



## A new genus of polystomatid parasitic flatworm (Monogenea: Polystomatidae) without free-swimming life stage from the Malagasy poison frogs

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### Abstract

*Madapolystoma* n. g. (Monogenea, Polystomatidae), is proposed for a new genus of polystomatid from the urinary bladder of the Malagasy poison frogs of the genus *Mantella* (family Mantellidae), with the description of one new species. This is the second anuran polystome to be described from Madagascar. The parasites are small with a maximum body length of less than 3 mm. The two gut caeca have a few diverticulae but no prehaptoran anastomoses and are confluent posteriorly. The haptor bears six well-developed suckers and one pair of hamuli. A single small ovary lies in mid-body while the single follicular testis lies posteriorly in the body. Vaginae are present. The new genus is unique among all known polystomes in that it apparently has no free-swimming ciliated larvae but instead is characterized by advanced intra-uterine development with larvae developing hamuli and even suckers while still *in utero*. Based on molecular phylogenetic data, the closest relative of *Madapolystoma* is the genus *Eupolystoma* from Africa. Madagascar has a very diverse anuran fauna with a 100% level of endemicity at the species level, and more *Madapolystoma* species from other mantellid hosts await description.

**Key words:** Monogenea, Polystomatidae, *Madapolystoma*, *Mantella*, Madagascar

### Introduction

Madagascar's unique biota evolved under isolation for an extended period of time (Goodman & Benstead 2003; Glaw & Vences 2007). As initial part of the Gondwana supercontinent the island has been separated from India for *ca* 96–84 Myr (Storey *et al.* 1995; Briggs 2003) and from Africa for *ca* 165–158 Myr (Rabinowitz *et al.* 1983; Briggs 2003). Various hypotheses have been postulated to explain the species rich and endemic anuran diversity of Madagascar. Some of the endemic frog lineages dispersed directly from Africa during the Late Cenozoic (Vences *et al.* 2003; Yoder & Nowak 2006) while the most species rich endemic family Mantellidae and endemic representatives of the Microhylidae appear to be more ancient and have reached Madagascar in the Late Cretaceous or Early Cenozoic (Van Bocxlaer *et al.* 2006; Van der Meijden *et al.* 2007). Madagascar is ranked high in terms of species diversity, endemicity and threats to species ever since Myers *et al.* (2000) identified 25 geographical areas as biodiversity conservation 'hot spots'. This island is ranked as the second most important "hot spot" in terms of biodiversity conservation. The current inventory of scientifically named Madagascan anurans includes about 265 species, however this is a significant under-estimation and the true species diversity comprises at least 373 but possibly as many as 465 species (Vieites *et al.* 2009).

Polystomatids are endoparasitic monogenetic flatworms infecting aquatic and amphibious tetrapods with the majority in anurans and freshwater chelonians. Thus far 23 genera have been recognised within the Polystomatidae, containing more than 200 species. Polystomes most likely arose 425 Myr ago during the transition

between actinopterygians and sarcopterygians (Verneau *et al.* 2002). Whereas some anuran polystomes like *Polystoma* Zeder, 1800 and chelonian polystomes like *Polystomoides* Ward, 1917 and *Neopolystoma* Price, 1939 have cosmopolitan distributions, most others are restricted to single continents or geographical realms. The genus *Metapolystoma* Combes, 1976 is only known from Africa and Madagascar, *Protopolystoma* Bychowsky, 1957 from Africa as well as from feral populations of its host *Xenopus*, and *Eupolystoma* Kaw, 1950 from Africa and India.

