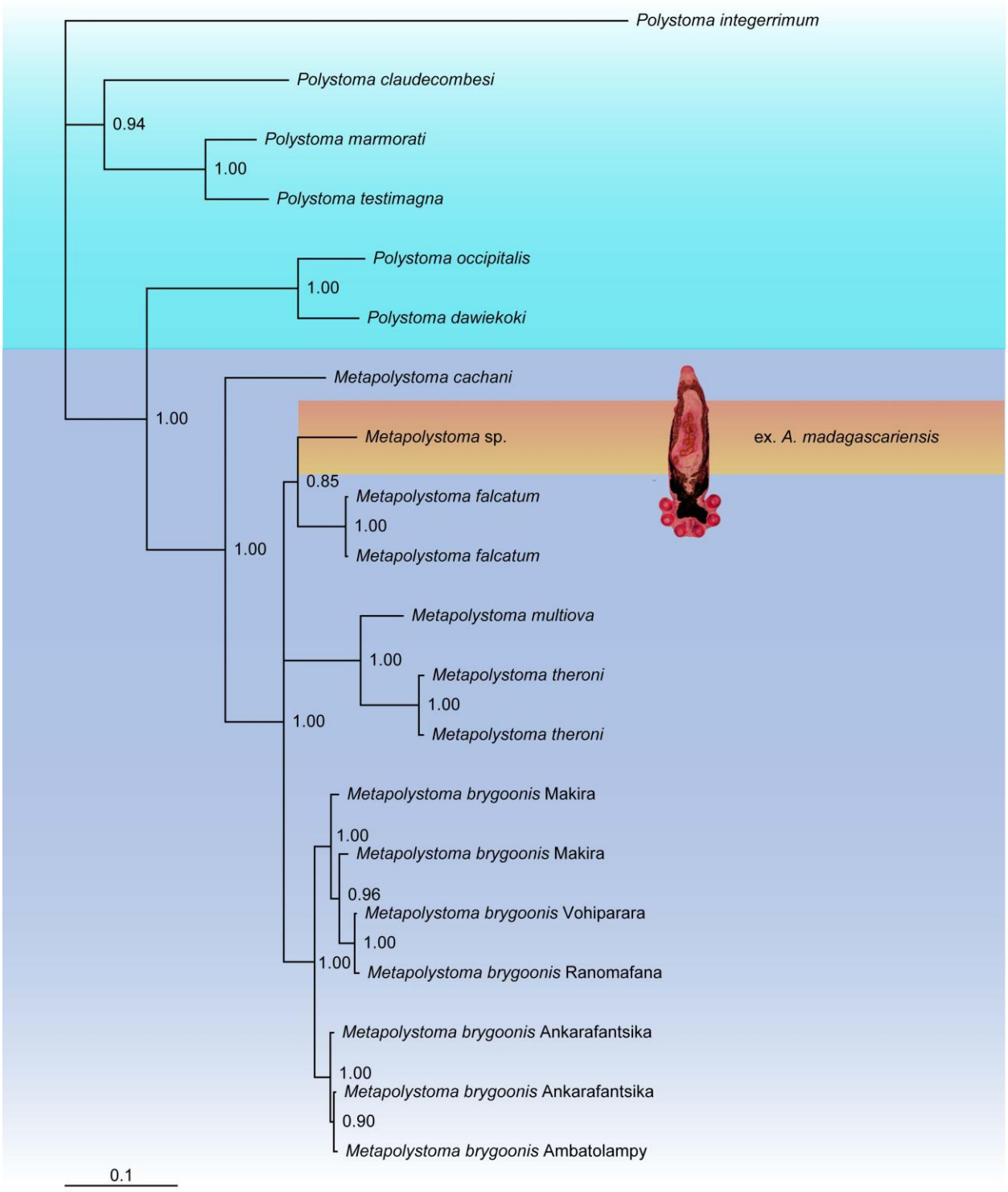


Figure 5.4: Bayesian tree inferred from concatenated 18S, 28S and COI gene sequences. Node values indicate Bayesian posterior probabilities.

The divergence estimates with standard deviations and total differences between *Metapolystoma* sp. ex. *A. madagascariensis* and *M. falcatum* are 0.052% ±0.001, 1 for 18S, 0.141% ±0.001, 2 for 28S and 9.372% ±0,018, 28 for COI. These differences exceed the species

level threshold of 0.07% for 28S and 2% for COI as demonstrated by Du Preez *et al.* (2007), suggesting that *Metapolystoma* sp. ex. *A. madagascariensis* is a new species.

5.3.3 *Metapolystoma* sp. (Fig. 5.5)

5.3.3.1 Taxonomic summary:

Class Monogenea van Beneden, 1858
Order Polystomatidea Lebedev, 1988
Family Polystomatidae Gamble, 1896
Genus *Metapolystoma* Combes, 1976

5.3.3.2 Type host:

Aglyptodactylus madagascareniensis (Duméril, 1853), Mantellidae (Fig. 5.1b).

5.3.3.3 Site in host:

Adults in urinary bladder, some juveniles in the Muller ducts and urinary bladder.

5.3.3.4 Type material:

Morphological descriptions based on 13 mature and 2 juvenile parasites (Holotype 583, Paratypes NMBP 584–586). The holotype (Fig. 5.5) and paratypes NMBP 585 and NMBP 586, originate from Andasibe, Madagascar, while paratype NMBP 584 originated from Sahasarotra, Madagascar. Type material deposited in the Parasitic Worm collection, National Museum, Aliwal Street, Bloemfontein 9301, South Africa.

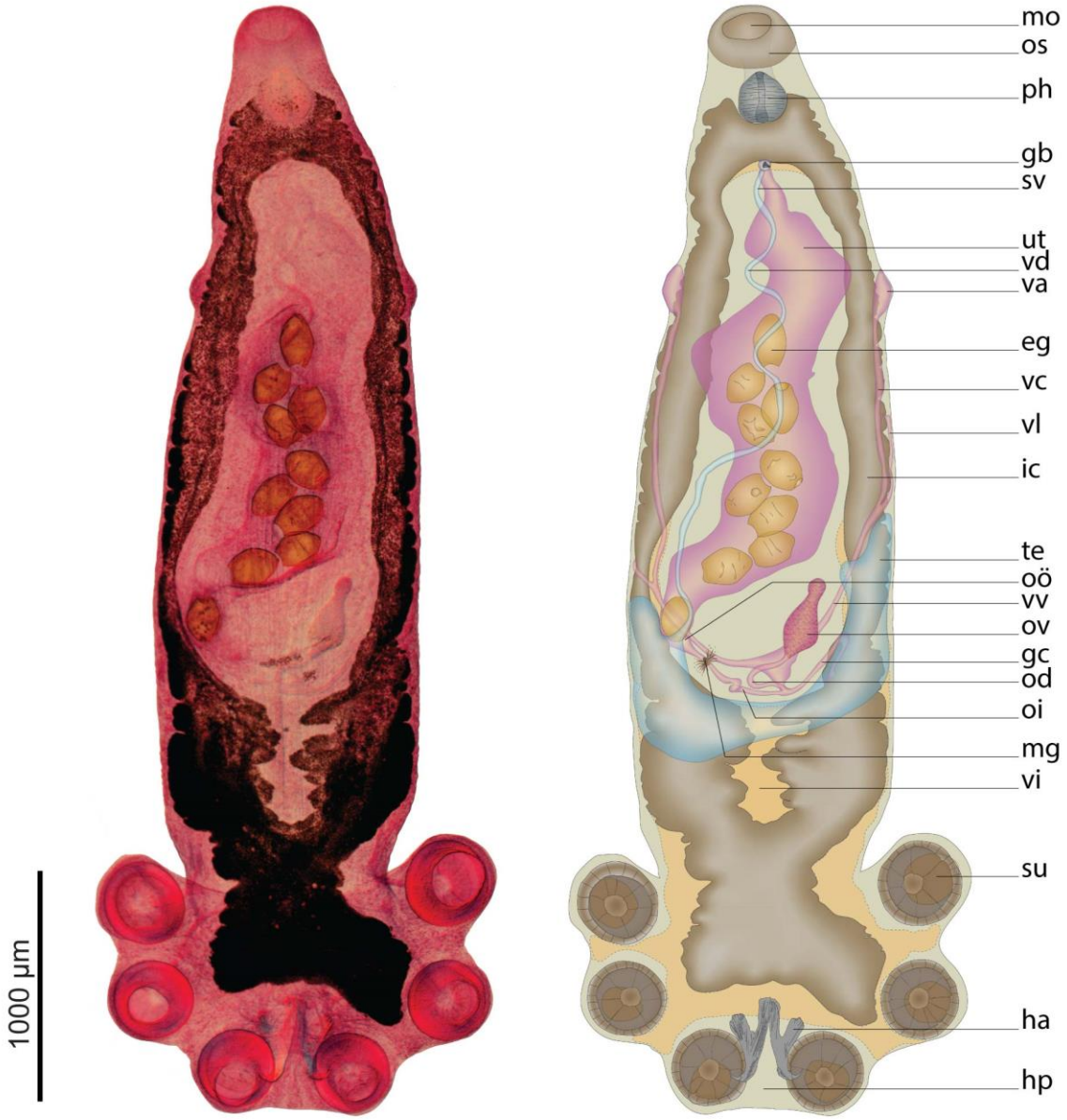


Figure 5.5: a-b) Ventral view of *Metapolystoma* sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito-intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mo, mouth; mg, Mehlis gland; od, oviduct; oi, oö-vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vc, vaginal canal; vd, vas deferens; vi, general position of vitelline (not visible on type); vl, vitelline duct; vv, vitello-vaginal canal.

5.3.3.5 Voucher material:

Remaining specimens in the polystome collection of Louis du Preez, North-West University, Potchefstroom, South Africa.

5.3.3.6 Type locality:

Holotype (NMBP 583) and (Paratypes NMBP 585–586) from Andasibe, Madagascar (Fig. 5.1), (-18.938753S; 48.414471E). Paratype (NMBP 584) from Sahasarotra, Madagascar (Fig. 5.1) (-16.866948S; 49.500559E).

5.3.3.7 Zoobank registration:

The Life Science Identifier (LSID) of the article is: xxx. The life science identifier (LSID) of the new name *Metapolystoma* n. sp. Landman *et al.* is: xxx.

5.3.4 Description

Measurements were obtained from 13 mature, and two juvenile bladder parasites. All measurements are presented in micrometres. Body pyriform (Fig. 5.5), dorsoventrally flat, 3,508–5,042 (4,517 ±700; 4) long, 1,209–1,758 (1,515 ±228; 4) wide, with widest section at 53%–69% (58% ±7.2; 4) of total length measured from anterior end, body length 2.2–4.2 (3.1 ±0.9; 4) times greater than width. Width at vagina 1,041–1,340 (1,210 ±149; 4). Mouth 228–284 (250 ±26; 4) in diameter, sub-ventral surrounded by false oral sucker. False oral sucker interspersed with mucous gland cells (Fig. 5.6A). Medial pharynx length 251–297 (282 ±21; 4) greater than width 226–284 (257 ±27; 4), imbedded with glandular cells throughout its length (Fig. 5.6B). Intestine bifurcates at 14%–17% (16% ±1; 4) of total length from anterior most point, converging posteriorly at 58%–74% (69% ±8; 4) of total length, from anterior most point, extending into haptor; no prehaptoral anastomoses. Lateral diverticula length equal to width. Medial diverticula only posterior to ovary, length greater than width.

Ovary 349–427 (378 ±43; 3) long, 136–190 (169 ±28; 3) wide, elongate, not lobed, positioned posterior to midbody, 7%–9% (8% ±1; 3) of body length, 2–3 (2 ±0.3; 3) times longer than wide. Oviduct 422–491 (455 ±35; 3) long, 18–49 (34 ±9; 3) wide. Mehlis' glands large, surrounding the base of the oötype (Fig. 5.6C). Uterus large, occupying one quarter of body proper, saclike, surrounded by accessory glands at genital bulb connection (Fig. 5.6D), containing up to 61 ovoid, operculate eggs 187–242 (211 ±12.6; 43) long, 104–182 (126.7 ±21.3; 49) wide, some contain fully developed oncomiracidia; some hatched intra-uterine oncomiracidia present. Two parallel vaginae 195–270 (229 ±27; 7) long, 93–177 (137 ±33; 7) wide, on lateral margins, bearing multiple marginal openings formed by branching vaginal canal (Fig. 5.6E), vaginal vestibule cup shaped, 24%–27% (25% ±1.2; 4) from anterior. Vitellaria extended throughout most of body, stretching in between haptoral suckers, surrounding the female reproductive organs. Genito-intestinal canal prominent 469–706 long, 22–51 (33 ±9; 2) wide, situated posterior to ovary.

Testis follicular, encapsulated in connective tissue that forms a tube (Fig. 5.6F), U-shaped, mainly positioned posterior to the ovary with two lateral processes extending forward along the lateral line past the ovary to a position about 25% from anterior end of body proper, ventral to intestine. Lateral fields of testis pushed into posterior position when the uterus is filled with eggs, sometimes causing the testis to be asymmetrical. Vas deferens widens anteriorly to form sinuous semen vesicle 14.1–32.6 (22 ±2; 3) wide, 38–88 (62 ±25; 3) long, narrowing towards genital bulb, opening in common genital opening. Genital pore opening ventral, directly posterior to intestinal caeca bifurcation, 5%–15% (12% ±5; 4) of total length from anterior most point, genital bulb muscular 59–72 (66 ±4; 7) in diameter, surrounded by glandular cells, armed with a genital crown 21.6–27.4 (23.5 ±2; 6) in diameter, with seven genital spines 26.67–31.97 (29.73 ±1.69; 17) long (Fig. 5.7a).

Posterior haptor 1,208–1,280 (1,239 ±32; 4) long, 1,647–2,055 (1,852 ±168; 4) wide, 24%–34% (28% ±4.6; 4) of body length, bearing three pairs of cup-shaped haptoral suckers equal in diameter 296–526 (387 ±51; 27). Well-developed hamuli between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 5.7b), handle length X 340–475 (391 ±42; 14), guard length Y 263–392 (315 ±27; 15), and hook length Z 57–69 (62.5 ±3.6; 21); some hamuli with posterior crest (Fig. 5.7b right). Marginal hooklets placed as for other polystomes: pairs one and two between hamuli, marginal hooklet pairs three to five embedded in suckers, pairs six to eight between anterior suckers. Marginal hooklet pairs one 29.6–33.6 (31.7 ±1.3; 8) long (Fig. 5.7c top) and eight 25.9–30.0 (27.5 ±1.6; 8) long (Fig. 5.7c bottom), larger than pairs two to seven 17.8–22.2 (20.6 ±1,2; 12) long (Fig. 5.7c middle). Hamulus guard, handle and head deeply grooved for muscle attachment (Fig. 5.8a-d). Marginal hooklet pairs one (Fig. 5.9a) and eight (Fig. 5.9b) laterally grooved between hook tip and guard for muscle attachment, hooks two to seven not grooved (Fig. 5.9c).

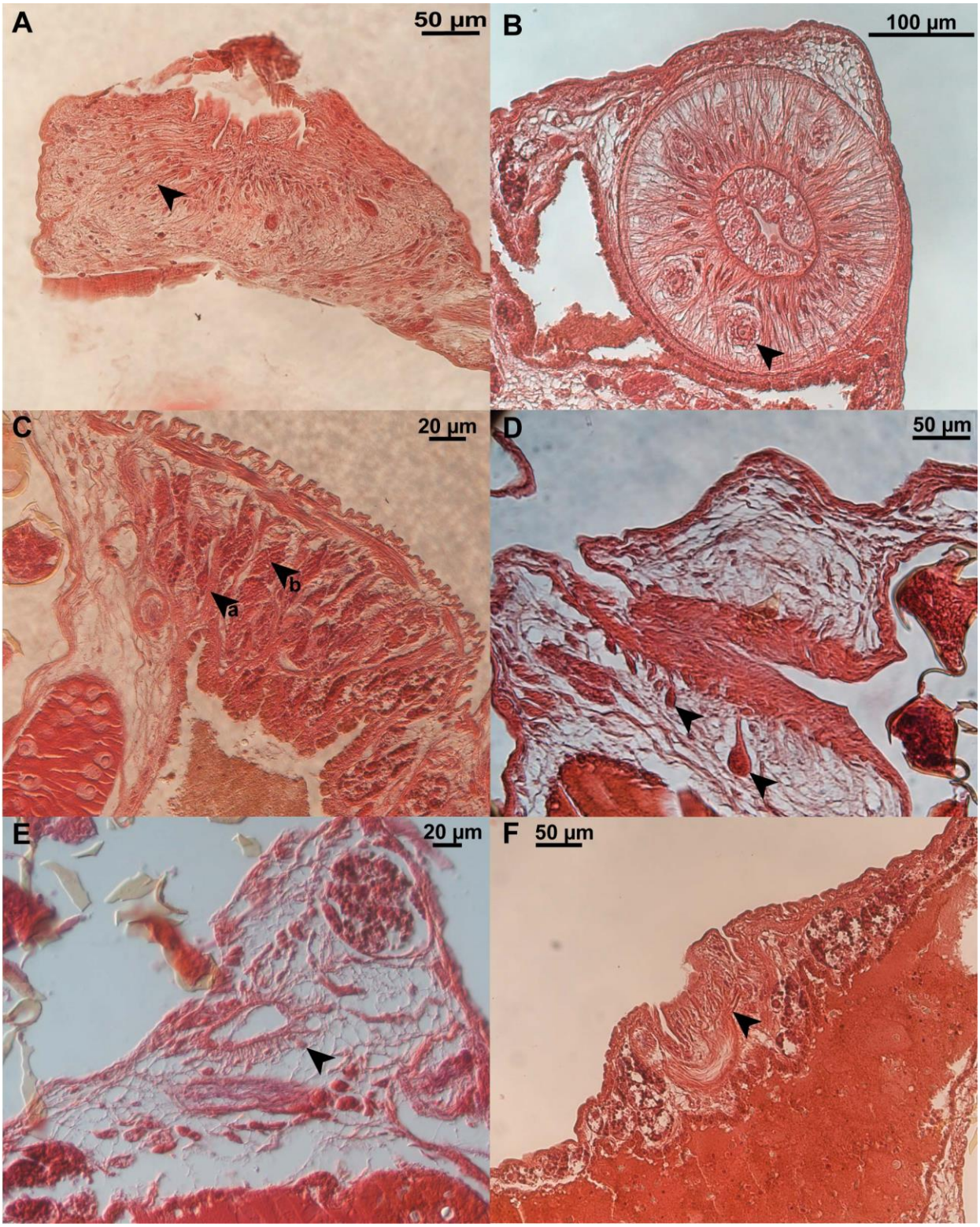


Figure 5.6: Light microscope micrographs of histology sections through *Metapolystoma* sp. A) Sagittal section through false oral sucker interspersed with mucous gland cells (arrow). B) Cross-section through pharynx imbedded with glandular cells (arrow). C) Sagittal section through testis showing testis follicles (arrow b) surrounded with connective tissue (arrow a). D) Sagittal section through genital bulb surrounded with glandular cells (arrow). E) Sagittal section through Mehlis gland (arrow). F) Sagittal section through vagina with the vaginal canal branching out (Arrow) to form multiple vaginal pores.

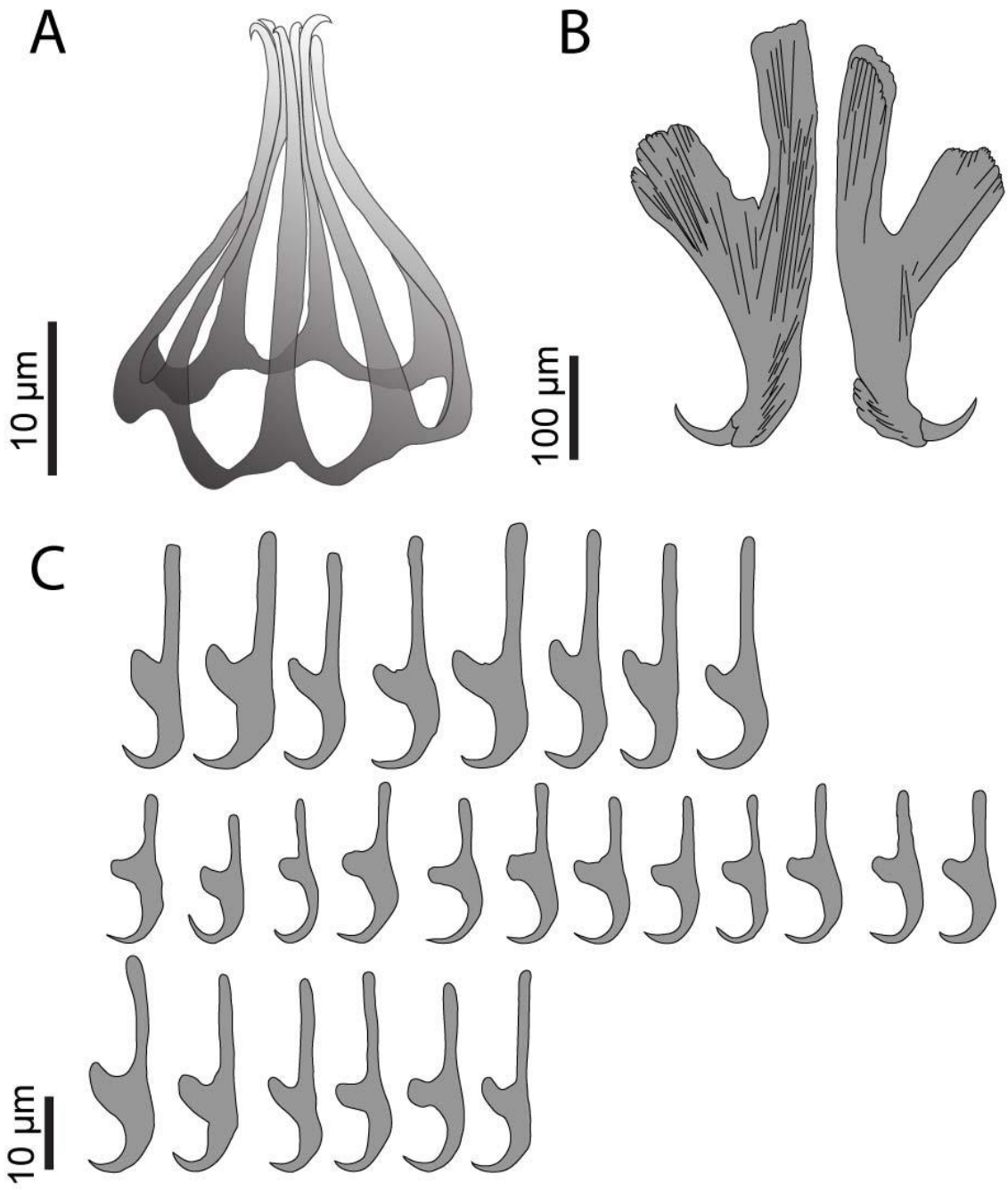


Figure 5.7: *Metapolystoma* sp. A) Marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes. B) Hamuli from holotype. B) Genital crown from holotype.

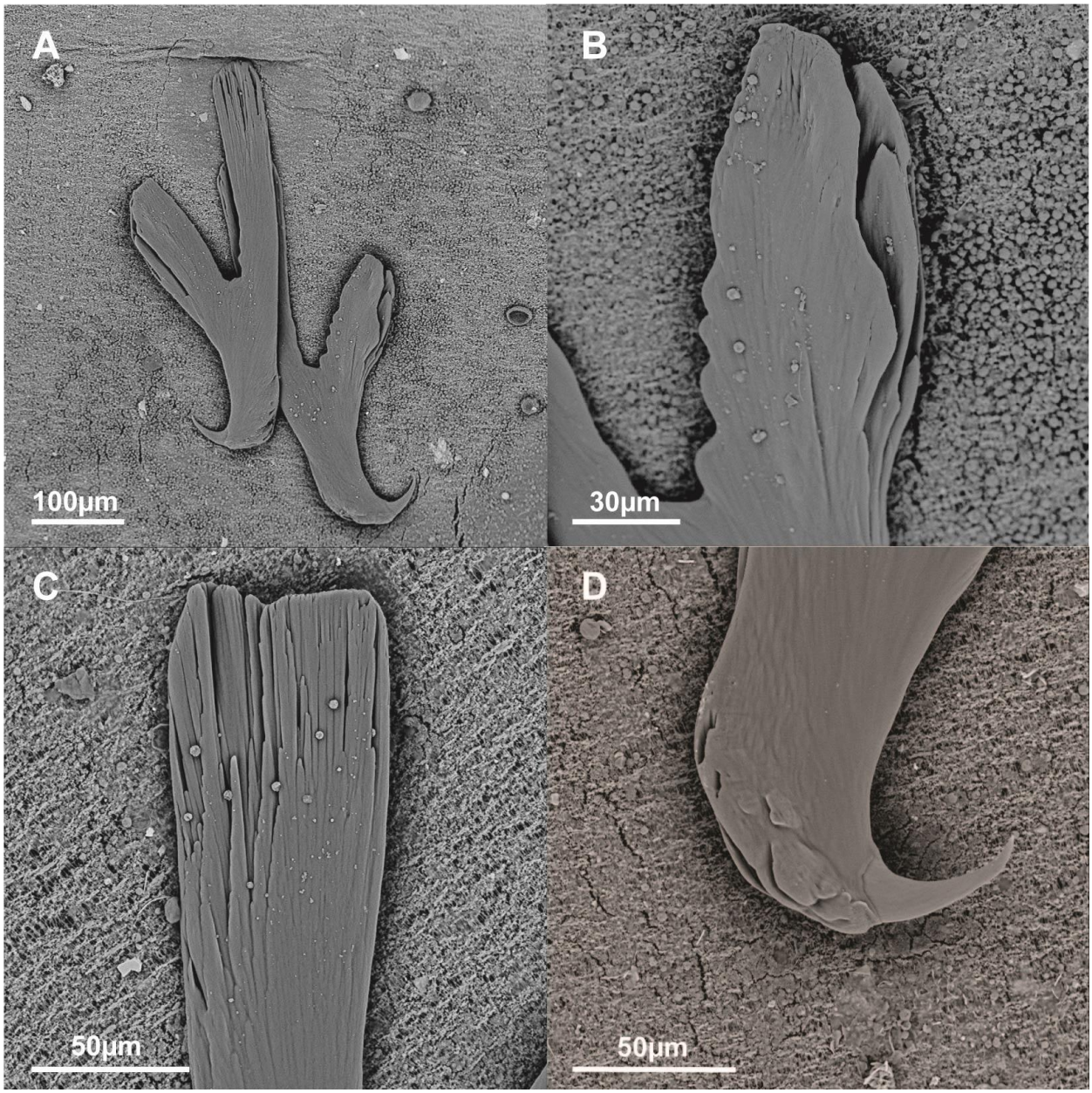


Figure 5.8: Scanning electron micrographs of *Metapolystoma* sp. showing A) Hamuli. B) Deep grooves in hamulus guard for muscle attachment. C) Deep grooves in hamulus handle for muscle attachment. D) Deep grooves in hamulus head for muscle attachment.

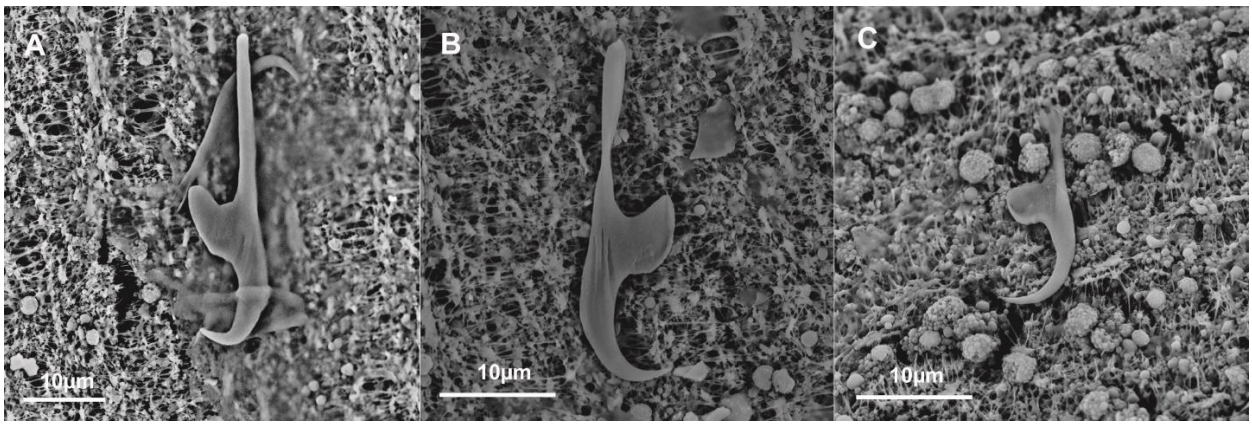


Figure 5.9: Scanning electron micrographs of *Metapolystoma* sp. showing A) Lateral grooves on marginal hooklet one for muscle attachment B) Lateral grooves on marginal hooklet eight for muscle attachment. C) The absence of lateral grooves for muscle attachment on marginal hooklets two to seven.

5.3.5 Egg and marginal hooklet morphometrics

Egg morphometric measurements separated *Metapolystoma* sp. from *M. brygoonis*, *M. falcatum*, *M. theroni, M. multiova*, and *M. vencesi* with no overlap in the scatterplot (Fig. 5.10). Egg morphometric measurements, however, did not separate *Metapolystoma* sp. from *M. ansuanum, M. cachani*, *M. porosissimae*, or *M. vencesi.*

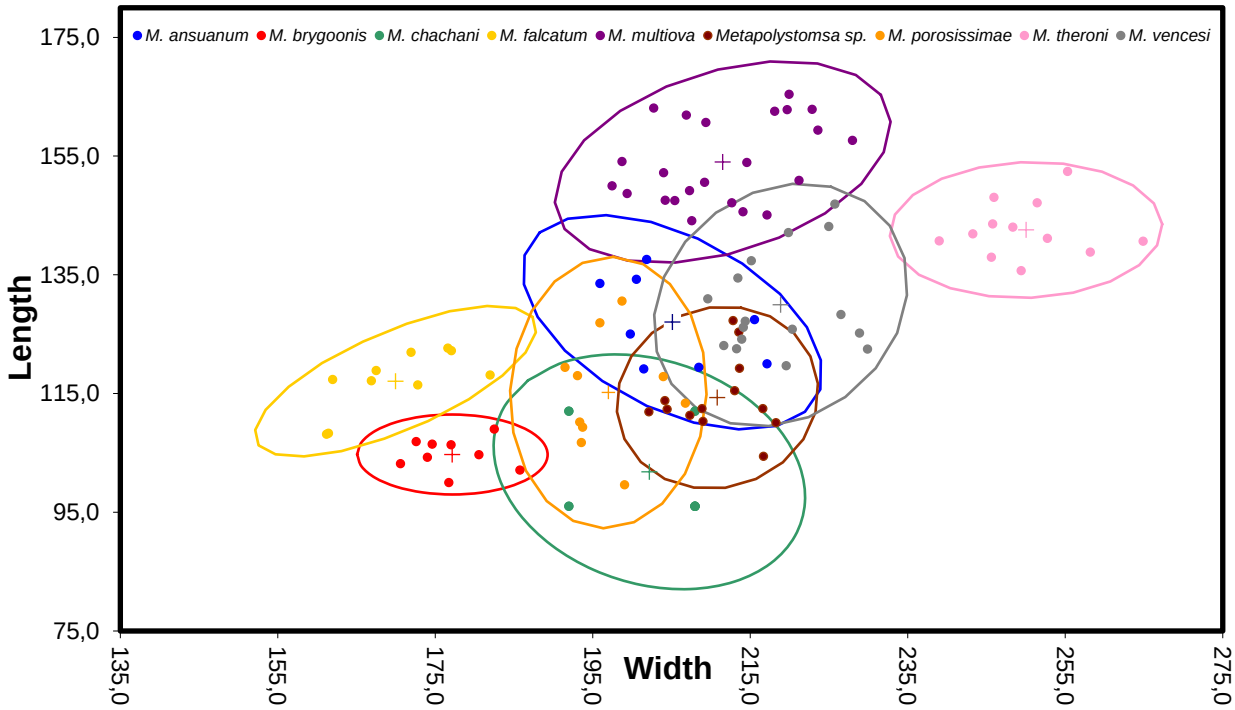


Figure 5.10: Scatter diagram of egg length plotted against egg width for all known *Metapolystoma* spp. and *Metapolystoma* sp. The ellipses represent 100% of the confidence interval about the mean.

Marginal hooklet morphometric measurements separated *Metapolystoma* sp. from *M. falcatum* with no overlap in the scatterplot (Fig. 5.11). Marginal hooklet morphometric measurements, however, did not separate *Metapolystoma* sp. from *M. multiova*, *M. porosissimae*, *M. theroni*, or *M. vencesi*.

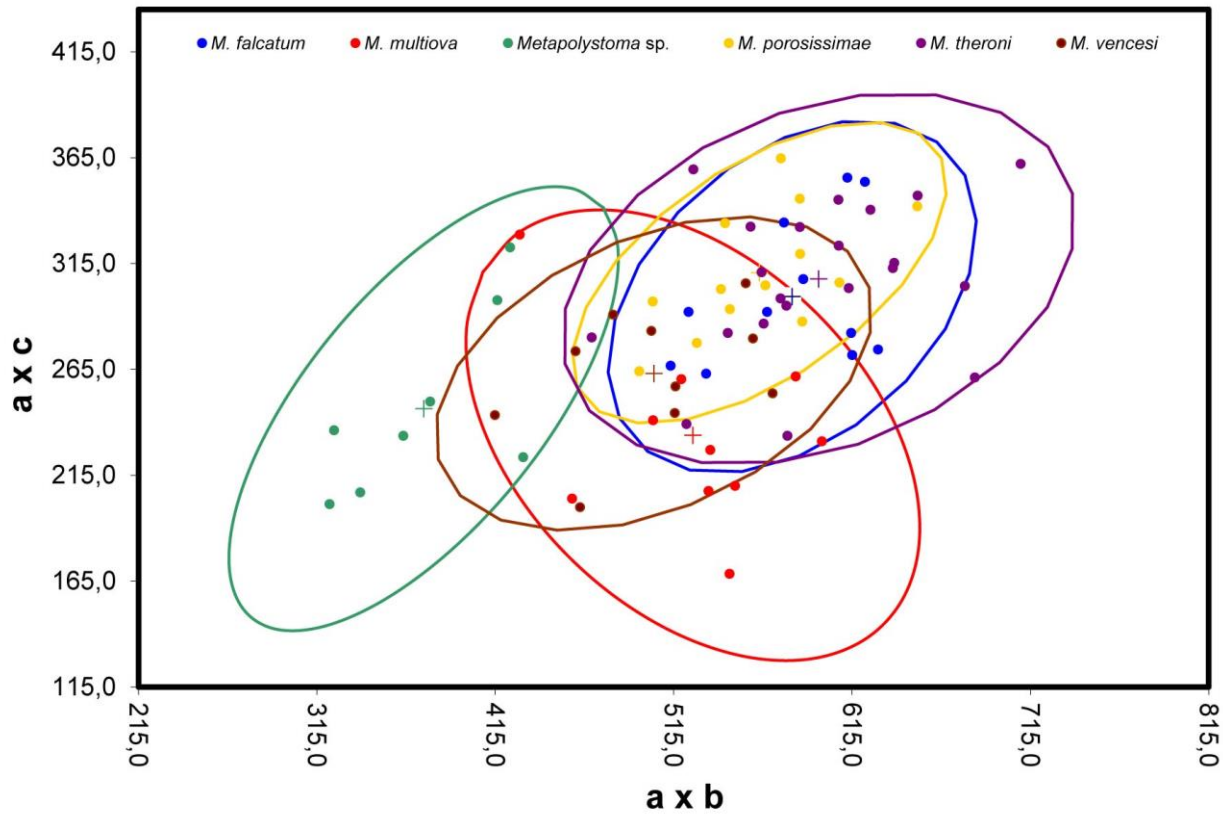


Figure 5.11: Scatter diagram of a × c plotted against b × c (see figure 2.2C) for *Madapolystoma* spp., and *Metapolystoma* sp. and *M. isaloensis* n. sp. The ellipses represent 100% of the confidence interval about the mean.

5.3.6 Remarks

The phylogenetic position of *Metapolystoma* sp. (Fig. 5.4) places it within *Metapolystoma* (Polystomatidae). Genetic divergence estimates between *Metapolystoma* lineages support its description as a new species. *Metapolystoma* sp. differs from all other *Metapolystoma* species in that it has a saclike uterus, whereas other metapolystomes have tubiform uteri that convolute between the oötype and the genital pore. Furthermore, *Metapolystoma* sp. differs from all other metapolystomes except *M. theroni* on the number of genital spines (7). Egg morphometrics (Fig. 5.10) and hamulus hook length, however, separate *Metapolystoma* sp. (57–69) from *M. theroni* (50–55).

5.4 Discussion

There are now seven *Metapolystoma* species known from Madagascar and two from Africa. It can, however, be expected that more *Metapolystoma* species await discovery from *Aglyptodactylus* species, since only one of the six *Aglyptodactylus* species was investigated for these parasites. *Aglyptodactylus madagascariensis* is a predominantly terrestrial species that occurs in the eastern forests of Madagascar and spends most of its time between leaf litter during the daytime and in and around temporary pools at night (Glaw & Vences, 2007). These frogs are explosive breeders that breed following heavy rains (Glaw & Vences, 2007).

Although unanswered ecological questions regarding *Metapolystoma* and its phylogenetic nested position in *Polystoma* resulted in the questioning of its validity as a taxon (Bentz *et al.*, 2001; Olson & Tkach, 2005; Verneau *et al.*, 2002), both phylogenetic and morphological results group metapolystomes together (Landman *et al.*, 2021; Verneau *et al.*, 2009a). Furthermore, as with *Metapolystoma*, *Polystoma* has both a neotenic branchial and mature branchial to bladder cycle. Ovoviviparity has been observed in specimens of *Polystoma integerrimum* and *Polystoma pelobatis* (Euzet and Combes, 1965). This ovoviviparous cycle is, however, not part of these parasites normal reproductive cycle. *Polystoma integerrimum*, similar to *P. pleobatis*, has a short annual egg productive and deposition cycle of only four days per year (Tinsley, 1983). One egg may not get expelled by the end of the parasite's annual reproductive cycle (Combes, 1968; Tinsley, 1983). In such an instance, the egg will develop and hatch *in utero*, after which the oncomiracidium will exit the genital atrium and infect the same host as the parent. Metapolystomes, however, present true ovoviviparity where eggs develop and hatch prior to egg deposition to reinfect the same hosts (Murith, 1981a). One specimen of *B. madagascariensis,* for instance, was infected with 23 recently established oncomiracidia, 19 subadult parasites and one mature parasite (Unpublished data from the study of Landman *et al*. (2021)). In *B. doulioti*, one frog was infected with five small parasites in the Muller ducts and one mature parasite and six subadult parasites in the urinary bladder, suggesting multiple reinfection events (Unpublished data from the study of Landman *et al*. (2021)). This difference between the life cycles of these two genera provide further evidence supporting the validity of *Metapolystoma* as taxon. Furthermore, from a phylogenetic perspective, both *Metapolystoma* and *Wetapolystoma* Gray, 1993 are nested within *Polystoma* (Verneau *et al*., 2009a), suggesting that *Polystoma* might be a complex that requires further investigation.

Although great emphasis has been placed on Madagascar as a biodiversity hotspot (Myers *et al.*, 2000), its parasite diversity, including that of amphibian polystomes, is generally underexplored. This is especially true when taking into consideration Madagascar's rich amphibian diversity, which includes 356 species (Frost, 2018) of which 83 species have been

investigated for polystomes at a few selected localities (Verneau *et al.*, 2009a). These studies led to the discovery of 10 described amphibian polystome species, four undescribed *Madapolystoma* haplotypes (Landman *et al.*, 2018) and nine unidentified madapolystomes from different novel hosts. Another *Metapolystoma* sublineage within the current *M. brygoonis* awaits further investigation (Landman *et al.*, 2021). At the current rate of species extinction many species are becoming extinct before they are formally described (Costello *et al.*, 2013; Pimm *et al.*, 2006; 2014). The biodiversity of Madagascar is particularly threatened as a result of forest fragmentation and habitat loss (Ganzhorn *et al.*, 1999; Sussman, 1974). The hosts of some undescribed Malagasy polystome species, which were discovered during our studies are now Endangered. In fact, 80 (26%) of Madagascar's 311 assessed anurans are listed as being Endangered and 21 (7%) as Critically Endangered (IUCN, 2021). Consequently, due to collecting permit restrictions, the parasite diversity of these frogs will probably never be described. Extinction of any species has a ripple effect, as each species is a package of the host with its species-specific parasites. With amphibians being the most threatened vertebrate class and their numbers continuously declining (Gascon, 2007), this statement is true for their polystomatid parasites as well. Especially since amphibian polystomatids are generally considered to be host specific (Bentz *et al.*, 2001; Murith, 1981b; Tinsley, 1983). With parasites forming such an important part of biodiversity and ecology, the in-depth study of polystomes has the added advantage that in the process we learn more about the host itself. This is especially true when taking into consideration that the phylogeny of polystomes provide insights into the biogeographical origin of their amphibian hosts and ultimately aids in their conservation (Berthier *et al.*, 2014; Verneau *et al.*, 2009b). Madagascar is globally ranked twelfth with regard to amphibian richness (Andreone *et al.*, 2005). Furthermore, it is expected that many more Malagasy polystome species await description (Landman *et al.*, 2018). We therefore emphasize the need for parasite surveys in Madagascar to better conserve its biodiversity. We recommend that, as with this study, museum material should be investigated for parasites. The investigation of museum specimens will provide localities for further field investigations. Parasites that were dissected from museum material should be used as supplementary material since such specimens are usually not usable for molecular studies, and of poor quality for morphological investigation.

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CHAPTER 6:
GENERAL DISCUSSION



The true alchemists do not change lead into gold,

they change the world into words.

-William H. Gass

6.1. New species and morphology

This chapter is aimed at integrating the information gained from this study and to discuss the important general conclusions thereof. The broad aim was to provide information on the Malagasy polystomatid flatworms and to contribute to their known diversity by describing several new species. Each chapter dealt with an aspect of the Malagasy polystomatid diversity. The findings of this study shed some light on Madagascar as a biodiversity hotspot, not only for the anuran hosts for but their parasites as well.

Since parasitism is a common phenomenon, widely distributed, and affecting nearly every life form sometime during its lifespan (Kennedy, 1984), it comes as no surprise that many new parasite species are regularly being discovered. Madagascar's high amphibian diversity (Myers *et al.*, 2000), whose parasites have thus far been poorly studied, makes the discovery of eight new polystomatids unsurprising.

Identification of polystomes on a morphological level is often hard (Tinsley, 1974), especially in cryptic species such as *Madapolystoma* and usually requires detailed morphological examination and molecular tools for identification. Even with the aid of high-end microscopes, it is sometime challenging to identify polystomes to species level. Molecular tools are often needed to confirm identifications.

For *Madapolystoma* species, marginal hooklet morphometrics proved to be particularly helpful in distinguishing species from one another. Marginal hooklets one to eight were found to be of equal length, as reported for species of *Eupolystoma* and *Kankana*. This is in contrast with the usual situation encountered in species of *Polystoma* and most other polystomatids where the posterior-most hooklet pair is significantly larger than the rest (Aisien *et al.*, 2011; Du Preez, 2011; 2013; du Preez & Kok, 1993; 1995; Du Preez *et al.*, 2002; Tinsley, 1973; 1974). Therefore, this measurement may be a good characteristic for species delimitation in *Madapolystoma*. Marginal hooklet morphometrics were thus useful in separating newly described madapolystomes from one another, and from all the other known *Madapolystoma* species (Fig. 3.5) with high confidence (95%). Genital spine number and length are also useful characteristics for species identification in *Madapolystoma*.

For *Metapolystoma*, on the other hand, marginal hooklet morphometrics are of little taxonomic value. With the exception of *Metapolystoma* sp. ex. *A. madagascariensis,* scatterplots of different *Metapolystoma* species overlap and thus do not aid as taxonomic distinguishing characteristics (Fig. 5.10). Egg morphometrical measurements, however, proved to be informative characteristics for *Metapolystoma* species delimitation (Figure 5.9). Hamulus

dimensions and especially hamulus hook length, proved to be helpful to separate most *Metapolystoma* species from one another. As with *Madapolystoma*, genital spine numbers and length are also informative characteristics. Likewise, genital crown diameter has also been found to be a good characteristic for species delimitation with a low standard variability.

The value of sclerotised structures in the description of soft-bodied parasites such as polystomes has been emphasised (Du Preez & Maritz, 2006) and although some taxonomists advocate that polystomes should not be flat fixed (Platt *et al.*, 2011), it is of utmost importance to observe sclerotised structures in a flat orientation. Fixing specimens under cover slip pressure does not affect the measurement of sclerites or smaller rigid structures such as the oral sucker, genital bulb or even, in some taxa, the haptoral suckers (Platt *et al.*, 2011). When sufficient material is available we recommend that i) a specimen be fixed in high quality ethanol or a fixative such as RNAlater for DNA extraction; ii) some of the specimens be heat-killed by placing them in a drop of water on a microscope slide that is then heated from below with a butane lighter until the parasite stops moving followed by fixation in 10% NBF; and iii) for the remainder of the specimens to be heat killed and fixed in 10 % NBF under coverslip pressure. Body measurements and placement of organs should be studied from the unflattened specimens while sclerites should be measured in flattened specimens. In instances where a limited number of specimens are available, it is recommended that specimens should be fixed flat under coverslip pressure.

6.2 *Madapolystoma*

Species of *Diplorchis, Eupolystoma*, *Kankana*, *Neodiplorchis*, *Parapolystoma*, *Pseudodiplorchis* and *Sundapolystoma* all have extended uteri (Du Preez *et al.*, 2003; Rahariviololoniaina *et al.*, 2011) allowing for the accumulation of large numbers of eggs and/or *in utero* development. Therefore, repeated re-infection of a single host may occur either during breeding events, after releasing larvae, or following an internal life cycle inside the host. In the latter case the oncomiracidium does not leave the host but attaches to the bladder wall alongside its parent inside the urinary bladder. In *Eupolystoma* it has been shown that both ciliated and unciliated oncomiracidia are produced (Combes *et al.*, 1973; Fournier & Combes, 1979). Ciliated oncomiracidia are destined to leave the host to swim and find another host, while unciliated oncomiracidia are destined for an internal cycle. In all the above-named species, where larvae are excreted with the frog's urine (external life cycle), the life cycle is completed in water where a ciliated oncomiracidium is produced to infect the next host. In the case of *Madapolystoma*, they have thus far, only been found in host species of *Blommersia*, *Guibemantis* and *Mantella.* All three of these species deposit their egg clutches terrestrially or semi-terrestrially. Terrestrial egg deposition, therefore, imposes a number of problems for polystomes since water would not be available for either their eggs to hatch in or oncomiracidia to swim in. Furthermore, due to their

external life cycles, ciliated oncomiracidia are mainly adapted to infect tadpoles. Thus, the breeding behaviour of the above-named frogs would have left open, an unutilised terrestrial niche for polystome parasites. For polystomes to have filled this niche, they would have had to undergo certain adaptations. The first of these is that the life cycle of the Polystomatidae is characterized by a free swimming, ciliated oncomiracidium (Smyth & Wakelin, 1994) which would have been of little value in a terrestrial breeding habitat. This problem is circumvented by *Madapolystoma* with a larval phase that develops *in utero*, as with *Eupolystoma*, but the embryo develops into a near complete adult parasite with haptoral suckers and hamuli (Fig. 3.3).

During a field trip to Madagascar in February 2006, a frog egg mass overhanging a pool was collected and inspected under a stereo microscope. A small metapolystome embryo was observed on the egg mass. It is therefore hypothesised that the developing embryo of *Madapolystoma* spp. may leave the host during breeding events and stay on the egg mass until another frog visits the clutch. The embryo then enters the cloaca and migrates to the accessory bladder from where it migrates to the urinary bladder. Frog visits to egg clutches can take part in various forms. For example, as with *Guibemantis torneri*, which is infected with a *Madapolystoma* species, the male stays behind after mating to guard the clutch (Glaw & Vences, 2007) (Fig. 6.1). In some frog species it is known that the male or female regularly return to egg clutches to keep the clutch moist by urinating on them (Fig. 6.1). It is further possible, since some mantelids display tactile courtship behaviour as explained in section 1.3.1, that madapolystomes can be transmitted directly between frogs in amplexus (Fig. 6.1). Further, madapolystomes may also have internal life cycles as is the case with *Eupolystoma*. These modes of transfer between hosts, however, are only hypotheses and require investigation in order to be confirmed.

Advanced embryonic development would enable juvenile parasites to attach themselves to adult frogs for reinfection outside the water after they had been expelled from a host frog. For the parasite to overcome the risk of being expelled from the urinary bladder when the now infected frog urinates, haptoral suckers and hamuli are employed to secure its position. Even though, as mentioned above, marginal hooklet pairs one and eight are smaller than the norm, this smaller hook size would be of little importance for attachment to the host, since marginal hooklets are mainly used to attach to tadpoles which don't seem to be part of the parasite's life cycle.

Madapolystomes are K-strategists that produce few offspring with a lot of energy investment. If the above-mentioned modes of transfer prove to be the case, then this reproductive strategy makes sense. Since parasite transmission would be direct, many offspring would not be needed compared to other polystomes that need to find a host in large water bodies. Transmission would be more successful because of direct contact and transmission between hosts or contact between egg clutches.

Since polystome life cycles and maturation are synchronised with that of their hosts, advanced *in utero* development in madapolystomes makes sense. Most polystomatids produce chitinous yellow eggs that incubate in the water body after being released from the host. In species where *in utero* development is the norm (i.e. *Eupolystoma*, *Kankana*, *Pseudodiplorchis* and *Wetapolystoma* (see: Gray, 1993; Raharivololoniaina *et al.*, 2011; Tinsley, 1990) eggs are not encapsulated in a yellow rigid shell, but rather a semi-transparent flexible membrane. This allows for direct maintenance of developing larvae through parental nutrients. This direct maintenance also means that the developing F1 parasite is exposed to the adult frog's hormones, causing them to mature faster (Miretski, 1951). In the most advanced *in utero* developing juveniles, a darker cluster of cells is visible in the middle of the parasites (Fig. 3.3). We hypothesise that this cluster of darker cells is a developing F2 embryo, implying true vivipary. In a terrestrial habitat, viviparity has the advantage of a shorter maturation period, which would be even shorter in the case of *Madapolystoma* since the F2 generation is exposed to an adult frog's hormones from a young age. *Madapolystoma* hosts that were collected from February to March in Madagascar (frog breading season) were infected with a few adult madapolystomes while juvenile parasites were abundant. This ratio implies that the adult parasite has a short lifespan, and that it probably dies after giving birth. A fast developing F1 and F2 embryo would therefore sustain the population.

Madapolystomes seem to be well adapted to find hosts that meet their physiochemical requirements. Some madapolystomes are less host specific, as seen with *Madapolystoma biritika* that parasitises three different *Mantella* species. This reduced host specificity is an indication of the parasite being able to identify suitable hosts. This is most probably the result of host switching which is important for diversification in an evolutionary sense. The maximum reported number of eggs and developing embryos in a single individual of *Madapolystoma* spp. is 32 (Du Preez *et al.*, 2010). Furthermore, the mean intensity of madapolystomes found in Malagasy frogs is low which imposes a low nutritional burden on the host. No physical damage to the host by the parasite has been seen during dissection either. Tinsley (1995; 1999) demonstrated that the immune response of *Scaphiopus couchi* is able to reduce the initial number of *Pseudodiplorchis americanus* infections to much lower numbers. Since the infective larvae of *Madapolystoma* is further developed and stronger than the infective oncomiracidium of *P. americanus,* the effect of the host immune response on the parasite is likely to be reduced. Furthermore, parasites are able to survive inside the host until they are able to reproduce, suggesting that they have a form of protection against the host's immune system or that the host's immune system is unable to eradicate them.

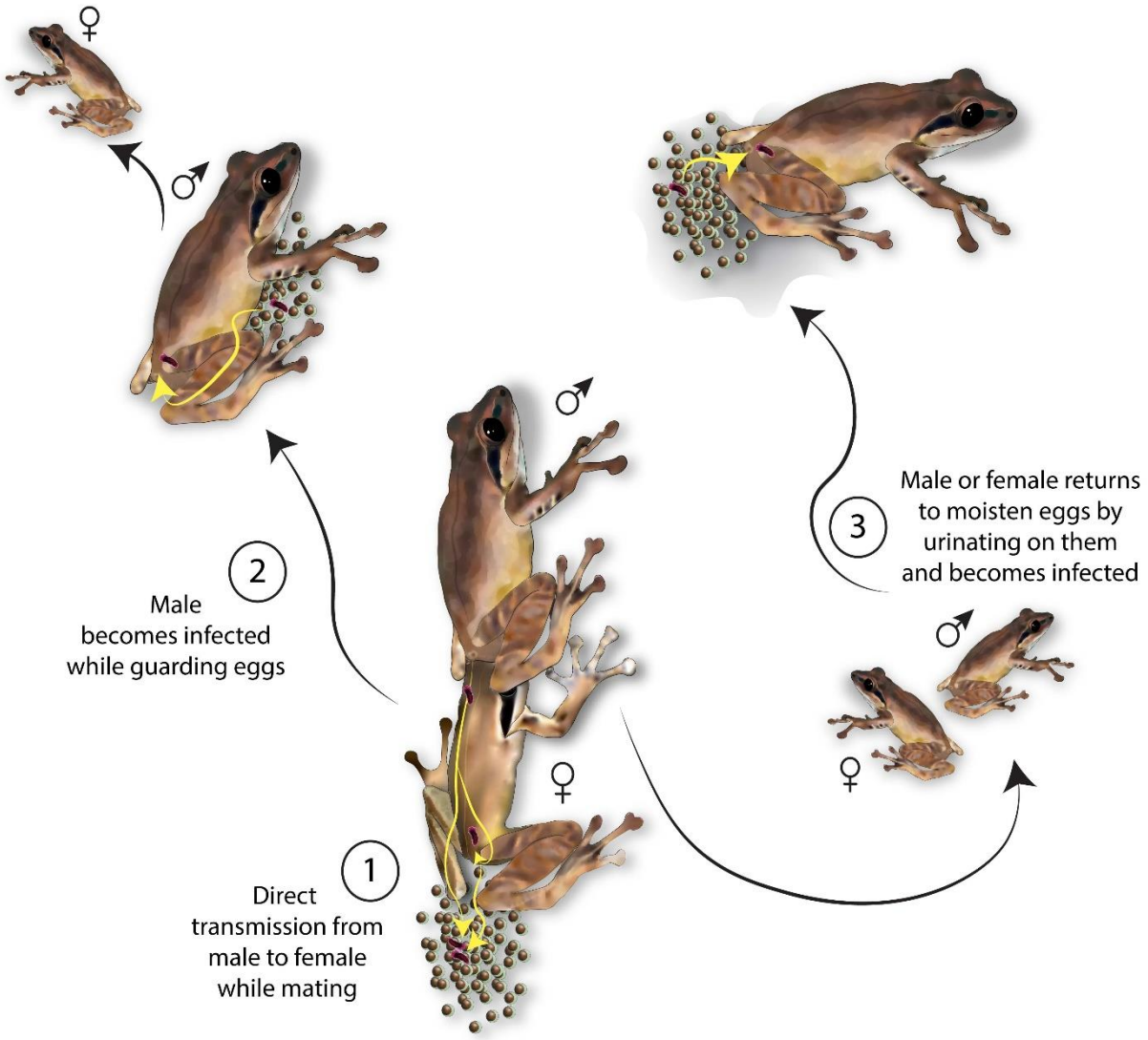


Figure 6.1: Possible modes of host infection for *Madapolystoma* species.