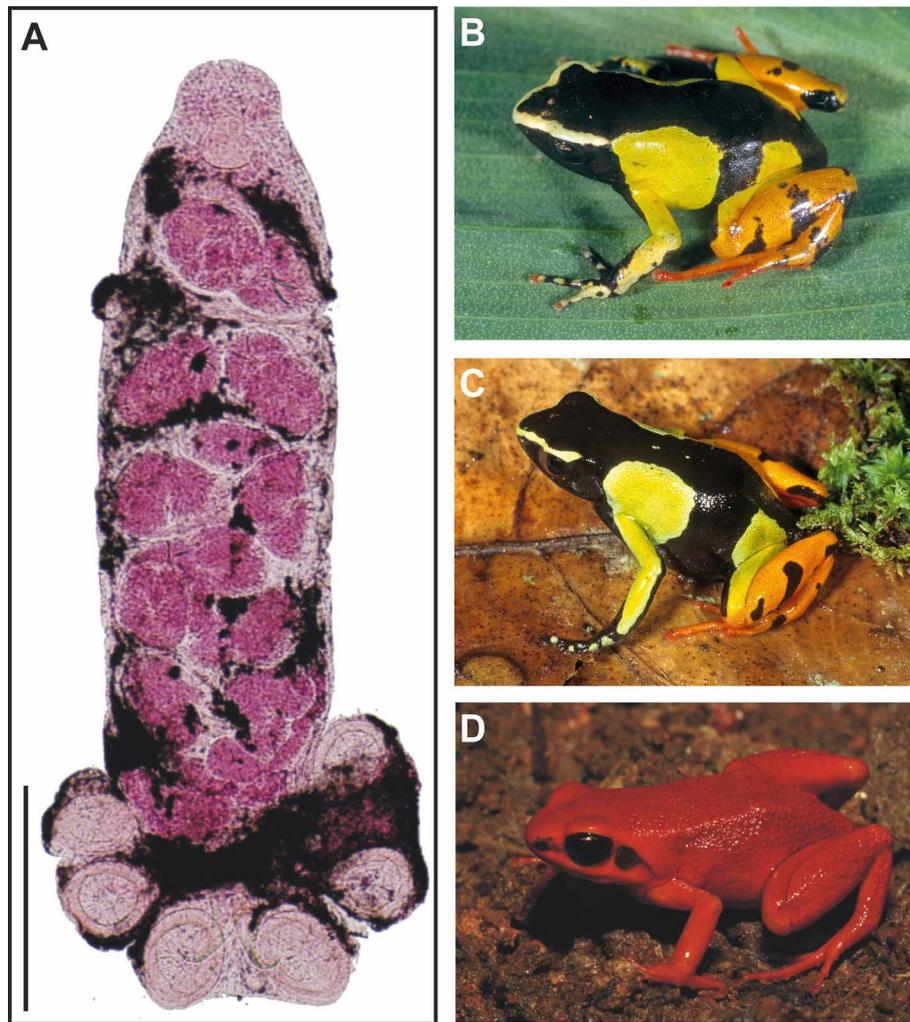
Of the 13 genera of anuran polystomes only *Metapolystoma* was until recently known from Madagascar with a single described species *Metapolystoma brygoonis* Euzet & Combes, 1964 known from *Ptychadena mascareniensis* (Duméril & Bibron). During a schistosome research visit to Madagascar in November 2001, the first author (LdP) discovered a new polystome that did not fit into any of the known polystome genera. Subsequent visits to Madagascar during 2005, 2006, 2007 and 2008 revealed that 25 out of the 86 anuran species that were screened were infected with polystomes. A molecular analysis in which 14 polystomes from 12 Malagasy host species were included revealed that the polystomes found in the host families Mantellidae and Ptychadenidae belong to two separate clades with one forming a lineage within *Metapolystoma* and the second corresponded to the undescribed genus (Verneau *et al.*, 2009). In contrast to the estimated origin of the lineage within *Metapolystoma* (ca 14–2 Myr ago) the undescribed genus may have colonised Madagascar in the Late Mesozoic or Early Cenozoic (Verneau *et al.* 2009). Only very limited numbers of adult specimens were available for a formal description but in the light of the fact that the anuran genus *Mantella* is a CITES-listed genus with numerous species threatened by extinction, the chances of obtaining more mature parasites at this stage is small. We thus decided to go ahead and establish the new genus and this paper serves as the formal description of this new genus and species.