6.3 *Metapolystoma*

The long dispute regarding the validity of *Metapolystoma* as a taxon has now turned in its favour. It is known that *Metapolystoma* is nested in *Polystoma* and that previous molecular studies could not conclude on its validity since only one sequence of *M. brygoonis* was available for phylogenetic analysis (Bentz *et al.*, 2001). In order to validate *Metapolystoma* as a taxon, that was only based on homoplastic characters (Verneau *et al.*, 2009b), Bentz *et al.* (2001) suggested that other metapolystomes should be added to the phylogenetic analysis. An addition of 14 sequences from *Metapolystoma* morphotypes to phylogenies (Figs 4.14 and 5.11) maintain the nested position of *Metapolystoma* in *Polystoma*, however, all *Metapolystoma* morphotypes cluster together in phylogenies. It is estimated that *Metapolystoma* originated in Africa 14 million years ago from where it underwent a transoceanic dispersal to Madagascar where this lineage diverged

five million years ago (Verneau *et al.*, 2009b). Furthermore, the African *Polystoma* originated from an ancestral European *Polystoma* species that colonised Africa during a single colonisation event (Bentz *et al.*, 2001). Africa currently has the richest diversity of *Polystoma* species. It is further suggested that some African polystomes might rather be generalists as opposed to specialists and that host ecology and physiology are the forces that drive speciation from generalists (Bentz *et al.*, 2001). These ecological factors include aspects such as host habitat, breeding periods and tadpole longevity (Bentz *et al.*, 2001). The Ptychadenidae is one of the groups that *Polystoma* radiated onto and is known as a successful and widespread genus in Africa that is exceptionally suitable to host polystomes (Du Preez & Kok, 1992a). Furthermore, *Ptychadena* is undergoing an explosive radiation (Perret, 1979). Thus, if representatives of *Ptychadena* are rapidly radiating, it is possible that *Polystoma* might be mirroring their evolution (Bentz *et al.*, 2001). This evolutionary mirroring not only explains the high African *Polystoma* diversity, but also the speciation of *Metapolystoma* from *Polystoma*. Homoplasticity, as argued by Bentz *et al.* (2001) for the invalidity of *Metapolystoma*, therefore, rather provides an explanation for the evolutionary driving force behind its speciation. Additional evidence for the validity of *Metapolystoma* as a taxon is found in differences that occur between the life cycles of *Metapolystoma* and *Polystoma*. *Metapolystoma* presents true ovoviviparity (Murith, 1981a), while *Polystoma* only presents ovoviviparity in situations of incomplete egg expulsion (Combes, 1967). Another morphological distinct taxon, *Wetapolystoma* is also nested in *Polystoma* (Verneau *et al., 2009a*). Thus, it is evident that these two genera either diverged from *Polystoma*, or that *Polystoma* is a complex that requires further investigation.

For future studies, the case for the validity of *Metapolystoma* as a taxon can be strengthened with the molecular analysis of *M. porosissimae* and *P. sodwanensis*. If these two parasites are molecularly distinct from one another, and separately cluster to their current genera on a phylogeny, it would disprove reproductive plasticity within a single species over changing environments. Furthermore, the wide distribution of the two *M. brygoonis* lineages and their hosts, over both arid and forest habitats, might also resolve this question on a morphological level. If parasites from the same species from both arid and forest habitats have the same reproductive morphology, the theory on reproductive plasticity would also be disproved.

The monophyly of Malagasy metapolystomes (Figs 4.14 and 5.11) suggests that there was a single colonisation event of an ancestral African metapolystome to Madagascar. After the transoceanic arrival of *Metapolystoma* in Madagascar, a host switch took place from the African *Ptychadena* host to a Malagasy frog (Verneau *et al.*, 2009a). It is possible for this host switch to have taken place to *B. doulioti*, since its metapolystome, *M. falcatum,* is the phylogenetically closest relative (Figs 4.14 and 5.11) to *M. brygoonis*, which in turn, is the closest relative to African

metapolystomes. The Boophinae consists of only one genus, namely *Boophis*. These treefrogs are distributed throughout all the various habitat types within Madagascar and can be found breeding in either stagnant or running water during spring and summer (Glaw & Vences, 2007). Furthermore both *P. madagascariensis* and *B. doulioti* present pleisiomorphic reproductive strategies, overlap in distribution, and share the same breeding resources. This overlap in life strategies and resource utilisation would have provided ideal environmental conditions for a host switch to have taken place.

Based on molecular evidence, *Ptychadena mascareniensis* may comprise at least two separate lineages (Vences *et al.*, 2004; Zimkus *et al.*, 2017). These genetic differences within *P. mascareniensis* are supported by molecular evidence showing two distinct lineages for *M. brygoonis* (Fig. 4.14). Polystomes originated in the Palaeozoic age and co-evolved with their hosts (Héritier *et al.*, 2015; Verneau *et al.*, 2002; Verneau *et al.*, 2009a). Consequently, their phylogeny can provide information with regard to the diversification of their hosts over ancient and recent geological periods (Badets *et al.*, 2011). Furthermore, amphibian polystomes are generally considered to be host specific (Berthier *et al.*, 2014; Bourgat & Salami-Cadoux, 1976; Combes, 1966) and can be used as indicators of host identity. This second lineage of *M. brygoonis*, therefore, strengthens the case for the existence of a second *Ptychadena* species in Madagascar. Additional sampling would, however, be required for both the host and the parasite in the same localities. More samples of *M. brygoonis* from localities throughout the range of *P. mascareniensis* are needed to resolve this issue.

In context with the argument for polystomes as the ‘ideal parasite’ (Chapter 1.2), the host switch of metapolystomes from *Ptychadena* to *Boophis* and *Aglyptodactylus* hosts provides evidence that these parasites are well adapted to identify potential hosts. Furthermore, since *Metapolystoma*, in contrast to *Madapolystoma*, have a free-swimming larval stage that attaches to the gills of tadpoles in their life cycle, oncomiracidia would require additional attachment support. Attachment in this case is strengthened with marginal hooklet pairs one and eight being larger than pairs two to seven. Marginal hooklets one and eight in *Metapolystoma* sp. ex. *A. madagascariensis*, for instance, have lateral grooves for muscle attachment. These grooves are, however, not evident on marginal hooklets two to seven. Furthermore, the hamuli of *Metapolystoma* sp. ex. *A. madagascariensis* are also deeply grooved for muscle attachment. The muscle attachment sites on both the hamuli and marginal hooklets one and eight, therefore, suggest that these hooks play a larger function in attachment than hooklets two to seven. Furthermore, immature metapolystomes moving from the gills of infected tadpoles to the urinary bladder of the post-metamorphic froglet are small enough, in comparison to madapolystomes, to be able to enter the cloaca. These enlarged hooks in parasites that attach to tadpole gills plus a

smaller larval size, suggest that metapolystomes are physically adapted to the life cycle of the host to ensure successful infection. Additionally, the maturation of metapolystomes and the development of either mature or neotenic phenotypes are physiochemically linked to the life stage of the host. Either *Boophis* tree frogs in Madagascar are particularly well-suited hosts for metapolystomes, or metapolystomes might be exceptionally adaptable to infect and radiate onto other host species. This hypothesis is supported by the fact that metapolystomes have radiated and diversified into five different species in five different *Boophis* hosts over a relatively short evolutionary time (five million years, see Verneau *et al.*, 2009a). This diversity is not even representative of the metapolystome diversity in *Boophis* species, since only a few species were screened for these parasites at a few selected localities in the past. To crown the situation of *Metapolystoma* as an adaptable species, it is also found in a third host genus, *Aglyptodactylus*. It can therefore be expected that many more *Metapolystoma* species await discovery since Malagasy frogs are generally poorly studied for these parasites. As discussed in chapter 4, the situation for African metapolystome diversity is probably similar, and might even be greater, since African metapolystomes have had a longer time to diversify than those in Madagascar.

Metapolystomes are r-strategists that produce many offspring with low energy investment which can be seen where parasites produce large numbers of eggs. *Metapolystoma multiova* can hold more than 500 eggs in its uterus, with smaller and less developed offspring at birth in comparison to *Madapolystoma*. *Metapolystoma* hosts release their eggs in water with no parental care after oviposition. Contact for direct or indirect parasite transmission between hosts, as with *Madapolystoma*, is thus limited and requires parasites to search for dispersed hosts in larger areas than with *Madapolystoma*. This reproductive strategy with large numbers of offspring thus increases the probability of parasites encountering hosts. Even in the situation where only a few offspring are successful in finding and infecting hosts, evolutionary success is achieved since the parasite's genetic line has been carried over into a next reproductive generation. In comparison to *Madapolystoma*, differences in offspring energy investment would probably result in the same numbers of successful host infections and parasite survival. In some instances, up to 40 juvenile metapolystomes, or 15 adults have been observed to infect the urinary bladder of Malagasy hosts with no apparent effect on the host. This parasitic load is low in comparison to loads of more than 2000 parasites as seen in *Eupolystoma* (Du Preez *et al.*, 2003). This suggests a low nutritional burden on *Metapolystoma* hosts.

Since metapolystomes (bladder parasites), reach reproductive maturity with their hosts after two to three years of infection, and some frogs have been found with up to 15 adult parasites, it is evident that these parasites survive the host's immune defence. It can either be that the parasite has protection against the host's immune defence, or that the host's immune defence is

insufficient in eradicating these parasites. The latter is improbable, since it has been shown that the immune systems of anurans comprise almost all the components of those of mammals (Carey *et al.*, 1999).

With all these adaptations taken into consideration, species of both *Madapolystoma* and *Metapolystoma* meet all the necessary requirements, as discussed in chapter one, to be considered as 'ideal parasites'.

6.4 Why Malagasy parasite diversity is important for conservation

Global biodiversity is continuously declining (Waldron *et al.*, 2017). This decline is particularly evident in Madagascar where 44% of its natural forests were lost between 1953 and 2014 at a rate of 99 000 ha/year (Vieilledent *et al.*, 2018). Less than 11% of Madagascar remains forested (Benstead *et al.*, 2000). Apart from climate change, this rapid habitat decline is a result of anthropogenic activities such as slash-and-burn agriculture and pasture establishment (Scales, 2011). Most Malagasy amphibians are closely related to these forests and ecosystems (Ganzhorn *et al.*, 2001) and are becoming increasingly threatened (Andreone & Luiselli, 2003). In fact, amphibians are globally ranked as the most threatened vertebrate class with their numbers continuously declining (Gascon, 2007). Deforestation is not the only source of amphibian decline in Madagascar, many frogs are sought after for the pet trade (Andreone *et al.*, 2005). One such popular group of frogs in the pet trade are the Mantellidae (Andreone, 1994; Vallan, 2000). Mantelid frogs are particularly bound to forest habitats due to the specialisation of their breeding and ecosystem biology (Andreone, 1994; Vallan, 2000). Almost all mantellas are now listed as Cities II species as a result of pressures from habitat destruction and overutilization from the pet trade (Andreone *et al.*, 2005). Mantellas serve as hosts for madapolystomes, and since each species is a package of the host with its species-specific parasites, these parasites are also threatened. At the current rate of species extinction, many species are probably becoming extinct before they are even described (Costello *et al.*, 2013; Pimm *et al.*, 2006; Pimm *et al.*, 2014). As a result, some polystome species, including *Madapolystoma* species, which were discovered during the above-mentioned fieldwork or in archived museum collections (Table 1.1), will probably never be described as some of the hosts are now threatened.

Some biological threats to amphibians, such as chytrid infections, have been evaluated in Madagascar (Andreone *et al.*, 2008), however, the effect of polystomes on hosts as discussed in Chapter one is still largely unknown. Adverse effects of amphibian polystomes on their hosts have only been observed in *Scaphiopus couchi* infecting *Pseudodiplorchis americanus* (see Tinsley & Jackson, 2002). It is unknown whether other polystomes cause damage to their hosts. Parasites impose energy costs upon their hosts (Arnott *et al.*, 2000) and are generally considered to be

harmful, yet, paradoxically, some parasites may enhance host survival or growth (Arnott *et al.*, 2000; Lim & Green, 1991; Phares, 1996; Probst & Kube, 1999; Rothschild & Rothschild, 1939; Sorensen & Minchella, 1998). Furthermore, since polystomes co-evolved with their hosts, their phylogeny provides insights to the biogeographical origin of their amphibian hosts and ultimately aid in their conservation (Berthier *et al.*, 2014; Verneau *et al.*, 2009a). Therefore, if it was known whether polystomes influenced their hosts either positively or negatively, both instances would contribute to the conservation of Malagasy amphibians. More detailed parasite surveys in Madagascar are necessary, since it would be of little use to know the effect of these parasites on their hosts without an inventory of the hosts they infect.

Preferably new species should not be described from small sample sizes. Consequently, many undescribed species are collecting dust in biological collections at universities and museums while researchers hope to find more material to formally describe them. The reality is that since many parasite hosts are protected, hard to find, or funding, permits and access are limited, these specimens remain safely in the back of cupboards. Madapolystomes from endangered mantellas, as mentioned above, serve as an excellent example of such undescribed specimens. It is therefore recommended that, if only a few specimens are available, the material should be taxonomically described especially when molecular evidence can be provided. This type of description will make these species known to other scientists, who may have similar specimens in storage, that could provide additional information. Furthermore, if these species names are published, other scientists will be aware of them and be able to collect additional material when the opportunity presents itself. If these species are known, biodiversity estimates of countries such as Madagascar would be a truer representation than what is currently reported. Accurate biodiversity estimates are important since the spatial and phylogenetic pattern of species richness forms the basis on which large scale processes that drive biodiversity hinge (Edie *et al.*, 2017). Species cannot be protected if they are not known to science. Furthermore, if the existence of species remains unknown, it will not be noticed when their numbers decline or when they become extinct, which will leave conservationists oblivious to rising environmental threats. Parasites form an integral part of biodiversity and ecology. An in-depth study of polystomes has the added advantage that in the process we learn more about the host itself. Thus, the study of polystome parasites ultimately aids in the conservation of their hosts (Berthier *et al.*, 2014; Verneau *et al.*, 2009a).

In conclusion, new species are not only regularly discovered in Madagascar but on a global scale. On the other end of the scale, global biodiversity is declining, and many species become extinct before they are taxonomically described. This seems to be reflected in Madagascar's rate of habitat loss. The study of polystome parasites in Madagascar not only adds

new species to Madagascar's already high biodiversity but, importantly, provides insight into the hosts biology, ecology and geographic origins. This information forms an important part of conservation and can ultimately aid in the protection of both polystomes and amphibians. It is critically important to conduct more biodiversity studies in Madagascar, since we cannot conserve species that remain unknown.

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APPENDICES



I saw the angel in the marble and carved until I set him free.

- Michealangelo -

Appendix 1

Landman, W., Verneau, O., & Du Preez, L. H. (2018). First record of viviparity in polystomatid flatworms (Monogenea: Polystomatidae) with the description of two new species of *Madapolystom*a from the Madagascan anuran hosts *Blommersia domerguei* and *Mantella expectata. International Journal of Parasitology: Parasites and Wildlife,* 7, 343−354. doi.org/10.1016/j.ijppaw.2018.09.004.

Appendix 2

Landman, W.J., Verneau, O., Raharivololoniaina, L., du Preez, L.H. (2021). First record of *Metapolystoma* (Monogenea: polystomatidae) from *Boophis* tree frogs in Madagascar, with the description of five new species. *International Journal for Parasitology: Parasites and Wildlife*. 14, 161−178. 10.1016/j.ijppaw.2021.01.012.

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ABSTRACT

Two frog species, *Blommersia domerguei* and *Mantella expectata*, are reported as hosts for new species of *Madapolystoma*. Phylogenetic analyses and genetic divergences observed in the genus supported the distinction of two morphotypes infesting selectively each host species and morphological investigation combining marginal hooklet morphometrics, genital spine number and measurements further showed that polystomes from the two host species differed from each other and from all other known polystomes. *Madapolystoma magnahami* n. sp. and *Madapolystoma isaloensis* n. sp. are therefore described as two new species. Advanced *in utero* development was illustrated in both polystome species following the observation of well developed hamuli and two pairs of haptoral suckers in developing embryos. Inside some of these *in utero* embryos a F2 generation embryo was also observed. This is the first report of true viviparity among polystomatid flatworms.

1. Introduction

As a biodiversity hotspot Madagascar is ranked within the world's top three regions of conservation importance (Myers et al., 2000). The high species diversity and level of endemicity is ascribed to the isolation of the island over an extended period of time (Myers et al., 2000; Goodman and Benstead, 2005; Glaw and Vences, 2007). The separation of Madagascar from other landmass began during the breakup of Gondwana about 156–165 Mya (Rabinowitz et al., 1983) and ended when it separated from India about 84–94 Mya (Storey et al., 1995). Globally, Madagascar is ranked as the country with the twelfth highest amphibian species richness (Andreone et al., 2008). While the true species number of anuran species was reckoned to be close to 465 species (Glaw and Vences, 2007; Vieites et al., 2009; Glaw et al., 2010), 345 frog species are currently described from the island (Frost, 2018) with a near 100% endemicity. Only two non-native invasive species have been recorded, namely *Hoplobatrachus tigerinus* Daudin, 1802 and *Duttaphrynus melanostictus* Schneider, 1799 (Glaw et al., 2010; Moore et al., 2015).

In spite of the conservation status of Madagascar, the diversity and endemicity of the less prominent taxa are poorly known (Myers et al., 2000; Goodman and Benstead, 2005). As can be expected, the species richness is not restricted to the herpetofauna alone but also applies to their parasites (Wohltmann et al., 2007; Junker et al., 2010; Rocha et al., 2012; Kuzmin et al., 2013). Four genera of polystomes (Monogenea: Polystomatidae) have been described in Madagascar: (i) *Uropolystomoides* Tinsley and Tinsley, 2016 with a single species *Uropolystomoides chabaudi* (Euzet and Combes, 1965) from the chelonian host *Pelomedusa subrufa* (Lacépède, 1788); (ii) *Metapolystoma* Combes, 1976, with a single species *Metapolystoma brygoonis* (Euzet and Combes, 1964) from the anuran host *Ptychadena mascareniensis* (Duméril and Bibron, 1841); (iii) *Madapolystoma* Du Preez et al., 2010 with three species infecting frogs, namely *Madapolystoma biritika* Du Preez et al., 2010 from *Mantella madagascariensis* (Grandidier, 1872), *Madapolystoma cryptica* Berthier et al., 2014 and *Madapolystoma ramilijaonae* Berthier et al., 2014, from the same host *Guibemantis liber* (Peracca, 1893); (iv) *Kankana* Raharivololoniaina et al., 2011, with a single species *Kankana manampoka* Raharivololoniaina et al., 2011 from the

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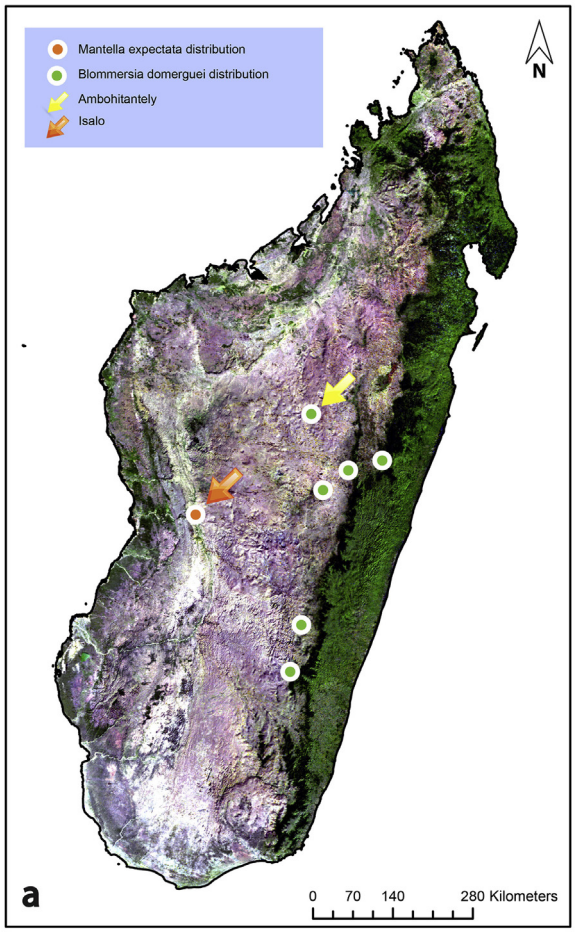


Fig. 1. a) Map of Madagascar with the distribution areas and sampling localities of the two investigated frogs; b) *Blommersia domerguei*; c) *Mantella expectata*. (Map-Library, 2007).

frog *Cophyla pollicaris* (Boulenger, 1888).

Because it has been shown that polystomes coevolved with their hosts since their origin in the Palaeozoic age (Verneau et al., 2002, 2009a; Héritier et al., 2015), investigating their phylogeny can provide relevant insights into the diversification of amphibians over ancient and recent geological periods (Badets et al., 2011). Out of the 345 known anuran species from Madagascar, 86 species from a few selected localities were screened for polystomes (Verneau et al., 2009b). At least twelve polystome morphotypes were identified from these amphibians, suggesting that a great number of polystome species from Madagascar still await description. It is therefore important to study their systematics and evolution as this particular frog-polystome association may not only provide significant information on the biogeographical origins of Malagasy frogs (Verneau et al., 2009b), but also, ultimately, aid in their conservation as discussed by Berthier et al. (2014) for the host *Guibemantis liber*.

In January 2005 during a herpetological survey conducted in Madagascar (Fig. 1a), two frog species examined for polystomes were found to be infected with two distinct *Madapolystoma* morphotypes. *Blommersia domerguei* Guibé, 1974 (Fig. 1b) was collected from the Ambohitantely Special Reserve (Fig. 1a) and *Mantella expectata* Busse and Böhme, 1992 (Fig. 1c) was collected in the Isalo region (Fig. 1a). Since the discovery of these two groups of parasites, the collection of additional specimens of *B. domerguei* and *M. expectata* has been hampered by administration difficulties for sampling amphibians in Madagascar, and because of the conservation status of the second species. Therefore, despite the low sample size, we now describe the two new species herein since it is unlikely that we will have the opportunity to collect additional material in the foreseeable future.

2. Material and methods

2.1. The hosts

Blommersia domerguei and *M. expectata* are both small frogs of the family Mantellidae, which is the most diverse amphibian family in Madagascar (Glaw and Vences, 2006). *Blommersia domerguei* is known from six small areas along the east coast of Madagascar (Fig. 1a). It occurs in swamps at a relatively high altitude (Glaw and Vences, 2007) and its conservation status is considered to be “Least Concern” (IUCN, 2017). Species in this genus lay their eggs against structures overhanging ponds or streams (Glaw and Vences, 2007). In contrast, *M. expectata* is listed as “Endangered” (IUCN, 2017) and is known from only a small geographical area in the dry sandstone massif near Isalo (Glaw and Vences, 2007) (Fig. 1a). The majority of *Mantella* species lay their eggs in excavated terrestrial nests. After flooding, tadpoles leave the nest and move to ponds or streams (Glaw and Vences, 2007).

2.2. Host and parasite sampling

Fifteen adult specimens of *B. domerguei* were collected in the Ambohitantely Special Reserve in Madagascar in January 2005. Frogs were collected by hand and temporarily kept in clear plastic bags containing plant material and water, until dissection. The six specimens of *M. expectata* used in this study were obtained from an exporter in Antananarivo who collected the frogs at Isalo during the same period. Prior to dissection, frogs were anesthetized and subsequently killed with MS222 (ethyl-4-aminobenzoate). Dissection and internal inspection were performed using a Nikon SMZ-645 dissecting microscope. The urinary bladder and kidneys were removed and inspected for worms in a small glass Petri dish containing 0.6% Ringers solution. Adult parasites were fixed in 10% buffered formalin under coverslip pressure while most of the subadult polystomes were mounted in ammonium picrate glycerine. Some of the juveniles were preserved in absolute ethanol for molecular studies. Adult polystomes were washed free of fixatives in tap water and stained overnight in a weak acetocarmine

solution, dehydrated, cleared in xylene and mounted in Canada balsam.

2.3. Sequence analysis

28S rDNA sequences of *Madapolystoma* spp. that were reported in Verneau et al. (2009b) and Berthier et al. (2014) were obtained from Genbank (Table 1). Sequences of *K. manampoka, Eupolystoma alluaudi* (de Beauchamp, 1913) and *Eupolystoma vanasi* Du Preez et al., 2003, were also selected for rooting the tree according to Raharivololoniaina et al. (2011). Sequence alignment was done with the help of ClustalW (Thompson et al., 1994) implemented in the MEGA software version 7 (Kumar et al., 2016) with regard to the 28S ribosomal secondary structure defined for polystome species (Badets et al., 2011; Héritier et al., 2015).

To depict the relationships within *Madapolystoma*, a Minimum Evolution (ME) tree was inferred from the MEGA software, based upon the calculation of the Kimura 2-parameter distance after excluding gaps and partially sequenced regions in the final alignment (complete deletion option). One thousand replications were completed to evaluate the robustness of the nodes. Finally, genetic divergences (uncorrected p-distances) as well as total differences were determined for species delimitation following the complete deletion option in MEGA-7.

2.4. Morphology and morphometry

Specimens were examined using a Nikon NiE compound microscope (Nikon, Netherlands) fitted with a Nikon DS-Ri1 digital camera. Morphological structures and organs were measured in micrometres using a Nikon NIS elements D software program. Marginal hooklets were measured and plotted according to the procedure of Du Preez and Maritz (2006), in order to discriminate distinct species.

3. Results

3.1. Phylogenetic relationships and genetic divergences

Regarding the ME tree (Fig. 2), a sister species relationship was unambiguously evidenced between *M. biritika* and the undescribed species of *Madapolystoma* from *B. domerguei*, with bootstrap support of 100%. Considering the 1.2% genetic divergence that was calculated between the two polystomes (Table 2), we consider that they are separate species according to the 28S species-level threshold defined by

Table 1
Polystome species investigated, host species, geographical locations and 28S Genbank accession numbers.

Polystome species	Host species	Location	Genbank Accession Number
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: Andasibe	JN800271
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: An'Ala	JN800272
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: Ranomafana	JN800273
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: Ranomanafakely	JN800274
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: An'Ala	FM897276
Madapolystoma ramilijaonae	*Guibemantis liber*	Madagascar: Andasibe	FM897277
Madapolystoma cryptica	*Guibemantis liber*	Madagascar: Tsaratanana	JN800275
Madapolystoma cryptica	*Guibemantis liber*	Madagascar: Andranomapanga	JN800276
Madapolystoma cryptica	*Guibemantis liber*	Madagascar: Ambohitantely	JN800277
Madapolystoma cryptica	*Guibemantis liber*	Madagascar: Makira	JN800278
Madapolystoma sp.	*Guibemantis liber*	Madagascar: Andrakata	JN800279
Madapolystoma sp.	*Guibemantis liber*	Madagascar: Montagne d'Ambre	JN800280
Madapolystoma biritika	*Mantella baroni*	Madagascar: Unknown locality	FM897278
Madapolystoma sp.	*Blommersia wittei*	Madagascar: Isalo	FM897273
Madapolystoma sp.	*Gephyromantis sculpturatus*	Madagascar: An'Ala	FM897274
Madapolystoma sp.	*Gephyromantis sculpturatus*	Madagascar: An'Ala	FM897275
Madapolystoma sp.	*Blommersia blommersae*	Madagascar: An'Ala	FM897271
Madapolystoma sp.	*Blommersia domerguei*	Madagascar: Ambohitantely	FM897272
Madapolystoma sp.	*Mantella expectata*	Madagascar: Isalo	FM897279
Kankana manampoka	*Cophyla pollicaris*	Madagascar: Ranomafana	HM854293
Eupolystoma vanasi	*Schismaderma carens*	South Africa	AM157200
Eupolystoma alluaudi	*Bufo* sp.	Togo	AM157199

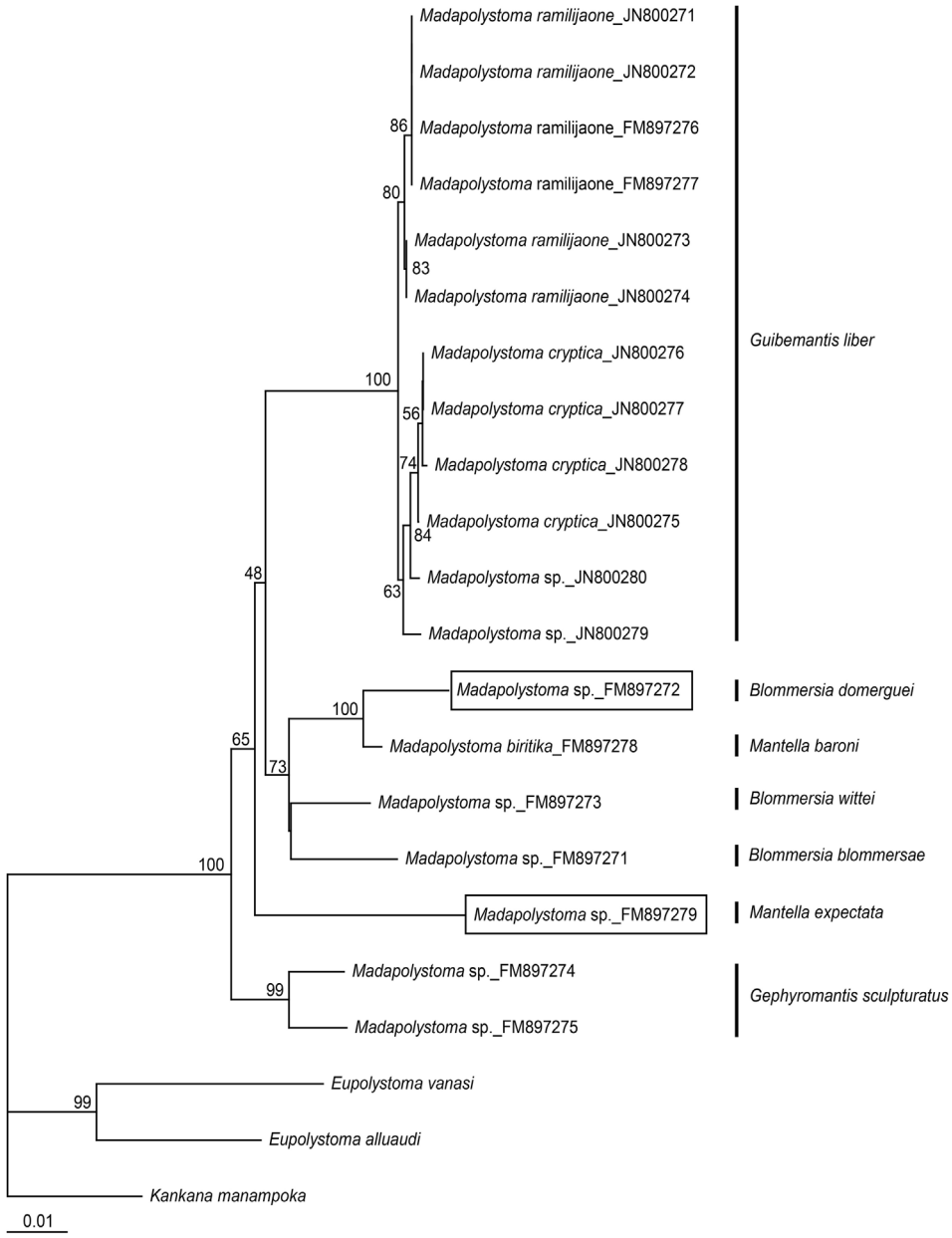


Fig. 2. Minimum Evolution tree for *Madapolystoma* spp. Numbers on nodes indicate bootstrap support values. *Madapolystoma* sp. from *B. domerguei* refers to *M. magnahami* n. sp. and *Madapolystoma* sp. from *M. expectata* refers to *M. isaloensis* n. sp.

Du Preez et al. (2007) for amphibian polystomes, which was estimated to about 0.07%. Furthermore, 22 substitutions were observed between these two polystomes following pairwise sequence comparisons, among which 11 corresponded to individual changes in the undescribed species of *Madapolystoma* from *B. domerguei*, suggesting it is a distinct species.

The undescribed species of *Madapolystoma* from *M. expectata* occupied a more basal position within *Madapolystoma*, being in an intermediate position between two undescribed polystomes from *Gephyromantis sculpturatus* (Ahl, 1929) and all other polystome spp., however with low bootstrap support (Fig. 2). Because the genetic divergences between this polystome and the remaining polystomes ranged from 5.46 to 6.32% (Table 2), it is likely that this polystome is also a separate species according to the 28S species-level threshold defined by Du Preez et al. (2007). Similarly, 70 to 81 substitutions were observed between this polystome and all others, among which 25 corresponded to unique changes, thus supporting our conclusion regarding its systematic status.

3.2. Taxonomic summary of *Madapolystoma magnahami* n. sp. (Fig. 3; Table 3)

3.2.1. Classification

Class Monogenea van Beneden, 1858, Order Polystomatidea Lebedev, 1988, Family Polystomatidae Gamble, 1896.

Genus *Madapolystoma* Du Preez et al., 2010.

3.2.2. Type host

Blommersia domerguei (Mantellidae).

3.2.3. Type locality

Ambohitantely Special Reserve, Madagascar (18,166667S; 47,273333E).

Table 2
Matrix of p-distances (upper right) and total differences (lower left) inferred from pairwise comparisons of 28S sequences.

	JN800271	JN800272	JN800273	JN800274	FM897276	FM897277	JN800275	JN800276	JN800277	JN800278	JN800279
*Madapolystoma ramilijaonae*_JN800271		0.0000	0.0016	0.0016	0.0000	0.0000	0.0055	0.0062	0.0062	0.0070	0.0062
*Madapolystoma ramilijaonae*_JN800272	0		0.0016	0.0016	0.0000	0.0000	0.0055	0.0062	0.0062	0.0070	0.0062
*Madapolystoma ramilijaonae*_JN800273	2	2		0.0000	0.0016	0.0016	0.0055	0.0062	0.0062	0.0070	0.0047
*Madapolystoma ramilijaonae*_JN800274	2	2	0		0.0016	0.0016	0.0055	0.0062	0.0062	0.0070	0.0047
*Madapolystoma ramilijaonae*_FM897276	0	0	2	2		0.0000	0.0055	0.0062	0.0062	0.0070	0.0062
*Madapolystoma ramilijaonae*_FM897277	0	0	2	2	0		0.0055	0.0062	0.0062	0.0070	0.0062
*Madapolystoma cryptica*_JN800275	7	7	7	7	7	7		0.0008	0.0008	0.0016	0.0055
*Madapolystoma cryptica*_JN800276	8	8	8	8	8	8	1		0.0000	0.0008	0.0062
*Madapolystoma cryptica*_JN800277	8	8	8	8	8	8	1	0		0.0008	0.0062
*Madapolystoma cryptica*_JN800278	9	9	9	9	9	9	2	1	1		0.0070
Madapolystoma sp._JN800279	8	8	6	6	8	8	7	8	8	9	
Madapolystoma sp._JN800280	7	7	7	7	7	7	4	5	5	4	7
*Madapolystoma biritika*_FM897278	55	55	53	53	55	55	55	56	56	57	56
Madapolystoma sp._FM897273	52	52	52	52	52	52	52	53	53	54	55
Madapolystoma sp._FM897274	61	61	61	61	61	61	61	62	62	63	62
Madapolystoma sp._FM897275	58	58	60	60	58	58	60	61	61	62	61
Madapolystoma sp._FM897271	59	59	57	57	59	59	59	60	60	61	60
Madapolystoma sp._FM897272	68	68	67	67	68	68	69	70	70	70	71
Madapolystoma sp._FM897279	76	76	74	74	76	76	77	78	78	78	78
*Kankana manampoka*_HM854293	110	110	108	108	110	110	109	110	110	109	111
*Eupolystoma vanasi*_AM157200	145	145	143	143	145	145	144	145	145	144	146
*Eupolystoma alluaudi*_AM157199	134	134	132	132	134	134	134	135	135	136	134

	JN800280	FM897278	FM897273	FM897274	FM897275	FM897271	FM897272	FM897279	HM854293	AM157200	AM157199
*Madapolystoma ramilijaonae*_JN800271	0.0055	0.0429	0.0406	0.0476	0.0453	0.0461	0.0531	0.0593	0.0859	0.1132	0.1046
*Madapolystoma ramilijaonae*_JN800272	0.0055	0.0429	0.0406	0.0476	0.0453	0.0461	0.0531	0.0593	0.0859	0.1132	0.1046
*Madapolystoma ramilijaonae*_JN800273	0.0055	0.0414	0.0406	0.0476	0.0468	0.0445	0.0523	0.0578	0.0843	0.1116	0.1030
*Madapolystoma ramilijaonae*_JN800274	0.0055	0.0414	0.0406	0.0476	0.0468	0.0445	0.0523	0.0578	0.0843	0.1116	0.1030
*Madapolystoma ramilijaonae*_FM897276	0.0055	0.0429	0.0406	0.0476	0.0453	0.0461	0.0531	0.0593	0.0859	0.1132	0.1046
*Madapolystoma ramilijaonae*_FM897277	0.0055	0.0429	0.0406	0.0476	0.0453	0.0461	0.0531	0.0593	0.0859	0.1132	0.1046
*Madapolystoma cryptica*_JN800275	0.0031	0.0429	0.0406	0.0476	0.0468	0.0461	0.0539	0.0601	0.0851	0.1124	0.1046
*Madapolystoma cryptica*_JN800276	0.0039	0.0437	0.0414	0.0484	0.0476	0.0468	0.0546	0.0609	0.0859	0.1132	0.1054
*Madapolystoma cryptica*_JN800277	0.0039	0.0437	0.0414	0.0484	0.0476	0.0468	0.0546	0.0609	0.0859	0.1132	0.1054

Table 2 (*continued*)

	JN800280	FM897278	FM897273	FM897274	FM897275	FM897271	FM897272	FM897279	HM854293	AM157200	AM157199
*Madapolystoma cryptica*_JN800278	0.0031	0.0445	0.0422	0.0492	0.0484	0.0476	0.0546	0.0609	0.0851	0.1124	0.1062
Madapolystoma sp._JN800279	0.0055	0.0437	0.0429	0.0484	0.0476	0.0468	0.0554	0.0609	0.0867	0.1140	0.1046
Madapolystoma sp._JN800280		0.0445	0.0422	0.0492	0.0484	0.0476	0.0546	0.0609	0.0851	0.1132	0.1069
*Madapolystoma biritika*_FM897278	57		0.0289	0.0461	0.0484	0.0312	0.0172	0.0554	0.0835	0.1054	0.0968
Madapolystoma sp._FM897273	54	37		0.0414	0.0453	0.0304	0.0398	0.0546	0.0781	0.1046	0.0952
Madapolystoma sp._FM897274	63	59	53		0.0187	0.0429	0.0570	0.0570	0.0710	0.0991	0.0874
Madapolystoma sp._FM897275	62	62	58	24		0.0476	0.0593	0.0617	0.0788	0.1062	0.0960
Madapolystoma sp._FM897271	61	40	39	55	61		0.0445	0.0546	0.0773	0.1046	0.0898
Madapolystoma sp._FM897272	70	22	51	73	76	57		0.0632	0.0929	0.1124	0.1038
Madapolystoma sp._FM897279	78	71	70	73	79	70	81		0.0937	0.1101	0.1062
*Kankana manampoka*_HM854293	109	107	100	91	101	99	119	120		0.0734	0.0601
*Eupolystoma vanasi*_AM157200	145	135	134	127	136	134	144	141	94		0.0625
*Eupolystoma alluaudi*_AM157199	137	124	122	112	123	115	133	136	77	80	– >

3.2.4. Site in host

Mature parasites were found in the urinary bladder while immature stages were found in both urinary and accessory bladders.

3.2.5. Level of infection

Of the 15 specimens of *B. domerguei* that were collected, ten frogs were infected by two mature and 27 juvenile parasites, of which nine were found in the accessory bladder (prevalence 67%; mean intensity 2.7).

3.2.6. Type-material

Morphological description based on two mature and 20 immature specimens. Two sexually mature specimens (holotype NMBP 474 and paratype NMBP 475) as well as six immature specimens (paratypes NMBP 476 –NMBP 481) from a single locality i.e., Ambohitantely Special Reserve (Fig. 1a). Types are deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.2.7. Voucher material

The remaining specimens were deposited in the polystome collection of the North-West University, Potchefstroom, South Africa.

3.2.8. Zoobank

The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:4C10D3CF-44C2-4DB4-90B9-648C1F1D0CE1. The LSID for the new name *Madapolystoma magnahami* n. sp. is urn:lsid:zoobank.org:act:EB537C95-A9E2-4BBE-BB0D-E68725F30D10.

3.2.9. Etymology

The species epithet *magnahami* is a combination of two latin words, namely *magna* and *hamus*, meaning respectively great and hook. This refers to the large marginal hooklets of this species that are larger than those of all the other known species of *Madapolystoma*.

3.2.10. Description

Measurements in micrometres for mature parasites are given in Table 3. Body pyriform with widest point about two-thirds from anterior extremity (Fig. 3a and b), Mouth subterminal and surrounded by false oral sucker. Pharynx longer than wide. Intestine bifurcates, converging posteriorly; no prehaptoral anastomoses. Testis position unclear but probably in posterior half of body proper as vas deferens extends into posterior half of body proper; vas deferens widens anteriorly to form seminal vesicle, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal caeca bifurcation; genital atrium muscular, armed with six genital spines. Genital spines of both adult parasites were not measurable but measurable in subadult specimens. Ovary position unclear but based on position of reproductive ducts probably in midbody. Two vaginae, on lateral margins, with marginal opening; vaginal vestibule cup-shape. No distinct vitellaria observed; few small clusters of what appear to be granular vitelline follicles in posterior half of body. Genito-intestinal canal present and prominent; situated behind confluent vitelline duct. Uterus sac-like holds five and eight embryos, respectively. Embryos not ciliated, encapsulated in thin membrane. Four embryos in advanced stage of development with two pairs of suckers and developing hamuli clearly visible (Fig. 3a and b). Darker patch of cells observed at midbody in more developed embryos (Fig. 3a). Embryos 224–391 long and 152–168 wide. Sucker pair 1 of embryos 39–64 in diameter and sucker pair two 41–47. Haptor of adult parasite with three pairs of suckers. Hamuli well developed; without deep cut between handle and guard (Fig. 3c). Marginal hooklet pairs 1 and 2 located along periphery between posterior-most pair of suckers while marginal hooklet pairs 3–5 imbedded in suckers; marginal hooklet pairs 6–8 located anteriorly in haptor between sucker pair 3. Posterior-most marginal hooklet 1 and marginal hooklets 2–8 almost of equal length

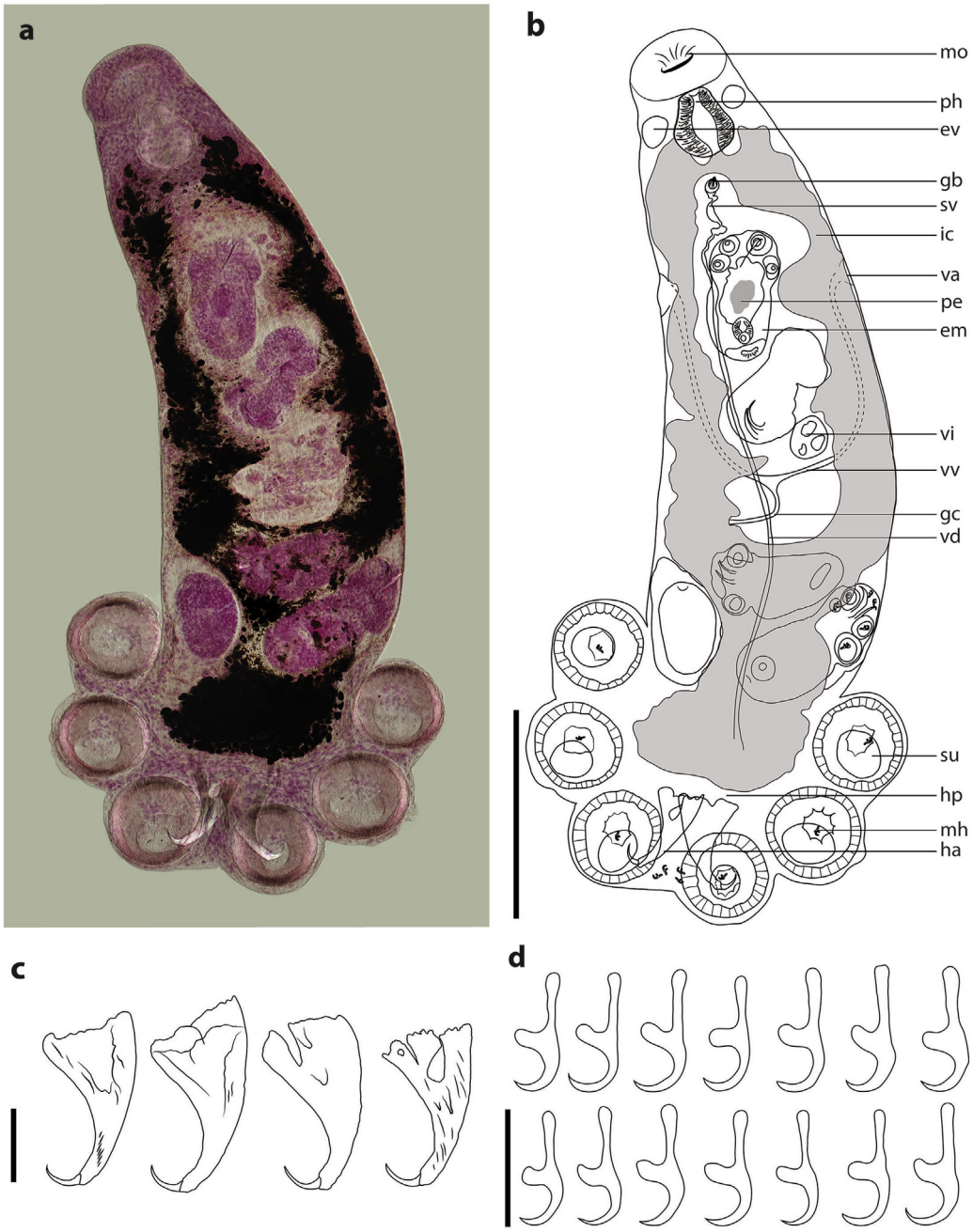


Fig. 3. a–b) Ventral view of *M. magnahami* n. sp. holotype. (c) Hamuli from mature specimens and (d) Marginal hooklets 1 (top) and 2–8 (bottom). Scale bars: B, 500 µm; C, 100 µm; D, 25 µm. Abbreviations: em, embryo; ev, excretory vessel; gb, genital bulb; gc, genito-intestinal canal; ha, hamuli. hp, haptor; ic, intestinal caecum; mh, marginal hooklet; mo, mouth; pe, potential embryo; ph, pharynx; su, sucker; sv, seminal vesicle; va, vagina; vd, vas deferens; vi, vitelline follicles; vv, vitelline duct.

(Fig. 3d).

3.3. Taxonomic summary of Madapolystoma isaloensis n. sp. (Fig. 4; Table 3)

3.3.1. Type host

Mantella expectata (Mantellidae).

3.3.2. Type locality

Isalo, Madagascar (coordinates not known).

3.3.3. Site in host

Mature parasite was found in the urinary bladder while immature stages were found in both urinary and accessory bladders.

3.3.4. Level of infection

All six specimens of *M. expectata* examined were infected by as many as nine subadult parasites. One mature and 24 juvenile polystomes were recovered. This resulted in a prevalence of 100%, with a mean intensity of 5.33.

3.3.5. Type material

Morphological description are based on one mature and 19 immature specimens. The type series comprises one sexually mature specimen (holotype NMBP 482) and six immatures (paratype NMBP 483–488) from a single locality, Isalo (Fig. 1a). Types are deposited in

Table 3
Body measurements in micrometres for all known *Madapolystoma* spp.

	Madapolystoma magnahami n. sp.	*Madapolystoma isaloensis* n. sp.	*Madapolystoma cryptica*	*Madapolystoma biritika*	*Madapolystoma ramilijaonae*
Total length	2179; 2340	2672	1151 (1027–1239)	2041 (1304–3041)	2948 (1493–3481)
Greatest width	507; 597	871	429 (411–439)	512 (420–597)	705 (602–857)
Haptor length - Body length to ratio	0.29	0.23	0.49	0.33	0.35
Width at vagina	414; 457	683		481 (369–548)	
Haptor length	550; 648	618	387 (337–424)	679 (594–788)	661 (567–771)
Haptor width	769; 934	877	475 (431–550)	882 (707–1046)	922 (765–1013)
Hamulus length X	239 (231–244)	173; 228	227 (215–239)	215 (190–238)	179 (163–195)
Hamulus length Y	204 (193–212)	183; 202	208 (197–219)	199 (175–223)	178 (154–193)
Hamulus hook length	48 (47–49)	46; 49	36 (31–39)	45 (34–50)	39 (31–47)
Oral disk	157; 161	134	71 (58–83)	160 (95–206)	123 (90–156)
Pharynx length	156; 166	149	94	136 (117–164)	175 (164–184)
Pharynx width	140; 153	130	58	119 (105–125)	154 (151–156)
Genital bulb diameter	15	27	31	20 (18–24)	33 (30–38)
Number of genital spines	6	7	7	8	5–8
Genital spine length	17 (16–17)	7.9 (7.4–8.4)	14	10.7 (10.5–10.9)	15 (14–16)
Sucker diameter	209 (193–221)	207 (201–216)	160 (122–186)	200 (160–255)	216 (188–244)
Maximum no of developing eggs or embryos *in utero*	5	0	11	1–32	21
Marginal hooklet 1 length	27.6 (26.6–28.4)	25.5 (24.6–26.6)	23.1 (21.6–24.7)	24.2 (21.4–26.1) 24.6 (20.4–26.9) 23.8 (21.2–26.1)	23.0 (20.6–26.2)
Marginal hooklet 2–8 length	27.0 (26.3–27.4)	25.8 (26.4–26.7)			

the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.3.6. Voucher material

The remaining specimens were deposited in the polystome collection of North-West University, Potchefstroom, South Africa.

3.3.7. Zoobank

The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:4C10D3CF-44C2-4DB4-90B9-648C1F1D0CE1. The LSID for the new name *Madapolystoma isaloensis* n. sp. is urn:lsid:zoobank.org:act:744DAD34-F102-4946-9135-813DD528118A.

3.3.8. Etymology

The species epithet refers to the type locality, Isalo.

3.3.9. Description

Measurements in micrometres for mature parasites are given in Table 3. Body elongate with widest point just anterior to the haptor (Fig. 4a and b); anterior mouth and posterior haptor with three pairs of suckers and pair of hamuli posteriorly between posterior-most sucker pair. Mouth subterminal surrounded by false oral sucker. Pharynx longer than wide. Intestine bifurcates, converging posteriorly; no prehaptoral anastomoses. Testis position unclear but probably posterior in body proper as vas deferens extends into posterior half of body proper; vas deferens widens anteriorly to form seminal vesicle, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal caeca bifurcation; genital atrium muscular; armed with seven genital spines. No distinct vitellaria observed; a few small clusters of what appears to be granular vitelline follicles in posterior half of body. Two vaginae, on lateral margins, with marginal opening; vaginal vestibule cup-shaped. Genito-intestinal canal present, prominent; situated behind confluent vitelline duct. Ovary position unclear but based on the position of reproductive ducts probably in midbody. Uterus sacciform, extending from genital bulb backwards full length of body proper. Haptor with three pairs of suckers. Two hamuli well developed; without cut between handle and guard (Fig. 4c). It was not possible to distinguish between marginal hooklets one and two on holotype but these were measured on juvenile paratypes (Fig. 4d). Marginal hooklet pairs 1 and 2 located along periphery between posterior-most pair of suckers; marginal hooklet pairs 3–5 imbedded in the suckers obscured and not measurable; marginal hooklet pairs 6–8 located anteriorly in haptor between sucker pair 3.

3.4. Marginal hooklet morphometrics

Marginal hooklet morphometric measurements separated *M. magnahami* n. sp. of *B. domerguei* from all the other known species of *Madapolystoma*, including *M. isaloensis* n. sp. of *M. expectata*, as no overlaps were evidenced in the scatterplot (Fig. 5). However, whereas marginal hooklet morphometric measurements separated *M. isaloensis* n. sp. from *M. ramilijaonae*, they did not clearly separate it from *M. biritika* and *M. cryptica* (Fig. 5).

3.5. Remarks

The phylogenetic position of the two undescribed species of polystomes found among Madagascan frogs clearly indicated they could be both assigned to the genus *Madapolystoma* of the family Polystomatidae. Regarding the genetic divergences estimated within *Madapolystoma* (Table 2) and private changes observed within each molecular lineage, i.e. *Madapolystoma* sp. of *B. domerguei* and *Madapolystoma* sp. of *M. expectata*, molecular results thus supported the morphological description of two new species, i.e. *M. magnahami* n. sp. from *B. domerguei* and *M. isaloensis* n. sp. from *M. expectata*. The most significant morphological characteristics that distinguish *M. magnahami* n. sp. and *M. isaloensis* n. sp. from each other and from the three other known *Madapolystoma* spp. (*M. biritika*, *M. madagascariensis* and *M. cryptica*) are the size and shape of marginal hooklets and the number and size of genital spines (Table 3).

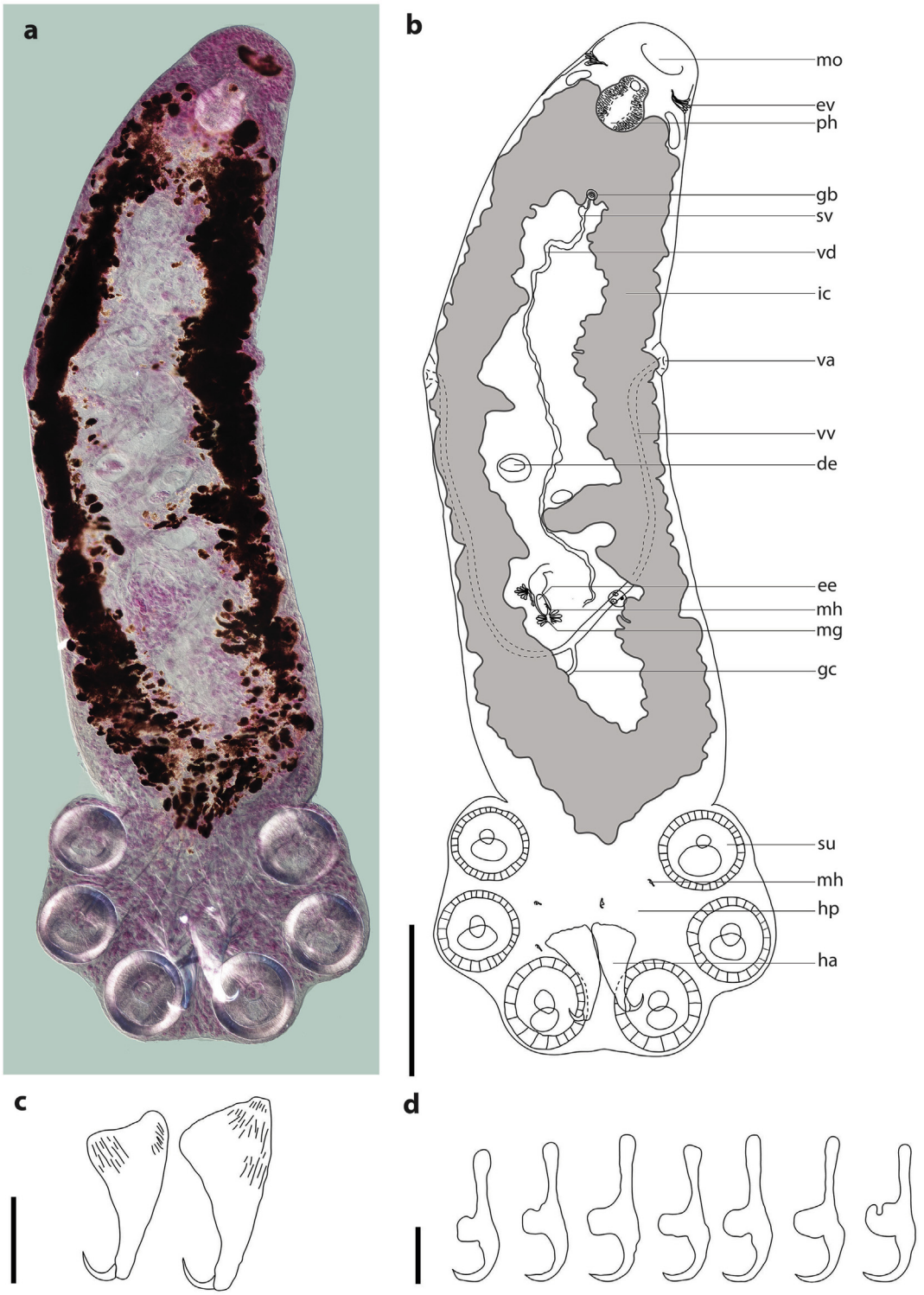


Fig. 4. a–b) Ventral view of *M. isaloensis* n. sp. holotype. (c) Hamuli from mature specimens and (d) Marginal hooklets 1–8. Scale bars: B, 200 µm; C, 100 µm; D, 20 µm. Abbreviations: de, developing embryo; ee, early embryo; ev, excretory vessel; gb, genital bulb; gc, genito-intestinal canal; ha, hamuli. hp, haptor; ic, intestinal caecum; mh, marginal hooklet; mo, mouth; ph, pharynx; su, sucker; sv, seminal vesicle; va, vagina; vd, vas deferens; vi, vitelline follicles; vv, vitelline duct.

4. Discussion

In species of *Madapolystoma* marginal hooklets C1–C8 were found to be of equal length. This phenomenon has also been reported for species of *Eupolystoma* and *Kankana*. This is in contrast with the usual situation encountered in species of *Polystoma* and most other polystomes where the posteriormost hooklet pair is significantly larger than the rest (see for instance Tinsley, 1973, 1974; Du Preez and Kok, 1993, 1995; Du Preez et al., 2002; Aisien et al., 2011; Du Preez, 2011, 2013). Therefore, this measure may be a good character for species delimitation in *Madapolystoma*. Marginal hooklet morphometrics (Fig. 5) were thus useful in separating *M. magnahami* n. sp. from *M. isaloensis* n. sp. and from all the other known *Madapolystoma* spp. with 95% confidence. *Madapolystoma magnahami* n. sp. currently has the largest marginal hooklets of

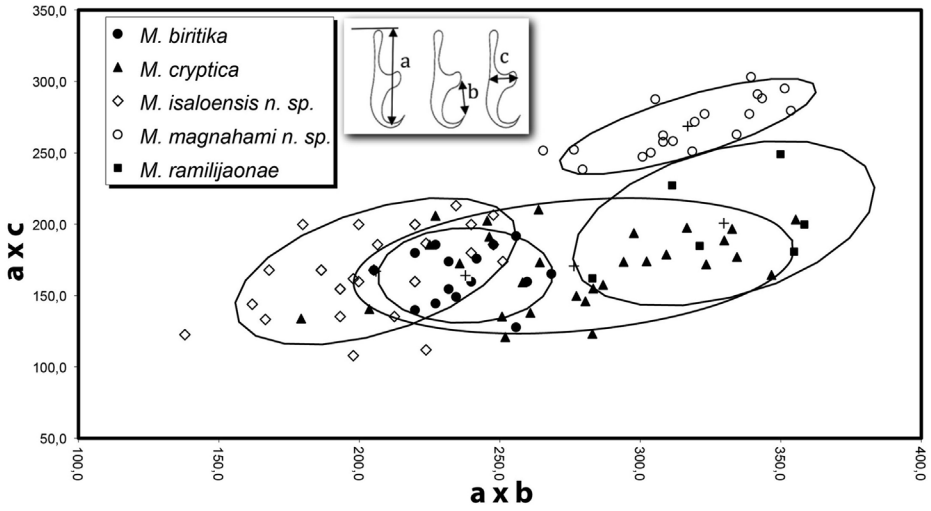


Fig. 5. Scatter diagram of a × c plotted against b × c for all known *Madapolystoma* spp., *M. magnahami* n. sp. and *M. isaloensis* n. sp. The ellipses represent 95% of the confidence interval about the mean.

all known species in the genus. However, the measurements of marginal hooklets were not able to isolate *M.* isaloensis n. sp. from *M. cryptica* nor *M. biritika*. They did however separate *M. isaloensis* n. sp. from *M. magnahami* n. sp. and *M. ramilijaonae.* Regarding the number of genital spines in *M. magnahami* n. sp., although it overlapped with that reported in *M. ramilijaonae*, the length of genital spines was larger on average than for *M. ramilijaonae* (Berthier et al., 2014). Though the number of genital spines recorded in *M. isaloensis* n. sp. overlapped with that reported in *M. cryptica*, length of genital spines was larger than that of *M. cryptica* (Berthier et al., 2014).

The value of sclerotized structures in the description of soft-bodied parasites such as polystomes has been emphasised (Du Preez and Maritz, 2006) and although some taxonomists advocate that polystomes should not be flat fixed (Platt et al., 2011), it is of utmost importance to observe sclerotized structures in flat orientation. Fixing specimens under cover slip pressure does not affect the measurement of sclerites or smaller rigid structures such as the oral sucker, genital bulb or even, in some taxa, the haptoral suckers (Platt et al., 2011). When sufficient material is available we recommend that (1) a specimen be fixed in high quality ethanol or a fixative such as RNALater for DNA extraction; (2) some of the specimens be heat-killed by placing them in a drop of water on a microscope slide that is then heated from below with a butane lighter until the parasite stops moving followed by fixation in 10% buffered formalin and (3) remainder of the specimens to be fixed in 10% buffered formalin under coverslip pressure. Body measurements and placement of organs should be studied from the unflattened specimens while sclerites should be measured in flattened specimens. However, in instances where a limited number of specimens are available, such as here we do recommend fixing specimens flat under coverslip pressure.

Species of *Diplorchis, Eupolystoma*, *Kankana*, *Neodiplorchis*, *Parapolystoma*, *Pseudodiplorchis* and *Sundapolystoma* all have extended uteri (Du Preez et al., 2003; Raharivololoniaina et al., 2011) allowing for the accumulation of large numbers of eggs and/or *in utero* development. Therefore repeated re-infection of a single host may occur either during breeding events, after releasing larvae, or following an internal life-cycle inside the host. In the latter case the oncomiracidium does not leave the host but attaches to the bladder wall alongside its parent inside the urinary bladder. In *Eupolystoma* it has been shown that both ciliated and unciliated oncomiracidia are produced (Combes et al., 1973; Fournier and Combes, 1979); ciliated oncomiracidia are destined to leave the host to swim and find another host, while unciliated oncomiracidia are destined for an internal cycle. In *Madapolystoma* the *in utero* development is taken a step further in that no ciliated oncomiracidia are produced. Embryos develop gradually into juvenile parasites. In *M. magnahami* n. sp. hamuli and two pairs of suckers (Fig. 3a and b) were observe. In the most advanced *in utero* developing juveniles of *M. magnahami* n.sp. a darker cluster of cells is visible in the middle of the parasites (Fig. 3a). We hypothesize that this cluster of darker cells is a developing F2 embryo, implying true vivipary. To confirm whether *Madapolystoma* is viviparous, histological serial sectioning would be necessary; however no specimens were available for histology. True viviparity has been well documented for the teleost monogenean *Gyrodactylus* (see: Tinsley, 1983; Harris, 1983, 1985; Bakke et al., 2002; Cable and Harris, 2002) and reported for the anuran monogenean *Gyrdicotylus* (see Harris and Tinsley, 1987; Jackson and Tinsley, 1994). Du Preez et al. (2010) and Berthier et al. (2014) reported advanced development of embryos with the presence of developing hamuli and suckers in the embryos but did not mention the development of F2 developing embryos within the F1 generation.

The presence of only a small number of developing embryos in species belonging to *Madapolystoma* indicates a unique reproductive strategy. All the known hosts for *Madapolystoma* namely species of *Blommersia*, *Guibemantis* and *Mantella* deposit their egg clutches terrestrially or semi-terrestrially. While species of *Blommersia* and *Guibemantis* attach their eggs to vegetation or other objects close to water, *Mantella* spp. deposit their eggs in hidden cavities on the ground (Glaw and Vences, 2007). During a field trip to Madagascar in February 2006, a frog egg mass overhanging a pool was collected and inspected under a stereo microscope. A small polystome embryo was observed on the egg mass. It has been documented that frogs laying eggs outside the water may return, at regular intervals, to urinate on the eggs to keep them moist. We therefore hypothesize that a developing embryo in *Madapolystoma* spp. may leave the host during such an event and stay on the egg mass until another frog visits the egg clutch, when it then enters the cloaca and migrates to the accessory bladder from where it migrates to the urinary bladder.

During stock piling of offspring *in utero,* the reproductive capacity of polystomatids is probably determined by body size (Tinsley, 1990). While the total annual egg production of *Polystoma integerrimum* (Fröhlich, 1791), with a length of 10 mm, may be as many as 4000 eggs produced in only a few days (Combes, 1972), in *Pseudodiplorcis americanus* (Rodgers and Kuntz, 1940) with a similar body length it rarely exceeds 300 (Tinsley, 1990). The maximum reported number of eggs and developing embryos in a single individual of *Madapolystoma* spp. is 32 (Du Preez et al., 2010). In the instance of *M. magnahami* n. sp. and *M. isaloensis* n. sp., with their very small body size of less than 2.5 mm, and *in utero* development to a very advanced stage, the annual offspring

production is probably very limited.

Well-defined testis tissue and ovaries could not be located in both *M. magnahami* n. sp. and *M. isaloensis* n. sp., in spite of careful examination with a high-end compound microscope. For species of *Gyrodactylus* it has been reported that the testis develops only after the first embryo is produced and that the female reproductive system develops after the male reproductive system (Bakke et al., 2007). Most polystomatids produce chitinous yellow eggs that develop in the water body after being released from the host. In species where *in utero* development is the norm (i.e. species of *Eupolystoma*, *Kankana*, *Pseudodiplorchis* and *Wetapolystoma* (see Tinsley, 1990; Gray, 1993; Raharivololoniaina et al., 2011) eggs are not encapsulated in a yellow rigid shell, but rather a semi transparent flexible membrane. This allows for direct maintenance of developing larvae through parental nutrients. Whereas vitellaria are distributed throughout most of the body proper in most polystomatids, it is significantly reduced and restricted to lateral fields in species displaying extensive *in utero* development of eggs. For some species of *Eupolystoma* and for *K. manampoka*, the closest relatives to species belonging to *Madapolystoma* (see Raharivololoniaina et al., 2011), the vitellaria are restricted to two narrow lateral streaks posteriorly in the body proper (Du Preez et al., 2003). The advanced *in utero* development as observed in *Madapolystoma* would involve direct maintenance of offspring by parental nutrients which explain the lack of vitellaria fields. According to Bakke et al. (2007), in viviparous forms the vitellaria never fully develop and never produce egg-shell precursor proteins. Vitelline cells in viviparous species appear to be reduced to patches of granular syncitia in the posterior part of the body (Cable et al., 1996). This is in accordance with what we observed for *M. magnahami* n. sp. and *M. isaloensis* n. sp.

Conflicts of interest

The authors declare that there was no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijppaw.2018.09.004.

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First record of *Metapolystoma* (Monogenea: Polystomatidae) from *Boophis* tree frogs in Madagascar, with the description of five new species

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ABSTRACT

Although Madagascar has more than 350 frog species of which all but two are endemic to the island, the known polystome (Monogenea: Polystomatidae) diversity parasitizing Malagasy frogs is low, encompassing five species of *Madapolystoma*, one species of *Kankana* and one *Metapolystoma*. Investigating the parasite diversity of frog parasites at selected Malagasy localities led to the discovery of undescribed polystomes. Five treefrogs, *Boophis albilabris*, *Boophis doulioti*, *Boophis luteus*, *Boophis madagascariensis* and *Boophis occidentalis* were found to be infected and are reported here as hosts for new *Metapolystoma* species. Morphological investigation, combining examination of body length, haptor length, genital bulb width, genital crown diameter, genital spine number, genital spine length, ovary length, egg length, hamulus length, hamulus guard length and hamulus hook length, revealed five distinct morphotypes. Phylogenetic analysis and genetic divergences obtained for three of the five morphotypes, support the distinction of new species. *Metapolystoma ansuanum* n. sp. is described from *B. luteus*, *Metapolystoma falcatum* n. sp. from *B. doulioti*, *Metapolystoma multiova* n. sp. from *B. occidentalis*, *Metapolystoma theroni* n. sp. from *B. madagascareniensis* and *Metapolystoma vencesi* n. sp. from *B. albilabris*. Finally, although the validity of *Metapolystoma* as taxon is not fully resolved yet, the phylogenetic position of the described species and their morphology provide clear evidence for new metapolystome taxa.

1. Introduction

Madagascar is well known for its high species diversity and endemicity, particularly in the cases of plants and vertebrates (Myers et al., 2000). When it comes to amphibian diversity, Madagascar is globally ranked in the top twelve (Andreone et al., 2008). Its unique herpetofauna serve as hosts for an equally unique and diverse assemblage of parasites (see Wohltmann et al., 2007; Junker et al., 2010; Rocha et al., 2012; Kuzmin et al., 2013; Landman et al., 2018). However, little is known about Madagascar's anuran polystome flatworm diversity, since only 86 of the 356 known frog species (Frost, 2020) have been screened for polystomes at a few accessible localities in the past (Verneau et al., 2009). Malagasy polystomes are currently represented by four genera encompassing a single chelonian and seven anuran species, all of these were found in the urinary bladder of their host. *Uropolystomoides chabaudi* (Euzet and Combes, 1965) from *Pelomedusa subrufa* (Bonnaterre, 1789) is the only polystome known from turtles. *Metapolystoma brygoonis* (Euzet and Combes, 1964) was the first polystome found within a Malagasy anuran host, as described from *Ptychadena mascareniensis* (Duméril and Bibron, 1841). Besides these two species, *Kankana manampoka* Raharivololoniaina et al. 2011 was described from *Cophyla pollicaris* (Boulenger, 1888). Five other species of *Madapolystoma* were reported from mantellids, namely *Madapolystoma biritika* Du Preez et al. (2010) from *Mantella madagascariensis* (Grandidier, 1872), *M. isaloensis* Landman et al. (2018) from *Mantella expectata* Busse and Böhme, 1922 and *M. magnahami* Landman et al. (2018) from *Blommersia domerguei* Guibé, 1974. *Madapolystoma cryptica* Berthier et al. 2014 and *M. ramilijaonae* Berthier et al. 2014 were conversely described from the same host species *Guibemantis liber* (Peracca, 1893).

Metapolystoma brygoonis was initially described as *Polystoma* and later elevated (Combes, 1976). This separation of *Metapolystoma* and *Polystoma* was based on morphological characters, including the large

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Table 1
Frog species under survey with their site of collection in Madagascar.

Frog species	No. of specimens collected	Locality
B. madagascariensis	30	Andasibe (Indri Reserve)
B. albilabris	6	Ambatolahy and Andringita massif
B. doulioti	22 frogs, 10 tadpoles	Ankarafantsika
B. luteus	9	Isalo (Cascade des Nymphes Special Reserve)
B. occidentalis	5	Isalo (Cascade des Nymphes Special Reserve)

extended uterus containing many eggs that fills the largest part of the body and the posterior position of the large ovary (Combes, 1976). The validity of *Metapolystoma* as a genus has however been under dispute ever since its nested position within *Polystoma* was demonstrated at the molecular level (Bentz et al., 2001; Verneau et al., 2002; Olson and Tkach, 2005). Up to the present, the three known *Metapolystoma* species have been described from grass frogs belonging to *Ptychadena*: *M. brygoonis* from *P. mascareniensis* in Madagascar, *M. cachani* (Gallien, 1956) from *Ptychadena longirostris* (Peters, 1870) in Ivory Coast and *Metapolystoma porosissimae* du Preez and Kok 1992, from *Ptychadena porosissima* (Steindachner, 1867) in South Africa. It was therefore likely that many Madagascan polystomes awaited discovery (Landman et al., 2018).

Among the 27 anuran genera found in Madagascar (Frost, 2020), *Boophis* Tschudi, 1838 is endemic to Madagascar and the Mayotte Island in the Comoros (Frost, 2020; Glaw and Vences, 2007), hence totalling 79 currently recognised species (Frost, 2020) representing a diverse and species-rich group within the Mantellidae Günter, 1859. During herpetological surveys conducted in Madagascar in 2005, 2006 and 2007, several species of *Boophis* from different localities were examined for polystome parasites. *Boophis albilabris* (Boulenger, 1888), *Boophis doulioti* (Angel, 1934), *Boophis luteus* (Boulenger, 1882), *Boophis madagascariensis* (Peters, 1874) and *Boophis occidentalis* Glaw & Vences, 1994 were found to be infected with five distinct, unknown *Metapolystoma* species. Since the collection of additional materials was constrained by permit restrictions and administrative difficulties in Madagascar, and because preliminary molecular and morphological data converged towards the same result, our objective was to describe new parasites collected from distinct frog species despite their small sample size.

2. Material and methods

2.1. Host and parasite sampling

Annually during February–March of 2005, 2006 and 2007, several *Boophis* species were collected in Madagascar (Table 1). Following collection by hand at night, frogs were individually kept overnight in clear plastic bags containing 50 ml of tap water. After 24 h, the water in which the frogs were kept was poured through two plankton sieves with mesh sizes of 500 and 100 µm respectively. The 500 µm sieve retained course debris and most of the faeces, while the 100 µm sieve retained parasite eggs and fine debris. The content of the 100 µm sieve was then washed into a Petri dish and inspected for the presence of polystome eggs using a dissecting microscope. Infected frogs were euthanized with MS222 (Ethyl-3-aminobenzoate methanesulfonate) and dissected for parasite investigation using a Nikon SMZ-645 dissecting microscope. Where no eggs were observed, two representatives per species per locality were dissected to check for the presence of non egg-producing polystomes. The kidneys, urinary bladder and accessory bladders were removed and inspected in a Petri dish containing 0.6% Ringers solution. For the purpose of molecular studies, some juvenile polystomes were preserved in absolute ethanol. The remainder of the juvenile polystomes were mounted in ammonium picrate glycerine or preserved in 10% NBF. Adult parasites were fixed in 10% NBF under coverslip pressure.

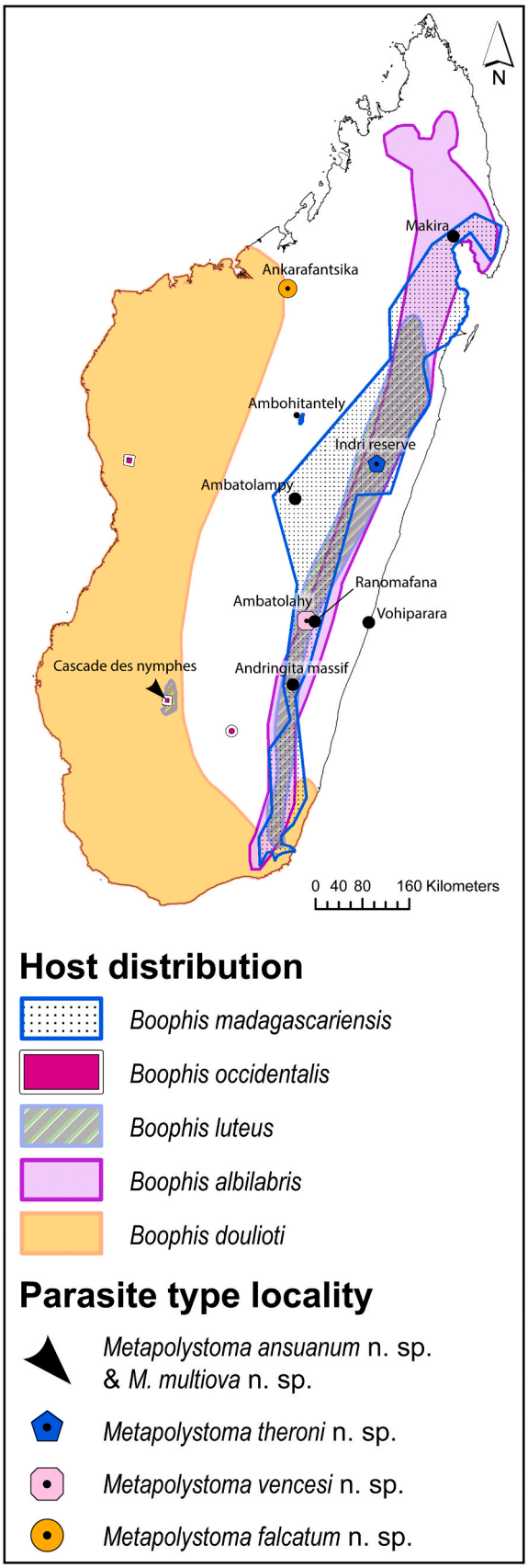


Fig. 1. Map of Madagascar showing parasite type localities and distribution areas of hosts.

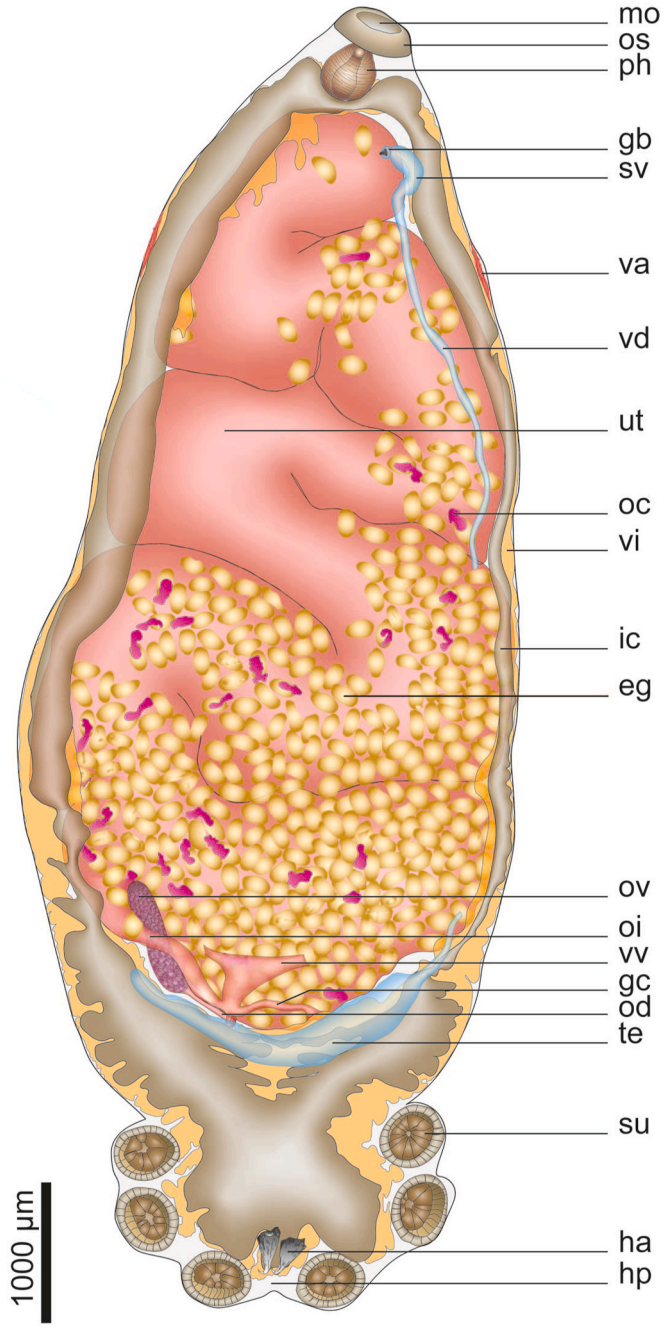


Fig. 2. Ventral view of *Metapolystoma vencesi* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vd, vas deferens; vi, vitelline; vv, vitello–vaginal canal.

Specimens were rinsed in water, stained with acetocarmine, dehydrated, cleared in xylene and mounted in Canada balsam. Five additional, fixed specimens of *B. albilabris* collected in 1972 by Mr. Blane at Andringita Massif, Madagascar, and archived at the Muséum National d'Histoire Naturelle of Paris, were dissected and parasites retrieved and processed as explained above. Host and parasite distribution maps (Fig. 1) were drawn in ArcMap 10.5.1 (Esri, California), and shape files for frog distributions were provided by the IUCN (IUCN, 2016a-e).

2.2. DNA extraction and amplification

All polystome DNA samples used in this study were sourced from Verneau et al. (2009) and Berthier (2011). According to these authors, DNA extractions were conducted in 100–150 µl of 10% Chelex 100 sodium (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) with the proteinase K 1 mg·mL−1 final concentration at 55 °C for the duration of 1 to 1,5 h. Enzymatic reactions were stopped at 100 °C for 15 min and DNA were stored at −20 °C until use. Amplifications of 18S, 28S and COI were conducted following the procedure described in Héritier et al. (2015), regardless of the gene of interest or primers used. The complete 18S rRNA gene and the partial 28S rRNA gene were amplified in two rounds with the respective combinations of primers F18/18Rg and 18F3/IR5 for 18S and LSU5'/IR16 and IF15/LSU3′ for 28S. Primer sequences are reported in Sinnappah et al. (2001), Verneau et al. (2009) and Héritier et al. (2015). COI was amplified using the combination of primers L-CO1p/H-Cox1p2 (Littlewood et al., 1997). All PCR products were sent to the Genoscreen Company (Lille, France) for purification and sequencing with their respective forward and reverse PCR primers. Sequences were read and edited with the software Geneious (Saint Joseph, Missouri, USA) to check chromatograms before use for phylogenetic and distance analyses.

2.3. Phylogenetic and distance analyses

All *Metapolystoma* sequences were edited and aligned independently using Clustal W implemented in MEGA version 7 (Kumar et al., 2016) under default parameters (Thompson et al., 1994). The alignment included five African *Polystoma* species and a single European *Polystoma* species, that is, *Polystoma integerrimum* (Frölich, 1791), which was used as an outgroup according to Bentz et al. (2001). They were subsequently concatenated in a single alignment for Bayesian analysis. The two ribosomal genes were treated as two separate partitions, and the COI genes as three distinct partitions according to their codon position. A two substitution rates model with a proportion of invariable characters was selected for the 18S partition, whereas a GTR + I model was selected for the 28S partition following the Akaike Information Criterion (AIC) implemented in Modeltest 3.06 (Posada and Crandall, 1998). Concerning COI, six types of substitutions and gamma rates each comprising four gamma rate categories were applied to the first and second positions, whereas six types of substitutions and invariable-gamma rates each comprising four gamma rate categories were applied to the third position. Evolutionary parameters were estimated independently for all five partitions. The Bayesian analysis was run using MrBayes 3.04b (Huelsenbeck and Ronquist, 2001), employing four chains running for ten million generations and sampled every 100 cycles. The Bayesian consensus tree was subsequently drawn after removing the first 10 000 trees (10%) as the burn-in phase, and viewed with TreeView version 1.6 (Page, 1996). Corrected pairwise distances were calculated independently for partial 18S, 28S and COI sequences using the Kimura 2-parameter model and 1000 bootstrap replicates in MEGA version 7 (Kimura, 1980).

2.4. Morphology and morphometry

Parasites were examined and photographed using a Zeiss Imager Axio10 compound microscope (Zeiss, Germany) fitted with a Zeiss Axio cam 305 camera (Zeiss, Germany). Morphological structures and organs were measured in micrometres using the Zeiss Zen Blue elements (Zeiss, Germany) software program. Hamuli were respectively measured from the apex behind the hook to the tip of the guard (Length X) and to the handle (Length Y), and the hook from tip to base (Length Z). Full-body images of type specimens were taken using a Nikon AZ100M microscope (Nikon, Netherlands) fitted with a low powered 1× objective. Illustrations were done in Adobe Illustrator CC (Adobe, California). Additional taxonomic measurements for *M. porosissimae* were taken from the type

Table 2
Body measurements in micrometres for all *Metapolystoma* spp. values.

Measurements	*Metapolystoma vencesi* n. sp.	*Metapolystoma falcatum* n. sp.	*Metapolystoma ansuanum* n. sp.	*Metapolystoma theroni* n. sp.	*Metapolystoma multiova* n. sp.
Number of mature specimens	6	1	2	1	2
Total length	9190	7871	2680–3952	9086	4915–5537
Greatest width	3496	3272	1035–1436	3208	2257–2419
Width at vagina	2315	1783	804–1001	2011	1469–2086
Haptor length – Body length ratio	0.16	0.19	0.23–0.25	0.18	0.18
Haptor length	1437	1492	669–912	1634	916
Haptor width	2344	2636	1166–1385	2572	1478
Hamulus length X	269–332 (299 ± 32; 3)	393–407	205	392–408	196–248 (215 ± 29; 3)
Hamulus length Y	225–302 (270 ± 40; 3)	303–314	179	252–257	121–174 (153 ± 23; 4)
Hamulus hook length Z	40–52 (47)	66–70	62	50–55	58.2
Oral disk	391	297	307–329	486	248–271
Pharynx length	302–420	327	256–308	401	302–405
Pharynx width	291–390	290	183–222	385	289–320
Genital bulb diameter	115	86	64–73	65	86
Ovary length	861	772	265–338	479	446
Ovary width	194	196	98–144	385	158
Number of genital spines	8	8	10	7	6
Genital spine length	31.5–33.4 (32.5 ± 0.6; 9)	24.3–31.6 (27.9 ± 1.78; 18)	24.5–28.5 (26.19 ± 1.16; 5)	28.9–34.9 (32.9 ± 4; 3)	40.4–43.7 (42 ± 1.4; 5)
Genital crown diameter	31.8	25.7	29.4–32.8	24.2–27.6 (25.8 ± 1; 10)	24.5
Sucker diameter	366–452(424 ± 37; 12)	435–498(468 ± 29; 6)	241–305(273 ± 25; 12)	404–424 (416 ± 8; 6)	329–356 (339 ± 10; 9)
Maximum number of eggs in utero	367	11	41	176	499+
Egg length	210–230 (219 ± 7; 16)	161–185 (171 ± 8; 11)	196–217 (205 ± 8; 8)	239–265(250 ± 7; 12)	198–228 (212 ± 9; 23)
Egg width	120–147 (130 ± 8; 16)	108–123 (117 ± 5; 11)	119–137 (127 ± 7; 8)	136–152(143 ± 5; 12)	144–165 (154 ± 7; 23)
Marginal hooklet 1 length	29.7–34.2 (32.5 ± 1.3; 13)	32.6–39.7 (36.9 ± 2.3; 13)		34.3–39.4 (936.2 ± 1.4; 21)	28.6–38.7 (33.8 ± 3.3; 11)
Marginal hooklet 2–7 length	20.8–25 (23.6 ± 1; 30)	21.8–27 (23.8 ± 1.52; 30)	22.3–24.79 23.5 ± 1.17; 4)	22.6–27.2 (24.8 ± 1.1; 44)	21.3–26.9 (24.7 ± 2; 17)
Marginal hooklet 8 length	27.4–32.4 (30.5 ± 1.6; 12)	28.8–33.5 (31.3 ± 1.5; 14)		30.5–34.1 (32.2 ± 1; 19)	28.4–36.3 (32.4 ± 2.8; 5)
Widest section from front	57%	66%	16–65%	51%	50%–57%
Times longer than wide	2.7	2.4	2.6–2.8	2.6	2.2–2.3

Measurement	*Metapolystoma porosissimae*[a]	*Metapolystoma brygoonis*[b]	*Metapolystoma cachani*[c]		
Number of mature specimens	15	10	10	3	57
Total length	4150–6710 (5870 ± 0.6; 10)	3400–4800 (4200)	4000	3880–5628 (4594)	3.680–7.250 (5.070)
Greatest width	1570–2360 (2040 ± 0.2; 11)	1220–1500 (1340)	1500	1120–2880 (1793)	1.160–2.880 (1.980)
Width at vagina	1.120–1.700 (1.460 ± 0.2; 11)	650–1030 (800)			880–1.760 (1.220)
Haptor length – Body length ratio	0.26–0.33 (0.29 ± 0.1; 10)	0.22–0.23 (0.22)	0.25	0.17–0.21 (0.19)	0.15–0.32 (0.23)
Haptor length	1.540–1.800 (1.670 ± 01; 12)	760–1070 (900)	1000	720–1072 (864)	910–1.500 (1.15)
Haptor width	2.010–2.660 (2.360 ± 0.2; 12)	1220–1760 (1530)	1500	1248–2800 (1858)	1.300–2.350 (1.730)
Hamulus length X	432–472 (447 ± 14.3; 26)	330–420 (380)	310	280–450 (348)	330–520 (430)
Hamulus length Y	**332.4–385.7 (361.2 ± 18.52; 13)**				
Hamulus hook length Z	**72–86.6 (78.3 ± 3.97; 15)**				
Oral disk	461–566 (521)	220–340 (270)			
Pharynx length	267–348 (310 ± 31.8; 10)				
Pharynx width	234–298 (262 ± 20.4; 10)				
Genital bulb diameter	**93–129.5 (105.7 ± 11.91; 7)**	80	25		
Ovary length	546–755 (651 ± 89.1; 11)	410–460 (440)			
Ovary width	**266**	90–160 (120)			
Number of genital spines	9–10	8	8		
Genital spine length	31/**21.83–25.97 (24.144 ± 1.46; 6)**	30–32 (31)			
Genital crown diameter	**25.18–26.96 (26.35 ± 0.57; 7)**				
Sucker diameter	461–566 (521 ± 30; 65)	260–420 (340)	350–400	272–580 × 288–544 (386 × 379)	300–500 (420)
Maximum number of eggs in utero	250	100–140	70–100	4	8–390 (170)
Egg length	201–229 (214 ± 7.2; 30)	160–200 (180)	190	176–208 (200)	122–159 (0.138)
	192–207 (197 ± 5.1; 10)				
Egg width	113–134 (128 ± 6.6; 30)	90–120 (100)	120	96–112 (101)	
	100–131 (115 ± 9.3; 10)				
Marginal hooklet 1	35				
Marginal hooklet 2–7	22				
Marginal hooklet 8	33				
Marginal hooklet 1–2		31 – 34 (33)	30		
Marginal hooklet 6–8		31			

Values: (Min – Max (Mean ±O; N)), numbers in bold indicate additional measurements taken from species described prior to the present study.
[a] From Du Preez and Kok (1992a).
[b] From Euzet and Combes (1964).
[c] From Gallien (1956) (left), Kulo (1981) (middle) and Murith (1981) (right).

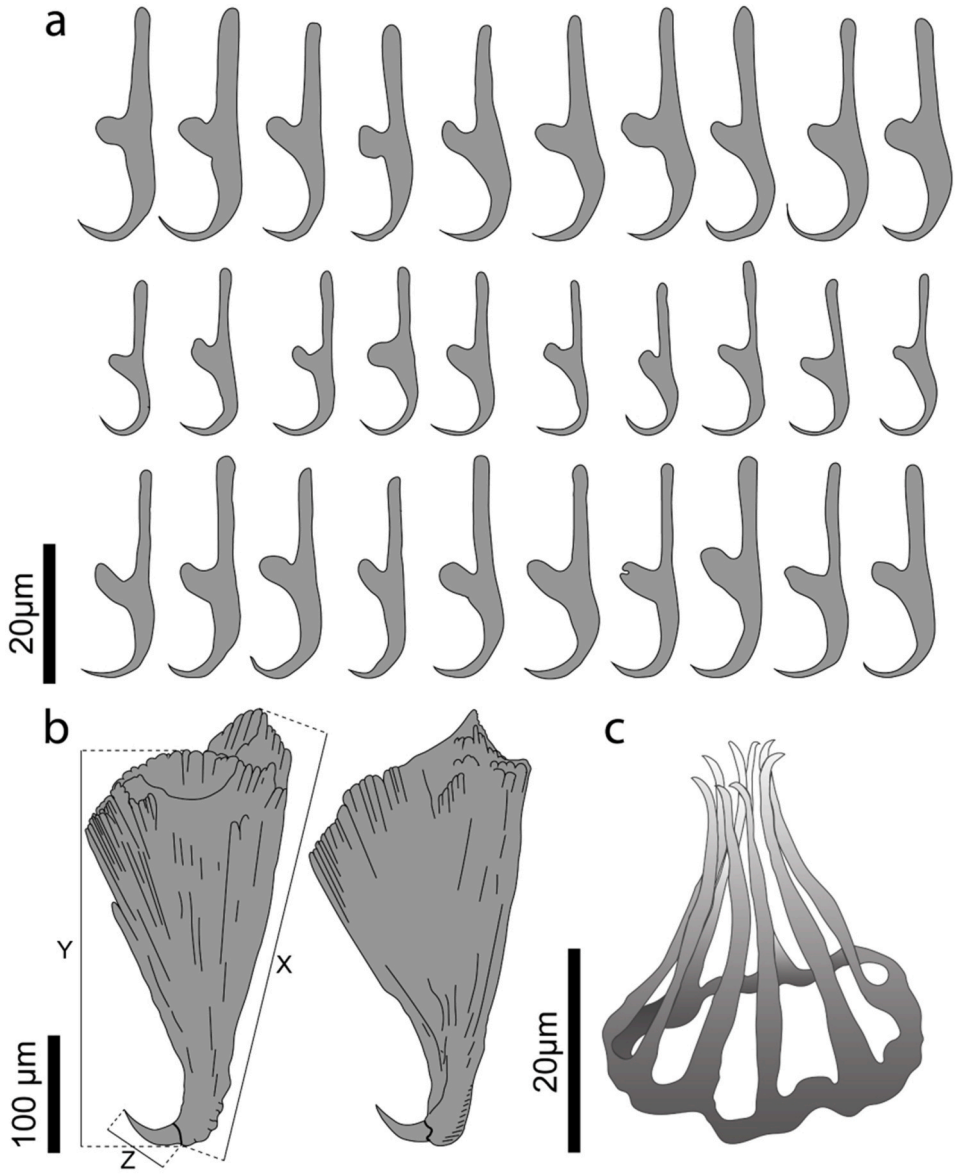


Fig. 3. *Metapolystoma vencesi* n. sp. from *Boophis doulioti*. **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c**, genital crown from holotype. Abbreviations: X, outer length; Y, inner length; Z, hook length.

series (Table 2).

3. Results

3.1. Taxonomic summary

Class: Monogenea van Beneden. 1858 Order: Polystomatidea Lebedev. 1988 Family: Polystomatidae Gamble. 1896 Genus: *Metapolystoma* Combes, 1976.

3.1.1. *Metapolystoma vencesi n. sp.* (*Fig. 2–3; Table 2*)

3.1.1.1. Type host. *Boophis albilabris* (Mantellidae)

3.1.1.2. Type locality. Ambatolahy, Madagascar (Fig. 1), (−21.2438667S; 47.4262167E).

3.1.1.3. Site in host. Urinary bladder.

3.1.1.4. Level of infection. One of six frogs examined from Ambatolahy was infected with one mature and four juvenile parasites (prevalence 16.6%). One of four frogs examined from Ambohitantely was infected with seven mature parasites (prevalence 25%). The prevalence for the combined sample was 20% and the mean intensity 6.5.

3.1.1.5. Type material. The morphological description is based on six mature, ten oncomiracidia and four juvenile parasites. Three specimens were sexually mature (Holotype NMBP 578; Paratypes NMBP 579–580) and two immature (Paratypes NMBP 581–582). The holotype and paratypes NMBP 581 and NMBP 582 originated from Ambatolahy, Madagascar, while paratypes NMBP 579 and NMBP 580 originated from Andringita Massif, Madagascar. Juvenile parasites and oncomiracidia were used for marginal hook measurements and drawings. The type material was deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301, South Africa.

3.1.1.6. Voucher material. Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

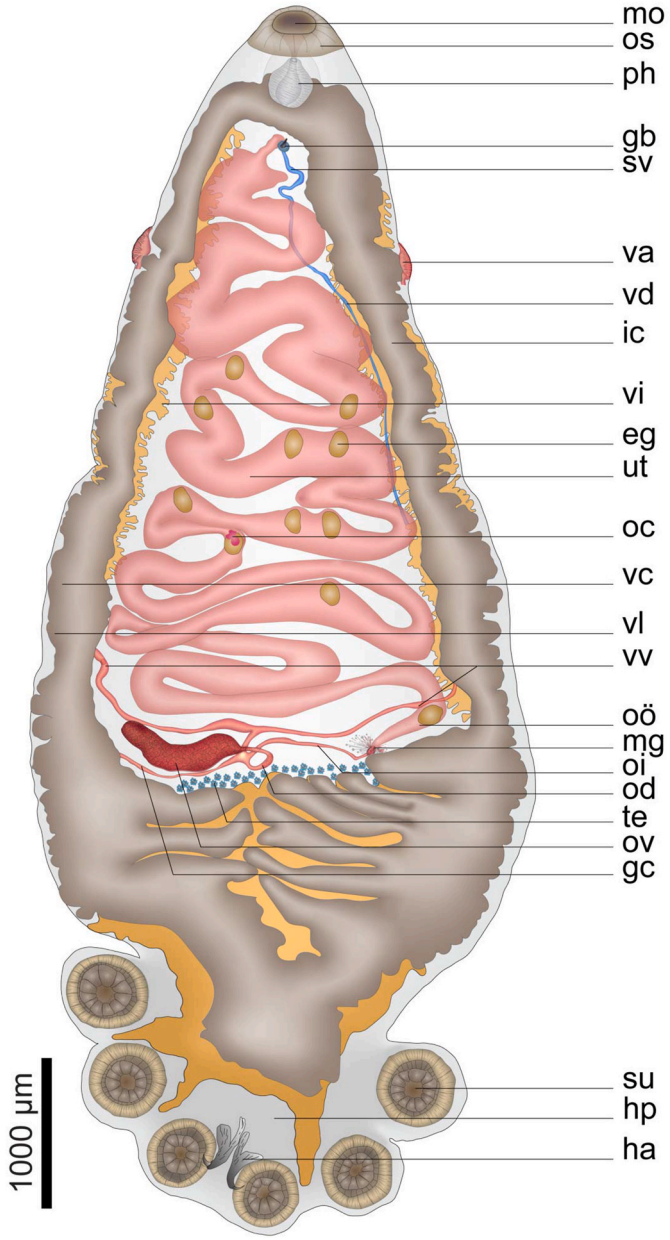


Fig. 4. Ventral view of *Metapolystoma falcatum* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vc, vaginal canal; vd, vas deferens; vi, vitelline; vl, vitelline duct; vv, vitello–vaginal canal.

3.1.1.7. Zoobank registration. The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48 EB-9EA4-881D43956065. The life science identifier (LSID) of the new name *Metapolystoma vencesi* n. sp. Landman et al. is: urn:lsid:zoobank.org:act:680E18A9-64D6-40C9-87D9-1AC5F28A1030.

3.1.1.8. Etymology. In recognition of Professor Miguel Vences, Technical University of Braunschweig, Germany, for his dedication to the study of Madagascan herpetofauna.

3.1.1.9. Description. Measurements reflected in Table 2. Body pyriform (Fig. 2), dorsoventrally flattened, widest section at 57% of total length from anterior end, body length 2.7 times greater than width. Mouth subventral, surrounded by false oral sucker. Posterior haptor 16% of body length, bearing three pairs of haptoral suckers of equal size. Marginal hooklets placed as for other polystomes: pairs one and two between hamuli, pairs three to five embedded in suckers, pairs six to eight found between anterior suckers, pairs one and eight larger than pairs two to seven (Fig. 3a). Well-developed hamuli between posterior-most haptoral suckers without cut between handle and guard (Fig. 3b). Medial pharynx length greater than width, positioned immediately posterior or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx, 8% of total length from most anterior point, converging posteriorly at position of 80% of total length from most anterior point, extending into haptor; no prehaptoral anastomoses. Lateral diverticula length equal to width, found only in last third of intestine. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, positioned in a narrow band posterior to the ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 20–32 (27 ± 5; 1) wide, 179 long, 2% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening on left ventral half, posterior to intestinal ceca bifurcation, 10% of total length from most anterior point. Genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing eight genital spines (Fig. 3c).

Ovary elongate, not lobed, positioned posterior to midbody, length 4.4 times greater than width, measuring 9% of body length. Oviduct 596 long, 29–53 (30 ± 7; 1) wide. Uterus massive and wide, occupying most of body proper, tubiform, convolute, containing 367 ovoid, operculate eggs; some with fully developed oncomiracidia. Hatched intrauterine oncomiracidia present. Mehlis' gland obscure. Two vaginae 180–420 long, 16–31 wide, on lateral margins, bearing multiple marginal openings, vaginal vestibule elongate, positioned at 18% from anterior. Vitellaria extended throughout most of body and haptor, surrounds female reproductive organs. Genito-intestinal canal prominent, 580 long, 40–133 (62 ± 28; 11) wide, situated posterior to ovary.

3.1.2. Metapolystoma falcatum n. sp. (Fig. 4-7; Table 2)

3.1.2.1. Type host. Boophis doulioti (Mantellidae).

3.1.2.2. Type locality. Ankarafantsika, Madagascar (Fig. 1), (16.115976S; 47.095631E).

3.1.2.3. Site in host. Gills of tadpoles and urinary bladder of mature frogs.

3.1.2.4. Level of infection. Three of 22 frogs collected were infected. One mature and 13 juvenile parasites were recovered, with a maximum of 12 recovered from a single host (prevalence 13.6%. mean intensity 4.7). Seven of 10 tadpoles collected were infected with a total of 13 neotenic parasites, while as many as three parasites were infecting a single host (prevalence 70%; mean intensity 1.85). Though the name neotenic for some polystomes can be confusing (see Badets and Verneau, 2009), it refers here to egg producing parasites that were recovered from the gills of tadpoles.

3.1.2.5. Type material. Morphological descriptions are based on one mature parasite, eight juveniles and seven neotenics. One sexually mature specimen (Holotype NMBP 560), four immature bladder parasites (Paratypes NMBP 561–564) and four neotenics (Paratypes NMBP 565–568), all from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.1.2.6. Voucher material. Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

3.1.2.7. Zoobank registration.

The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-

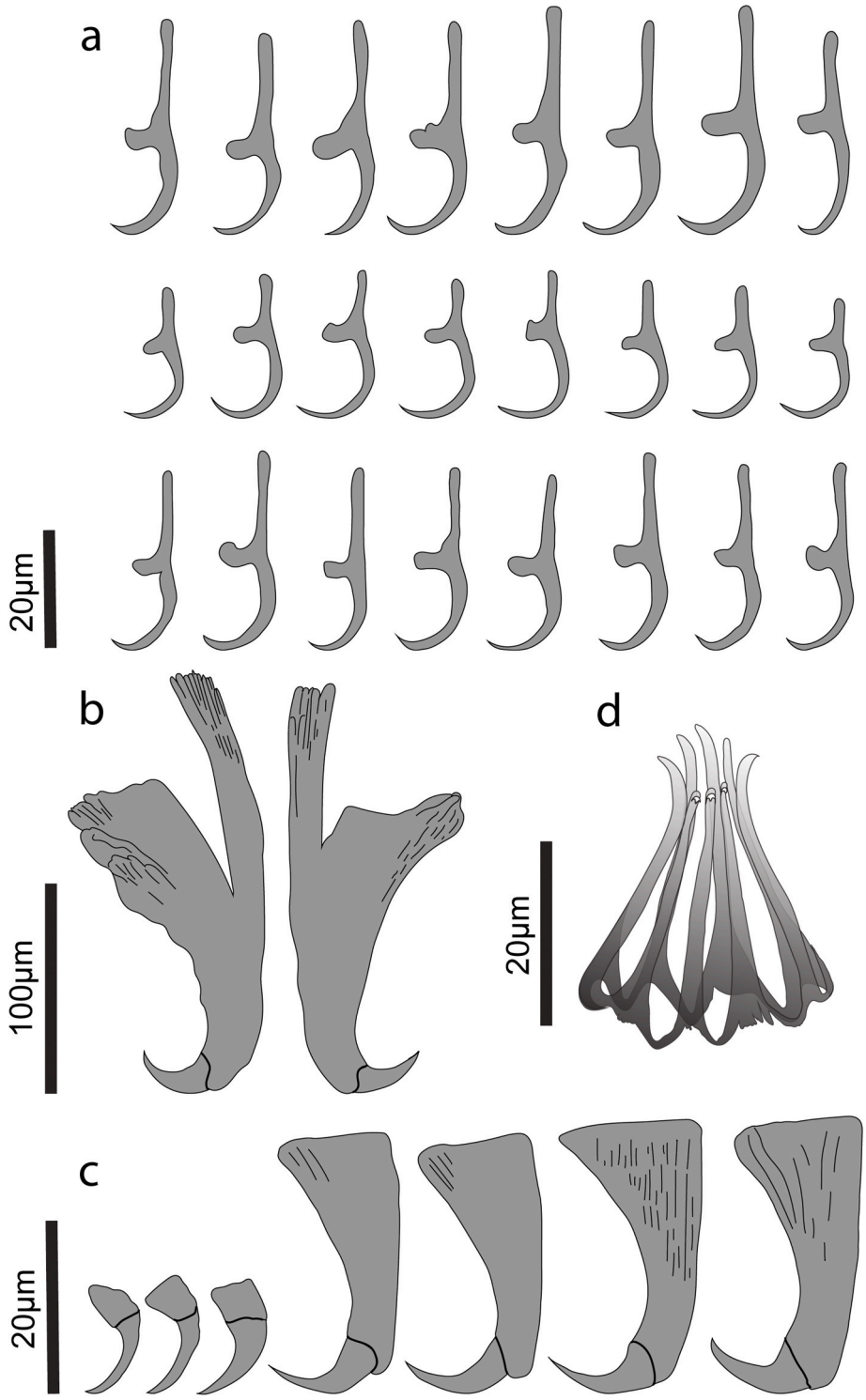


Fig. 5. *Metapolystoma falcatum* n. sp. from *Boophis doulioti*. **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c**, hamulus development; **d**, genital crown from holotype.

48 EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma falcatum* n. sp. Landman et al. is: urn:lsid:zoobank.org:act:FB1716E2-5C61–4C53-9A2F-BB364D6782A8.

3.1.2.8. Etymology. The species epithet refers to the exceptionally long curved tips of marginal hooklets two to seven (falcatum = sickle shaped, curved, hooked, armed with scythes).

3.1.2.9. Description. Mature parasite (Fig. 4–5).

Measurements reflected in Table 2. Body pyriform (Fig. 4), dorsoventrally flat, widest section at 66% of total length from anterior end, body length 2.4 times greater than width. Mouth sub-ventral, surrounded by false oral sucker. Posterior haptor 19% of body length bearing three pairs of haptoral suckers equal in size. Marginal hooklets placed as for other polystomes and as described for *M. vencesi* n. sp., pairs one and eight larger than pairs two to seven (Fig. 5a). Well-

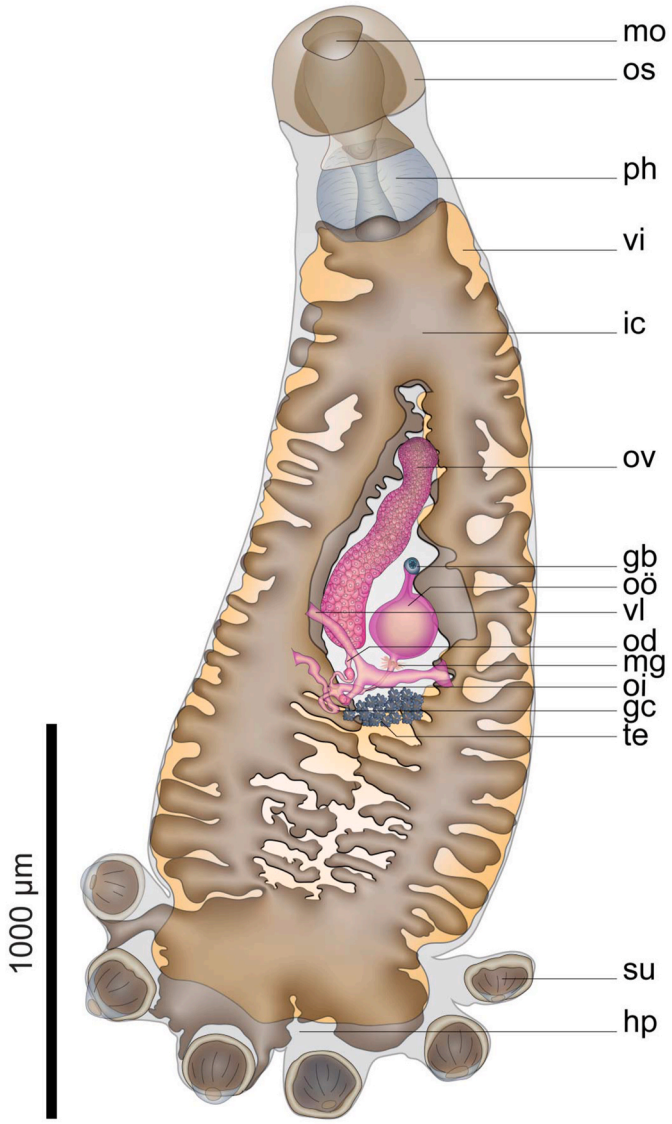


Fig. 6. Ventral view of *Metapolystoma falcatum* n. sp. from *Boophis doulioti*, neotenic form. Abbreviations: gb, genital bulb; gc, genito–intestinal canal; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; te, testis; ut, uterus; vi, vitelline; vl, vitelline duct; vv.

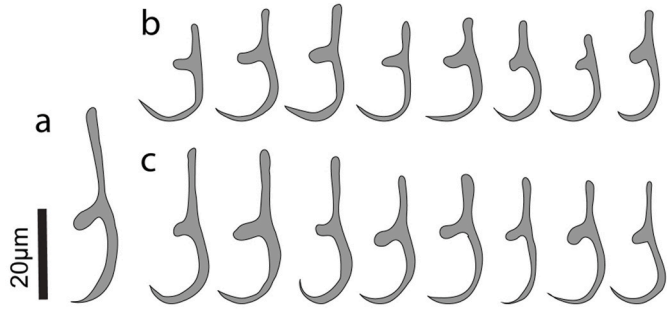


Fig. 7. *Metapolystoma falcatum* n. sp. from *Boophis doulioti*, neotenic form. **a**, marginal hooklets 2–7; **b,** marginal hooklets 8; **c**, marginal hooklet 1.

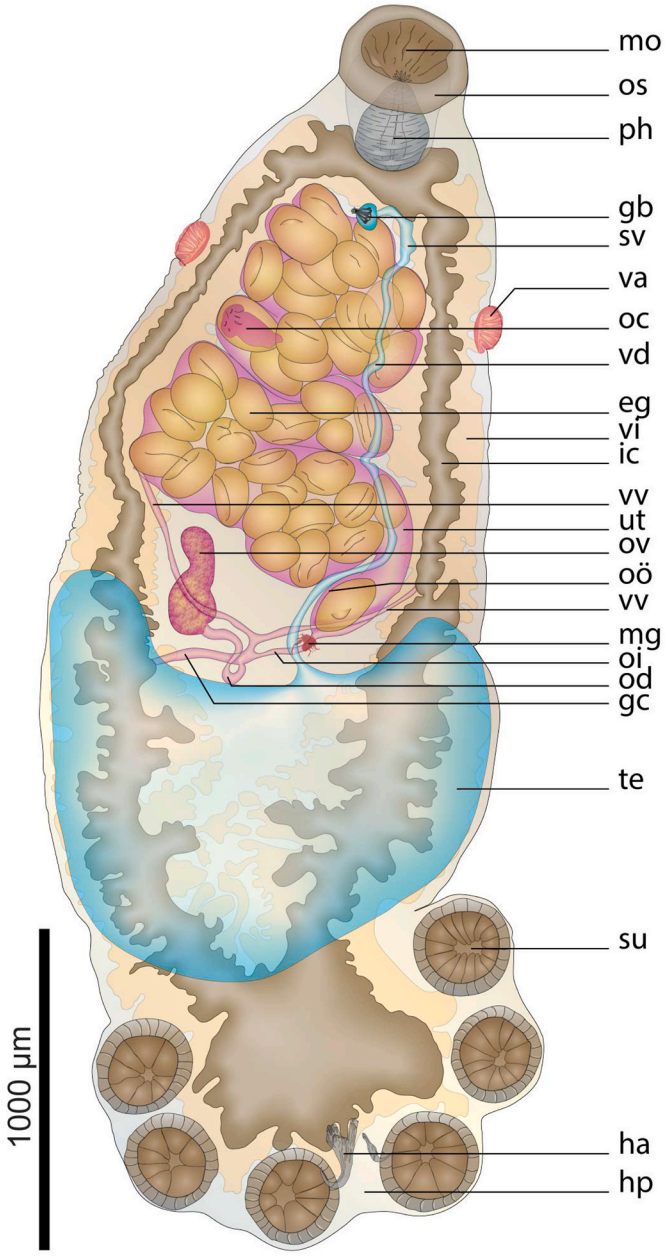


Fig. 8. Ventral view of *Metapolystoma ansuanum* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vd, vas deferens; vi, vitelline; vv, vitello–vaginal canal.

developed hamuli between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 5b). Hamuli development presented in Fig. 5c. Medial pharynx length greater than width, positioned immediately posterior to or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 11% from anterior, converging posteriorly at 82% from anterior, stretching in between haptoral suckers; no prehaptoral anastomoses. Intestine bears lateral diverticula, length equal to width. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, only a small section visible, positioned directly posterior to the ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 23–40 (30 ± 6; 1) wide, 301 long, measuring 4% of total length, narrowing towards genital bulb, opening

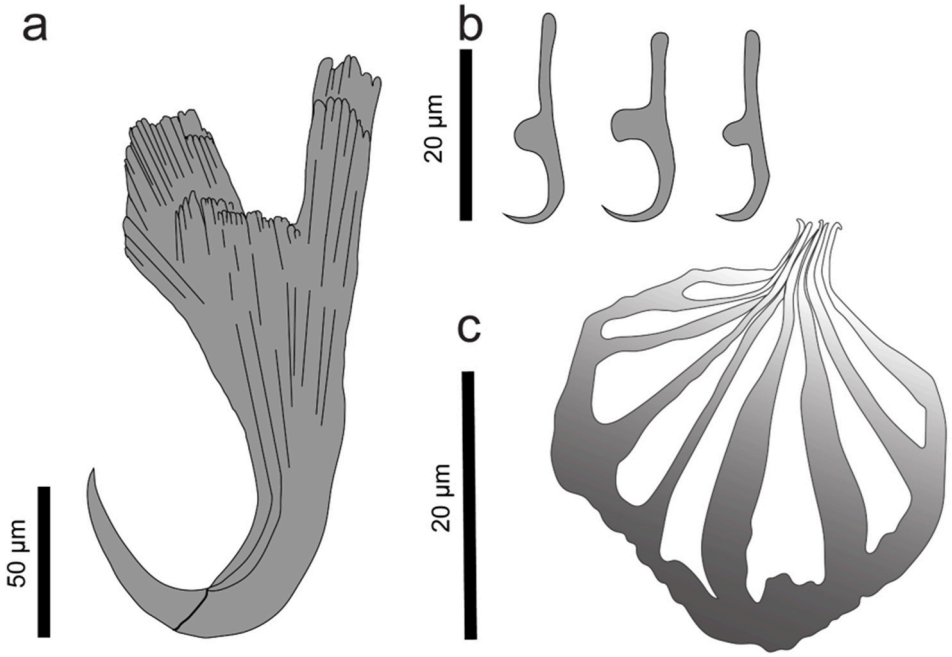


Fig. 9. *Metapolystoma ansuanum* n. sp. from *Boophis luteus*. **a**, hamulus from holotype; **b**, marginal hooklets 2–7; **c**, genital crown from holotype.

in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation at 12% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing eight genital spines (Fig. 5d).

Ovary elongated, not lobed, positioned posterior to midbody, length four times greater than width,10% of body length. Oviduct 411 long, 22–48 (30 7; 1) wide. Uterus massive and narrow, occupying most of the space between intestinal caeca, tubiform, serpentines between oötype and genital bulb. Eggs operculated. Holotype released 171 eggs with only 11 remaining in utero. Some eggs contain fully developed oncomiracidia. Mehlis' gland distinct. Two parallel vaginae 262–235 long, 102 wide, situated on lateral margins, bearing multiple marginal openings. Vaginal vestibule cup-shaped, 21% from anterior. Vitellaria dorsal to intestinal tract, extends throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal canal prominent, 943 long, 10–46 (23 ± 9; 1) wide, situated directly posterior to ovary.

Neotenic parasite (Fig. 6–7).

Measurements obtained from seven egg-producing neotenic parasites. Body pyriform (Fig. 6), dorsoventrally flat, ventrally concave, 1.419–3.307 (2.265 ± 667; 7) long. Maximum width 904–1.376 (1121 ± 368; 3). Body length 2–5 (3 ± 1; 6) times greater than width. Mouth 100–148 (124 ± 17; 5) in diameter, sub-ventral, surrounded by false oral sucker. Posterior haptor 313–526 (411 ± 89; 7) long, 541–1.166 (936 ± 305; 7) wide. Haptor length-body length ratio 0.13–0.24 (0.2 ± 0.04; 6), haptor bearing three pairs of haptoral suckers equal in size 103–226 (178 ± 32; 47). Hamuli absent. Marginal hooklets placed as for *M. vencesi* n. sp., pairs one 28.2 long (Fig. 7a), larger than pairs two to seven 22.6–27.7 (25.5 ± 1.5; 15), (Fig. 7b), pairs eight 27.5–35 (30.5 ± 3.155; 8) long (Fig. 7c). Medial pharynx length 199–312 (244 ± 45; 5) equal to width 207–312 (245 ± 41; 5), positioned immediately posterior or at the margin of false oral sucker. Intestine bifurcates at distance 27–30% (30% ± 2%; 3) from anterior, situated posterior to pharynx, converging posteriorly at 77–81% (80% ± 2.7%; 3) from anterior, stretching into area between haptoral suckers; no prehaptoral anastomosis. Intestine with lateral diverticula, length greater than width. Diverticula in posterior half longer than anterior. Medial diverticula only posterior to ovary, length greater than width, narrower than lateral diverticula.

Testis follicular, though only a small section was visible, positioned directly posterior to vitello-vaginal canal, ventral to intestine. Vas deferens not visible. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation, at distance 42–51% (46% ± 4%; 3) from anterior. Genital bulb diameter 31–51 (41 ± 9; 4), muscular, surrounded by glandular cells, armed with genital crown bearing eight to eleven genital spines 7.9–11.8 (9.8 ± 1; 10) long.

Ovary elongate, 256–580 (401 ± 120; 7) long, 67–117 (91 ± 19; 7) wide, not lobed, situated in middle of body, length three-six times greater than width, measuring 16–21% (17 ± 2.18%; 6) of body length. Oviduct 15 long, 6–28 (13 ± 6; 1) wide. Oötype 171–173 long, ovoid, containing a maximum of one ovoid, operculate egg 171–173 long, 132–142 wide. Uterus absent, eggs laid immediately after production. Mehlis' gland distinct. Vaginae absent. Vitellaria dorsal to intestinal tract, extended throughout most of body, except area occupied by female reproductive organs. Genito-intestinal canal prominent, 257 long, 7–47 (18 ± 8; 1) wide, situated posterior to ovary.

3.1.3. Metapolystoma ansuanum n. sp. (Fig. 8–9; Table 2)

3.1.3.1. Type host. Boophis luteus (Mantellidae).

3.1.3.2. Type locality. Cascade des Nymphes, Isalo National Park, Madagascar (Fig. 1), (22.46977S; 45.260701E).

3.1.3.3. Site in host. Urinary bladder.

3.1.3.4. Level of infection. One of nine frogs collected were infected with two mature parasites (prevalence 11.1%).

3.1.3.5. Type material. The morphological descriptions are based on two mature parasites (Holotype NMBP 569; Paratype NMBP 570) collected from the same locality in Cascade des Nymphes. The type material was deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.1.3.6. Zoobank registration. The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48 EB-9EA4-881D43956065. The LSID of the

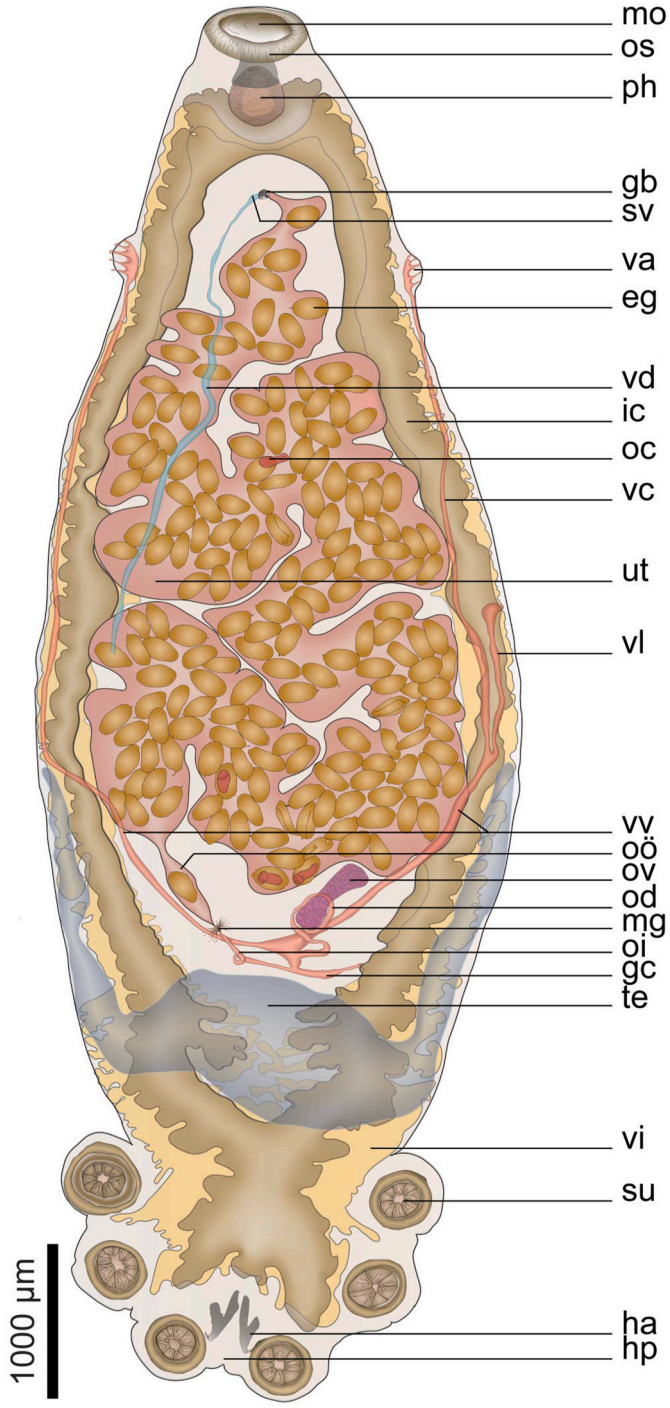


Fig. 10. Ventral view of *Metapolystoma theroni* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vc, vaginal canal; vd, vas deferens; vi, vitelline; vl, vitelline duct; vv, vitello–vaginal canal.

new name *Metapolystoma ansuanum* n. sp. Landman et al. is: urn:lsid:zoobank.org:act:4761BF6E-F309-4625-A7D7-CA64F3A3F6F8.

3.1.3.7. Etymology. This species is named for Mrs Anna-Susan van der Linde, known as Ansu, in acknowledgement of her teaching and inspiration of many secondary school pupils in the field of biology.

3.1.3.8. Description. Measurements reflected in Table 2. Body pyriform (Fig. 8), dorsoventrally flat, ventrally concave, widest section at position 16–65% from anterior end of body. Body length 2.6–2.8 times greater than width. Mouth sub-ventral, surrounded by false oral sucker. Posterior haptor measures 24% of total length, bearing three pairs of haptoral suckers of equal size. Well-developed hamuli positioned between posterior-most haptoral suckers with deep cut between handle and guard, hook length Z long relative to Length X (Fig. 9a). Marginal hooklets placed as for other polystomes: pairs one and two between hamuli, pairs three to five embedded in haptoral suckers, pairs six to eight positioned between anterior-most haptoral suckers (Fig. 9b). Medial pharynx length greater than width, positioned immediately posterior to or at margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 15–18% from anterior, converging posteriorly at 79–81% from anterior; no prehaptoral anastomoses. Lateral intestinal diverticula length equal to width in anterior half, length greater than width in posterior half. Medial diverticula only posterior to ovary.

Testis follicular, large, kidney-shaped, positioned posterior to ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 17–31 (26 ± 4; 1) wide, 422 long, measuring 11% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation at position 16–18% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing 10 genital spines (Fig. 9c).

Ovary, elongate, not lobed, positioned posterior to midbody, length 2.3–2.7 times greater than width, measuring 9–10% of body length. Oviduct 345 long, 19–39 (28 ± 6; 1) wide. Uterus large, occupying one-third of body proper, tubiform, convolute. Uterus contains 41 ovoid, operculate eggs, some contain fully developed oncomiracidia. Intrauterine oncomiracidia present. Mehlis' gland distinct. Two parallel vaginae, each 94–132 long (119 ± 18; 4), 66–81 (75 ± 8; 4) wide, found on lateral margins, bearing multiple marginal openings; vaginal vestibule cup-shaped at 21–26% from anterior. Vitellaria dorsal to intestinal tract, extending throughout most of body, except area occupied by female reproductive organs. Genito-intestinal canal prominent, 264 long, 30–40 (37 ± 4; 1) wide, situated directly posterior to ovary.

3.1.4. Metapolystoma theroni n. sp. (Fig. 10–11; Table 2)

3.1.4.1. Type host. Boophis madagascariensis (Mantellidae).

3.1.4.2. Type locality. Indri Reserve in Andasibe, Madagascar (Fig. 1), (18.930856S; 48.413611E).

3.1.4.3. Site in host. Urinary bladder.

3.1.4.4. Level of infection. Three of 30 frogs collected were infected with a total of one mature and 71 juvenile parasites, while as many as 40 parasites were infecting a single host (prevalence of 10%, mean intensity 24).

3.1.4.5. Type material. The morphological descriptions are based on one mature and 27 juvenile parasites. One sexually mature specimen (Holotype 573) and four immature ones (Paratypes 574–577), all from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.1.4.6. Voucher material. Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

3.1.4.7. Zoobank registration. The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48 EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma theroni* n. sp. Landman et al. is: urn:lsid:

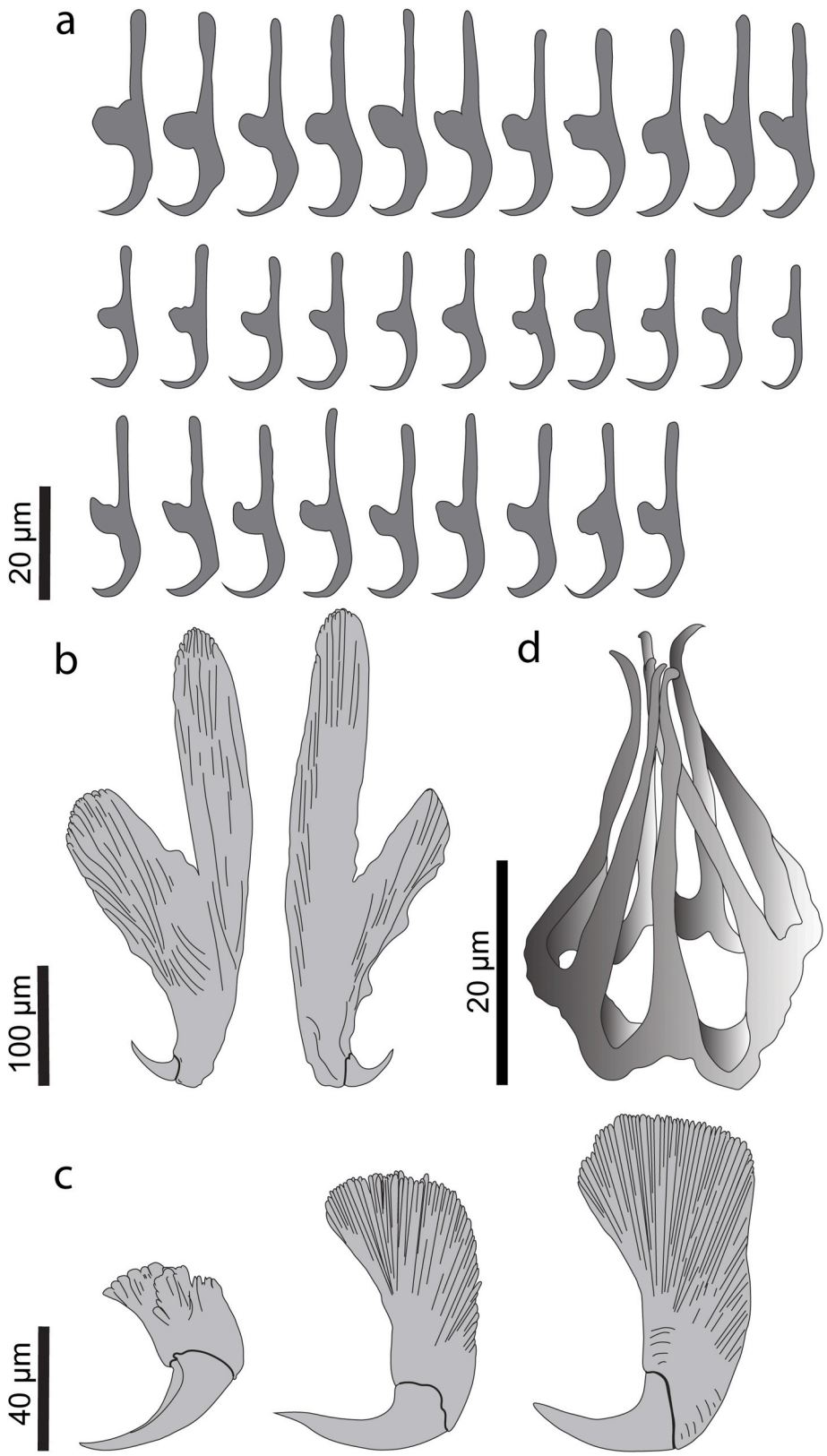


Fig. 11. *Metapolystoma theroni* n. sp. from *Boophis madagascariensis*. **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c,** hamulus development; **d**, genital crown from holotype.

zoobank.org:act:55203AE0-217D-45D0-B3C8-07DDE53FA9A3.

3.1.4.8. Etymology. This species is named in honour of emeritus Professor Pieter Daniel Theron at the North-West University, South Africa, in recognition of 54 years of inspiring teaching and dedication to the field of zoology.

3.1.4.9. Description. Measurements reflected in Table 2. Body pyriform

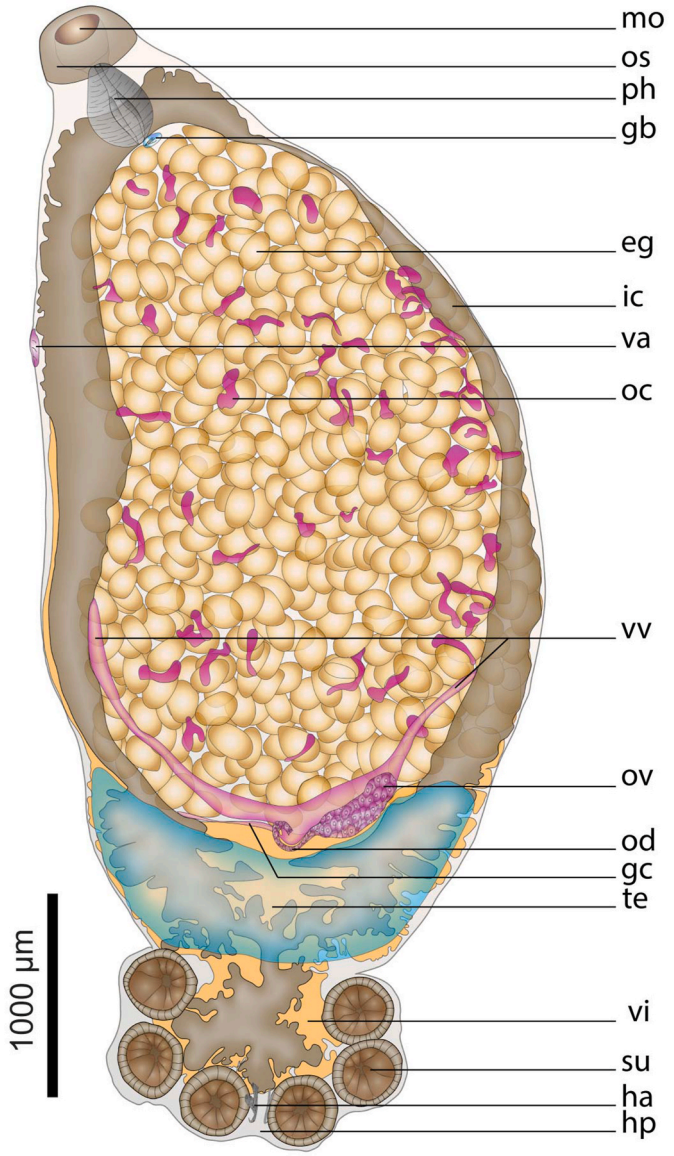


Fig. 12. Ventral view of *Metapolystoma multiova* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mo, mouth; oc, oncomiracidium; od, oviduct; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; te, testis; va, vagina; vi, vitelline; vv, vitello–vaginal canal.

(Fig. 10) dorsoventrally flat, widest section at 51% from anterior end, body length 2.6 times greater than width, mouth sub-ventral, surrounded by false oral sucker. Posterior haptor occupying 18% of total body length, bearing three pairs of haptoral suckers, equal in size. Marginal hooklets placed as for other polystomes, pairs one and two between hamuli, pairs three to five embedded in suckers, pairs six to eight in area between anterior-most suckers, pairs one and eight larger than pairs two to seven (Fig. 11a). Well-developed hamuli positioned between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 11b). Hamuli development presented in Fig. 11c. Medial pharynx length greater than width, positioned immediately posterior to or at margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 10% from anterior, converging posteriorly at 80% from anterior; no prehaptoral anastomoses. Lateral intestinal diverticula in first three quarters length equal to width, in last quarter length greater than width. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, u-shaped, mainly positioned posterior to the ovary with two lateral processes extending forward along the lateral line past the ovary up to one-third of the body proper, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 23–65 (46 ± 18; 1) wide, 122 long, measuring 1% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation, positioned 13% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing seven genital spines (Fig. 11d).

Ovary elongate, not lobed, positioned posterior to midbody, length 1.2 times greater than width, measuring 5% of body length. Oviduct 1167 long, 20–51 (33 ± 9; 1) wide. Uterus massive, occupying 50% of body proper, tubiform, serpentines between posterior connection at oötype and anterior connection at genital bulb, containing 176 ovoid, operculate eggs, some contain fully developed oncomiracidia. Hatched intrauterine oncomiracidia present. Mehlis' gland distinct. Two parallel vaginae 270–304 long, 132–177 wide, on lateral margins, with multiple marginal openings, vaginal vestibule cup-shaped at 18% from anterior. Vitellaria dorsal to intestinal tract, extended throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal canal prominent 457 long, 30–77 (52 ± 16; 1) wide, situated posterior to ovary.

3.1.5. Metapolystoma multiova n. sp. (Fig. 12–13; Table 2)

3.1.5.1. Type host. Boophis occidentalis (Mantellidae).

3.1.5.2. Type locality. Cascade des Nymphes, Isalo National Park, Madagascar (Fig. 1), (22.46977S; 45.260701E).

3.1.5.3. Site in host. Urinary bladder.

3.1.5.4. Level of infection. One of five frogs collected were infected with two adult parasites (prevalence 20%).

3.1.5.5. Type material. The morphological descriptions are based on two mature parasites. Two sexually mature specimens (Holotype 571, Paratype 572), both from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.1.5.6. Zoobank registration. The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48 EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma multiova* n. sp. Landman et al. is: urn:lsid:zoobank.org:act:6854087D-44B5–492B-9750-3647188F0F26.

3.1.5.7. Etymology. The species epithet is derived from Latin and related to the vast number of eggs (±500) carried by this species in contrast with other currently known species in the genus (multi = many + ova = eggs).

3.1.5.8. Description. Measurements reflected in Table 2. Body pyriform (Fig. 12), dorsoventrally flat, ventrally concave, widest section at 50–57% from anterior end of body. Body length 2.2–2.3 times greater than width. Mouth directed sub-ventrally, surrounded by false oral sucker. Posterior haptor measures 18% of total length bearing three pairs of haptoral suckers of equal size. Marginal hooklet pairs six to eight situated between anterior-most haptoral suckers, pairs one and eight larger than pairs two to seven, pairs one and two between hamuli, pairs three to five embedded in suckers (Fig. 13a). Well-developed hamuli situated between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 13b). Medial pharynx length greater than width, positioned immediately posterior to or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 13% from anterior, converging posteriorly at 79–85% from anterior; no

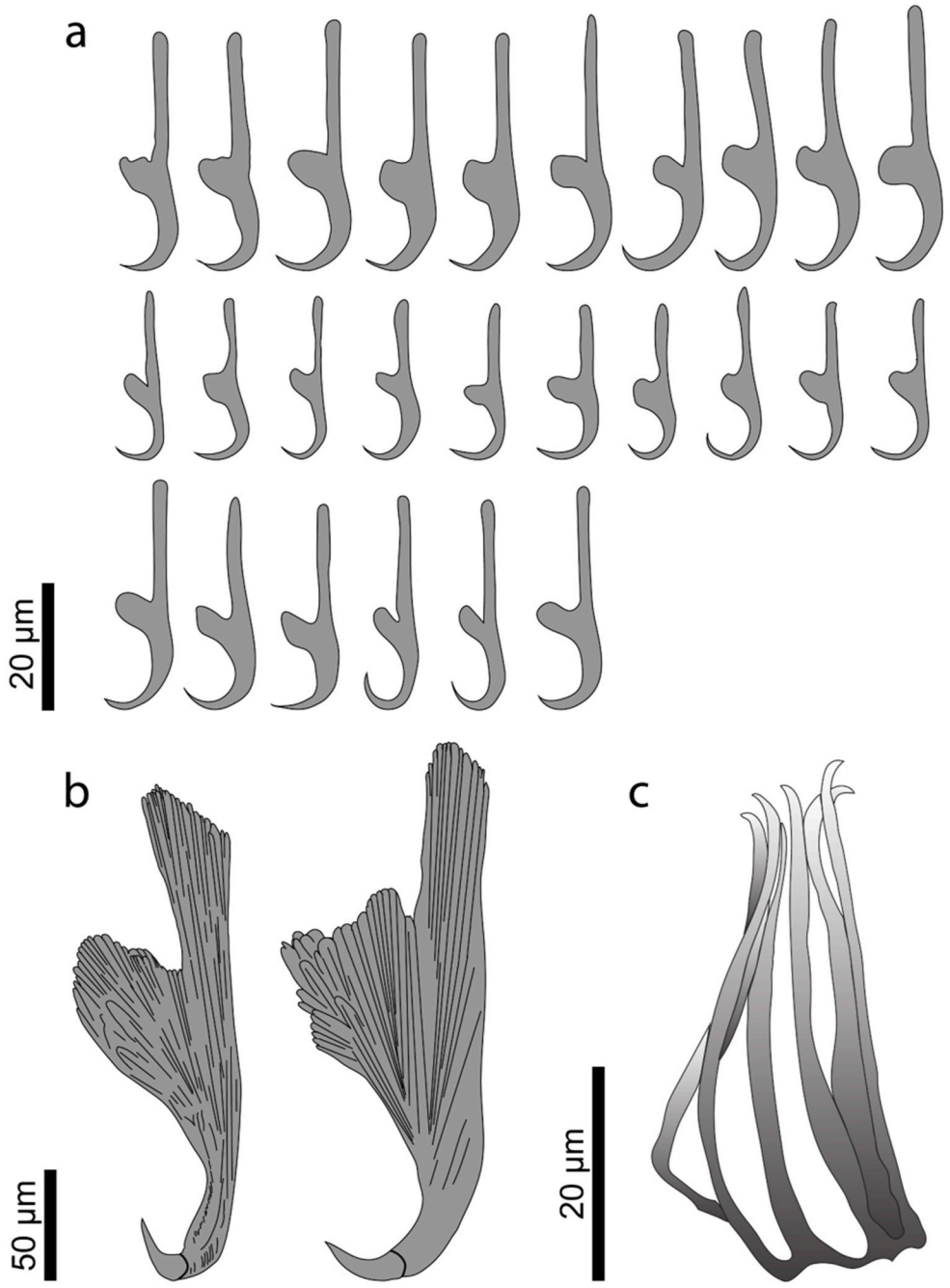


Fig. 13. *Metapolystoma multiova* n. sp. from *Boophis occidentalis*. **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype (left) and from paratype (right); **c**, genital crown from holotype.

prehaptoral anastomoses. Lateral intestinal diverticula situated in first third of intestine, where length is equal to width, absent in the second third while length is greater than width in the posterior third. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, sickle-shaped, posterior to ovary, ventral to intestine. Vas deferens obscured. Genital pore opening mid-ventrally situated on anterior margin of intestinal ceca bifurcation at 4% from anterior, genital bulb muscular, armed with genital crown bearing six genital spines (Fig. 13c).

Ovary elongate, not lobed, positioned posterior to midbody, length 2.8 times greater than width, measuring 8% of body length. Oviduct 988 long, 20–48 (31 ± 9; 1) wide. Uterus contains 336–499 ovoid, operculate eggs, some contain fully developed oncomiracidia, some hatched intrauterine oncomiracidia present. Mehlis' gland obscured. Two parallel vaginae, each 266–311 long, 58–62 wide, situated on lateral margins bearing multiple marginal openings. Vaginal vestibule cup-shaped, situated at 18% from anterior. Vitellaria dorsal to intestinal tract, extended throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal cannel prominent 633 long, 7–103 (24 ± 24; 1) wide, situated at level of ovary.

3.2. Genetic divergences between Metapolystoma species and parasite phylogeny

Some of the 18S, 28S and COI sequences used in this study were retrieved from GenBank, while the others were obtained and submitted under accession numbers MW053457, MW053458 and MW054236 to MW054249 (Table 3). The final alignment, which had resulted in 3.977

Table 3
Polystome species investigated, host species, locality and GenBank accession numbers for 18S, 28S and COI.

Polystome species	Host species	Locality	18S Accession number	28S Accession number	COI Accession number
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ambatolampy	**FM897287**[a]	**FM897270**[a]	**FM897300**[a]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ambatolampy	**MW054243**[g]	**JN800281**[b]	**MW053457**[g]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ankarafantsika	**MW054242**[g]	**MW054236**[g]	**JN800285**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Makira	**MW054246**[g]	**MW054239**[g]	**JN800288**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Makira	**MW054247**[g]	**MW054240**[g]	**JN800289**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ranomafana	**MW054245**[g]	**MW054238**[g]	**JN800286**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Vohiparara	**MW054244**[g]	**MW054237**[g]	**JN800287**[b]
Metapolystoma cachani	*Ptychadena longirostris*	Africa: Nigeria	**FM897280**[a]	**FM897262**[a]	**JN800294**[b]
Metapolystoma falcatum n. sp.	*Boophis doulioti*	Madagascar: Ankarafantsika	**MW054248**[g]	**JN800283**[b]	**JN800291**[b]
Metapolystoma falcatum n. sp.	*Boophis doulioti*	Madagascar: Ankarafantsika	**FM897286**[a]	**FM897269**[a]	**MW053458**[g]
Metapolystoma multiova n. sp.	*Boophis occidentalis*	Madagascar: Isalo	**FM897285**[a]	**FM897268**[a]	**FM897301**[a]
Metapolystoma theroni n. sp.	*Boophis madagascareniensis*	Madagascar: Andasibe	**FM897284**[a]	**FM897267**[a]	**FM897298**[a]
Metapolystoma theroni n. sp.	*Boophis madagascareniensis*	Madagascar: Andasibe	**MW054249**[g]	**MW054241**[g]	**JN800293**[b]
Polystoma claudecombesi	*Amietia delalandii*	South Africa	**FM897281**[a]	**FM897263**[a]	(–)
Polystoma dawiekoki	*Ptychadena anchietae*	South Africa	**AM051069**[c]	**AM157204**[d]	**AM913856**[e]
Polystoma integerrimum	*Rana temporaria*	France	**AM051071**[c]	**AM157206**[d]	**JF699306**[f]
Polystoma marmorati	*Hyperolius marmoratus*	South Africa	**AM051073**[c]	**AM157208**[d]	**AM913859**[e]
Polystoma occipitalis	*Hemisus marmoratus*	Ivory Coast	**AM051075**[c]	**FM897264**[a]	(–)
Polystoma testimagna	*Strongylopus fasciatus*	South Africa	**AM157194**[c]	**AM157217**[d]	**AM913860**[e]

[a] From Verneau et al. (2009).
[b] From Berthier and Verneau, unpublished.
[c] From Bentz et al. (2006).
[d] From Badets et al. (2011).
[e] From du Preez et al. (2007).
[f] From Rahariviololoniaina et al. (2011).
[g] Sequences reported in the present study; (–) Not available.

Table 4
Mean genetic distances (below diagonal) and total character differences (above diagonal) between species groups as inferred from comparisons of 18S rDNA sequences (1931 characters).

		1	2	3	4	5	6	7	8	9	10	11
1	*M. brygoonis*		1	2	2	2	11	10	13	10	9	25
2	*M. falcatum* n. sp. (B. d.)	0,001		1	1	1	10	9	12	9	8	24
3	*M. multiova* n. sp. (B. o.)	0,001	0001		0	2	11	10	13	10	9	25
4	*M. theroni* n. sp. (B. m)	0,001	0001	0,000		2	11	10	13	10	9	25
5	*M. cachani*	0,001	0001	0,001	0001		9	8	11	10	9	23
6	*P. occipitalis*	0,006	0005	0,006	0006	0,005		6	16	15	14	28
7	*P. dawiekoki*	0,005	0005	0,005	0005	0,004	0003		16	15	14	28
8	*P. claudecombesi*	0,007	0006	0,007	0007	0,006	0008	0,008		11	10	24
9	*P. marmorati*	0,005	0005	0,005	0005	0,005	0008	0,008	0006		1	25
10	*P. testimagna*	0,005	0004	0,005	0005	0,005	0007	0,007	0005	0,001		24
11	*P. integerrimum*	0,013	0013	0,013	0013	0,012	0015	0,015	0013	0,013	0,0126	

Abbreviations: B. d. = *Boophis doulioti*; B. o. = *Boophis occidentalis*; B. m. = *Boophis madagascariensis*

Table 5
Mean genetic distances (below diagonal) and total character differences (above diagonal) between species groups as inferred from comparisons of 28S rDNA sequences (1418 characters).

		1	2	3	4	5	6	7	8	9	10	11	12
1	*M. brygoonis* (SlA)	–	2	8	13	15	20	29	29	37	38	37	58
2	*M. brygoonis* (SlB)	0,001	–	6	11	13	18	27	27	35	36	35	59
3	*M. falcatum* n. sp. (B. d.)	0,006	0004	–	11	13	20	26	26	34	35	34	56
4	*M. multiova* n. sp. (B. o.)	0,009	0008	0,008	–	4	23	32	32	40	39	38	62
5	*M. theroni* n. sp. (B. m.)	0,011	0009	0,009	0003	–	27	34	34	40	39	38	62
6	*M. cachani*	0,014	0013	0,014	0016	0,019	–	32	33	41	42	41	68
7	*P. occipitalis*	0,020	0019	0,019	0023	0,024	0023	–	7	36	38	35	59
8	*P. dawiekoki*	0,021	0019	0,019	0023	0,024	0024	0,005	–	35	35	32	59
9	*P. claudecombesi*	0,027	0025	0,024	0,09	0,029	0030	0,026	0025	–	22	23	53
10	*P. marmorati*	0,027	0026	0,025	0028	0,028	0030	0,027	0025	0,016	–	5	53
11	*P. testimagna*	0,027	0025	0,024	0027	0,027	0030	0,025	0022	0,016	0004	–	52
12	*P. integerrimum*	0,042	0043	0,041	0045	0,045	0050	0,043	0043	0,039	0039	0,038	–

Abbreviations: SlA = sublineage A; SlB = sublineage B; B. d. = *Boophis doulioti*; B. o. = *Boophis occidentalis*; B. m. = *Boophis madagascariensis*.

characters, contained 19 taxa, among which 13 specimens of *Metapolystoma* and six specimens characterizing distinct species of *Polystoma*.

Minimum and maximum distances with standard deviation as estimated among *Metapolystoma* species were as follows. *M. falcatum* n. sp. (18S: 0.051% ± 0.0003–0.051 ± 0.0005%; 28S: 0.42% ± 0.001–1.42% ± 0.002; COI: 8.6% ± 0.016–15.68% ± 0.023); *M. multiova* n. sp. (18S: 0–0.1% ± 0.0007); 28S: 0.28% ± 0.001–1.64% ± 0.003; COI: 12.54% ± 0.020–13.78% ± 0.022); *M. theroni* n. sp. (18S: 0–0.1% ± 0.0007; 28S:

Table 6
Mean genetic distances (below diagonal) and total character differences (above diagonal) between specimens of *Metapolystoma* species as inferred from comparisons of COI sequences (323 characters).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*M. brygoonis* (Ankarafantsika)	–	1	2	21	21	13	17	27	27	40	39	41	26	36	35	38	55
2	*M. brygoonis* (Ambatolampy)	0,003	–	3	20	20	12	16	26	26	39	38	40	25	35	34	37	55
3	*M. brygoonis* (Ambatolampy)	0,006	0009	–	21	21	13	17	26	26	39	39	41	28	36	35	38	56
4	*M. brygoonis* (Vohiparara)}	0,068	0065	0,068	–	1	13	11	30	30	38	37	39	30	43	40	41	58
5	*M. brygoonis* (Ranomafana)	0,068	0065	0,068	0003	–	13	12	30	30	38	36	39	29	43	40	41	57
6	*M. brygoonis* (Makira)	0,041	0038	0,041	0042	0,042	–	8	25	25	37	34	36	22	35	32	36	54
7	*M. brygoonis* (Makira)	0,055	0051	0,055	0035	0,038	0025	–	30	30	38	36	38	29	40	37	40	55
8	*M. falcatum* n. sp. (B. d.)	0,089	0086	0,086	0100	0,099	0082	0,100	–	0	38	43	45	32	38	33	37	60
9	*M. falcatum* n. sp. (B. d.)	0,089	0086	0,086	0100	0,099	0082	0,100	0000	–	38	43	45	32	38	33	37	60
10	*M. multiova* n. sp. (B. o.)	0,137	0133	0,133	0129	0,129	0125	0,129	0129	0,129	–	40	38	40	49	37	42	61
11	*M. theroni* n. sp. (B. m.)	0,133	0129	0,133	0125	0,121	0114	0,121	0149	0,149	0138	–	4	36	48	41	45	64
12	*M. theroni* n. sp. (B. m.)	0,141	0137	0,141	0132	0,133	0121	0,129	0157	0,157	0130	0,013	–	40	50	41	46	65
13	*M. cachani*	0,085	0082	0,092	0099	0,096	0071	0,096	0106	0,106	0136	0,121	0136	–	35	31	34	51
14	*P. dawiekoki*	0,121	0117	0,121	0147	0,147	0117	0,135	0128	0,128	0170	0,166	0174	0,117	–	38	42	57
15	*P. marmorati*	0,117	0114	0,117	0136	0,135	0106	0,124	0110	0,110	0125	0,139	0139	0,103	0129	–	25	54
16	*P. testimagna*	0,128	0124	0,128	0139	0,139	0121	0,135	0124	0,124	0143	0,155	0158	0,113	0143	0,082	–	53
17	*P. integerrimum*	0,193	0193	0,198	0205	0,201	0189	0,193	0214	0,214	0218	0,231	0235	0,177	0202	0,189	0185	–

Abbreviations: B. d. = *Boophis doulioti*; B. o. = *Boophis occidentalis*; B. m. = *Boophis madagascariensis*

0.28% ± 0.001–1.93% ± 0.003; COI: 11.4% ± 0.019–15.68% ± 0.023). With regard to 18S, total differences between species showed no character difference between *M. multiova* n. sp. and *M. theroni* n. sp. while the total 18S differences among all other *Metapolystoma* spp. ranged from 1 to 2 (Table 4). Regarding total 28S differences (Table 5), values ranged from 4 to 27 among all *Metapolystoma* spp., while the lowest value occurred between *M. multiova* n. sp. and *M. theroni* n. sp. Finally, regarding total COI differences (Table 6), values ranged from 25 to 45 between all *Metapolystoma* spp. Though no 18S character difference was detected between *M. multiova* n. sp. and *M. theroni* n. sp., the high levels of genetic divergence between *M. falcatum* n. sp., *M. multiova* n. sp., *M. theroni* n. sp., *M. brygoonis* and *M. cachani* (as estimated on the basis of 28S and COI sequences) led us to consider three new *Metapolystoma* species.

When 18S, 28S and COI sequences were combined for Bayesian analysis (Fig. 14), *M. cachani* appears as the most basal species within *Metapolystoma* and Malagasy *Metapolystoma* form a clade. Within this clade, *M. multiova* n. sp. and *M. theroni* n. sp. are sister species; two sublineages are well differentiated within *M. brygoonis*. Sublineage A comprises polystomes found in localities at Makira, Ranomafana and Vohiparara (Fig. 1), while sublineage B comprises polystomes of the Ambatolampy and Ankarafantsika areas (Fig. 1). Because high levels of 28S and COI genetic divergence (Tables 5 and 6) were also observed these two sublineages (28S: 0.14% ± 0.0009; COI: 5.71% ± 0.011), it suggests that there could be two genetic entities within *M. brygoonis* infecting the same host species in Madagascar, that is, *Ptychadena mascareniensis*.

3.3. Remarks

The phylogenetic position of *M. falcatum* n. sp., *M. multiova* n. sp. and *M. theroni* n. sp. within the Polystomatidae (Fig. 14) confirms that they are members of *Metapolystoma* (Polystomatidae). Genetic divergence estimates among *Metapolystoma* taxa (Tables 4–6) sustain morphological descriptions, for these three species.

The lengths of *M. vencesi* n. sp. (9190) and *M. theroni* n. sp. (9086) differ from all other Malagasy metapolystomes, which vary in length from 2680 to 6710. *M. theroni* n. sp. has a haptor length of 1,634, separating it from all other known Malagasy metapolystomes. Haptor length of *M.falcatum* n. sp. (1492) and *M. vencesi* n. sp. (1437) overlap but differ substantially from *M. ansuanum* n. sp. (669–912), *M. brygoonis* (760–1070) and *M. multiova* n. sp. (916). Haptor length-body length ratio of *M. vencesi* n. sp. (0.16) separates it from all other species while, in the overlapping cases of *M. falcatum* n. sp. (0.19), *M. multiova* n. sp. (0.18) and *M. theroni* n. sp. (0.18), there is a marked difference from *M. ansuanum* n. sp. (0.23–0.25) and *M. brygoonis* (0.22–0.23), the latter two of which overlap in turn.

With a hamulus length X (Fig. 3b) of 269–332, *M. vencesi* n. sp. differs from all other known Malagasy metapolystomes, which range in length between 196 and 420. It is also the single species that has no separation between the hamulus handle and guard. *Metapolystoma brygoonis* (330–420), *M. falcatum* n. sp. (393–407) and *M. theroni* n. sp. (392–408) overlap but differ from *M. ansuanum* n. sp. (205) and *M. multiova* n. sp. (196–248), while the latter two also overlap. Hamulus hook length Z (Fig. 3b) separates all newly described metapolystomes from one another, ranging from *M. vencesi* n. sp. (40–52) with the smallest hooks to *M. falcatum* n. sp. (66–70) with the largest. Besides *M. ansuanum* n. sp. differs from all other metapolystomes in that it has an exceptional long hamulus hook relative to the rest of the hamulus and *M. falcatum* n. sp. differs from all others in that it has an exceptional long curved marginal hooklet tip on hooks two to seven.

With a genital bulb diameter of 115, *M. vencesi* n. sp. has the largest diameter ranging between 64 and 86. *Metapolystoma. brygoonis* (80), *M. falcatum* n. sp. (86), and *M. multiova* n. sp. (86) overlap but are separated from *M. ansuanum* n. sp. (64–73) and *M. theroni* n. sp. (65), while the latter two overlap. *Metapolystoma falcatum* n. sp. (26),

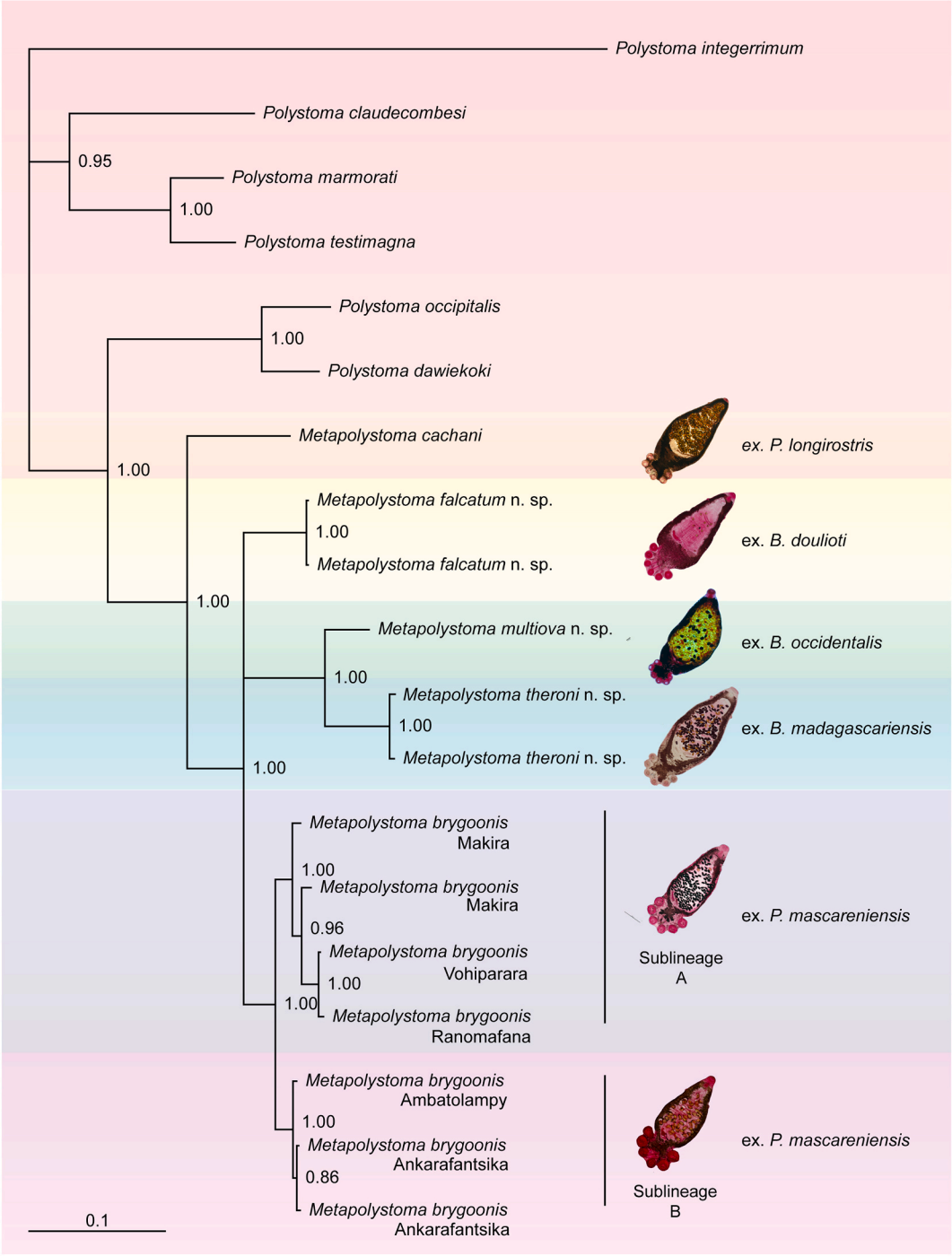


Fig. 14. Bayesian tree inferred from the analysis of concatenated 18S, 28S and COI gene sequences. Node values indicate Bayesian posterior probabilities.

M. multiova n. sp. (25), and *M. theroni* n. sp. (24–28) overlap in genital crown diameter but are separated from *M. ansuanum* n. sp. (29–33) and *M. vencesi* n. sp. (32), while the latter two overlap. *Metapolystoma brygoonis*, *M. falcatum* n. sp. and *M. vencesi* n. sp. all bear eight genital spines and differ from *M. ansuanum* n. sp., *M. multiova* n. sp. and *M. theroni* n. sp. that respectively bear ten, six and seven genital spines. With the greatest genital spine length of 40.4–43.7 *M. multiova* n. sp. is separated from all other known Malagasy metapolystomes, except *M. brygoonis*, which ranges between 24.3 and 34.9.

Metapolystoma brygoonis (410–460), *M. multiova* n. sp. (446) and *M. theroni* n. sp. (479) overlap in ovary length but differ from *M. ansuanum* n. sp. (265–338), *M. falcatum* n. sp. (772) and *M. vencesi* n. sp. (861), the latter which also differ from one another. With the greatest egg length of 239–265, *M. theroni* n. sp. differs from all other Malagasy metapolystomes, which range from 160 to 230. *Metapolystoma ansuanum* (196–217), *M. multiova* n. sp. (198–228), and *M. vencesi* n. sp. (210–230) overlap but differ from *M. brygoonis* (160–200) and *M. falcatum* n. sp. (161–185), the latter two which overlap.

To conclude, due to the small number of parasite specimens that were investigated, we can not exclude that the morphometric variability may represent intra specific variation. However, worms under investigation show a combination of morphological characters, namely shape of the hamulus handle and guard, shape of the hamulus hook, shape of the marginal hooklet tip on hooks and genital spine number that allow

the differentiation of five distinct metapolystome species.

4. Discussion

Metapolystoma was until now reported only from the Afrotropical realm anuran hosts belonging to *Ptychadena* (Ptychadenidae). *Ptychadena* is a successful and widespread genus in Africa being a well-suited host for polystomes (Du Preez and Kok, 1992a). Of the 56 known *Ptychadena* species (Frost, 2020), 11 are known to host polystomes, including 14 *Polystoma* and three *Metapolystoma* species (see Du Preez and Kok, 1992a). According to Verneau et al. (2009), *Metapolystoma* may have originated in Africa within the time window 19.8–4.3 million years ago (Mya) from ancestors close to *Polystoma* and further dispersed to Madagascar following natural transoceanic dispersal of the ancestor of *Pt. mascareniensis* at about 14.2–2.3 Mya (Verneau et al., 2009). Because *M. brygoonis* forms a sister group to all other members of Malagasy *Metapolystoma*, *M. cachani* being basal within *Metapolystoma*, a host switch was suggested from the ancestor of *Pt. mascareniensis* to ancestral *Boophis* (Mantellidae) (Verneau et al., 2009), which is confirmed in the present phylogenetic study. Even though *Ptychadena* and *Boophis* are not phylogenetically closely related, they both display pleisiomorphic reproductive modes, favouring the possibility of an ancestral host switch in Madagascar (see Verneau et al., 2009), and ultimately the diversification of *Metapolystoma* within *Boophis*. While *Boophis* is not the single representative of the family Mantellidae infected by polystomes in Madagascar, mantellid frogs of the two genera *Mantella* and *Blommersia* which both exhibit a derived mode of reproduction, are infected by polystomes of another genus, i.e. *Madapolystoma* (see Verneau et al., 2009; Du Preez et al., 2010; Landman et al., 2018). In addition, Verneau et al. (2009) reported another undescribed species of *Metapolystoma* from *Aglyptodactylus madagascariensis* (Duméril, 1853). Therefore, the five new *Metapolystoma* species described along this study clearly indicate that a larger diversity of metapolystomes can be expected. This is especially true since Madagascar is inhabited by 79 *Boophis* species (Frost, 2020) that may serve as hosts for *Metapolystoma* species. Furthermore, *M. brygoonis* can be divided in two separate lineages according to genetic differentiation (Fig. 14). These results strengthen the fact that *Metapolystoma* in Madagascar still continue to diversify and call for further sampling and investigation.

The validity of *Metapolystoma* has long been disputed. It was suggested that the uterine structure was achieved convergently within *Metapolystoma* (Tinsley, 1974), through the ability of the parasite to adapt to the ecology of its host (Kok and Seaman, 1987; Murith, 1981; Tinsley, 1983). Bentz et al., (2001) claimed it to be invalid and ascribed its morphological differences from *Polystoma* in terms of homoplastic characters. Nevertheless our phylogeny supports the monophyly of *Metapolystoma*, which is however nested within the paraphyletic *Polystoma*. If the morphological differences between *Metapolystoma* and *Polystoma* were the product of reproductive plasticity, *Metapolystoma* spp. would not have clustered together on a molecular level (see Fig. 14). The fact that they do, therefore, suggests that the long uterus may have been inherited by descent. It is however intriguing to note that two polystome species *M. porosissimae* and *P. sodwanensis*, which display completely different life-history strategies, can occur simultaneously in the same host species, i.e. *P. porosissima* in Africa (Du Preez and Kok, 1992b). *Metapolystoma porosissimae* displays a strategy where many eggs are stored in a large uterus, which is typical of polystomes that infest hosts within arid environments (Du Preez, 2015; Du Preez and Kok, 1992b). Conversely, *P. sodwanensis* has a small uterus containing only a few eggs, which is in line with a water-dependent host (Du Preez and Kok, 1992b). Even though morphological differences between the two species are distinct, the simultaneous occurrence of these two species in the same individuals of *Pt. porosissima* have reinforced the dispute (Du Preez and Kok, 1992b). Though the validity of *Metapolystoma* at this stage cannot be ruled out, a more in depth genetic investigation of the two African polystome species *M. porosissimae* and *P. sodwanensis* should help to conclude.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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