## Material and methods

During November 2001 the first author visited the Madagascar Exotic Park at Mandraka. Permission was obtained to screen a number of frogs for the release of polystome eggs which would indicate a positive infection. Species examined included *Mantella madagascareniensis* (10 specimens), *M. aurantiaca* (9 specimens), *M. laevigata* (7 specimens), *Boophis goudoti* (3 specimens), *Dyscophus guineti* (4 specimens), *Scaphiophryne pustulosa* (5 specimens) and *Ptychadena mascareniensis* (17 specimens). Frogs were removed from their display cages except *P. mascareniensis* that was collected among buildings on site. Frogs were grouped per species and placed in clear plastic jars, each containing about 5 mm deep tap water. Frogs were left overnight and the next morning water was poured through a 112 µm plancton sieve. The content of the sieve was then washed down into a Petri dish which was screened for polystome eggs. Polystome eggs were only retrieved from the container with the *P. mascareniensis* indicating a *Metapolystoma brygoonis* infection. From the container holding the *M. madagascareniensis* a single mature polystome was retrieved from the water. The specimen was transferred to a Petri dish containing dechlorinated tap water. Nine immature parasites were released from this parasite of which three were temporarily mounted in ammonium picrate and six were fixed in 96% EtOH for DNA extraction. Permission was obtained to dissect one *M. madagascareniensis* and it was found to be infected with one mature and five immature polystomes. A further four parasite larvae were released when the mature specimen was fixed under cover slip pressure. These larvae had no cilia and thus could not swim. The mature parasite and four juveniles were fixed under cover slip pressure in 70% EtOH while the remaining five specimens were fixed in 96% EtOH. During February 2008, a single specimen of *M. madagascareniensis* was collected at Besariaka near Moramanga. The specimen was dissected and found to be infected with 9 juvenile parasites. Of these two were fixed in 96% EtOH, three were flat fixed in 10% neutral buffered formalin and four were temporarily mounted in ammonium picrate. During January 2005, 17 specimens of *Mantella milotympanum* were obtained from a local dealer. These specimens almost certainly originated from the Fierenana area but the precise locality is unknown. Of the 17 specimens dissected no less than eight were found to be infected with between one and nine parasites (prevalence 47%, mean intensity 3.6). All of the parasites were juvenile except one mature specimen that was found with eight juveniles in one host. All parasite measurements are in micrometers.

During November 2005, a local collector supplied 11 specimens of *Mantella baroni* from Sahasoratra near Besariaka. This collection was fixed in EtOH and during February 2006 dissected for polystomes. One of the specimens was infected with three mature polystomes. One polystome specimen was stored in 96% EtOH while the

other two were stained and mounted. During January 2008 one of the authors (LR) dissected 10 specimens of *M. baroni* from the Moramanga area. One of the specimens was infected with one mature polystome. This parasite was fixed under cover slip pressure in 10% neutral buffered formalin, stained and mounted in Canada balsam.



**FIGURE 1.** *Madapolystoma biritika* n. g., n. sp. A, micrograph of the type specimen. Scale: 500 $\mu$ m; B, *Mantella madagascariensis* (specimen from Besariaka); C, *M. baroni* (specimen from An'Ala); D, *M. milotympanum*.

Our molecular sampling included 13 polystome specimens that were recovered from seven distinct mantellid species of Madagascar (see Table 1). Two specimens from the genus *Eupolystoma* were also analysed for rooting the tree (see Verneau *et al.* 2009). Polystomes were dried and incubated for 1.5 hours at 55°C in 100–150  $\mu$ l of Chelex 10% and Proteinase K (final concentration 1 mg ml<sup>-1</sup>). DNA extractions were stopped at 100°C for 15 minutes and DNA samples were preserved at -20°C until use. Amplifications of a DNA fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene were performed with Forward L-CO1p, 5'-TTTTTGGGCATCCTGAGGTTTAT-3' and Reverse H-Cox1p2, 5'-TAAAGAAAGAACATAATGAAAATG-3' primers (Littlewood *et al.* 1997), yielding a PCR product of approximately 440bp. Because PCR was not successful in all cases, we designed two other primers Forward COI-FB, 5'-TGGTATAATTAGTCATATATG-3' and Reverse COI-RB, 5'-AACAAACAAAYCAAGAATCATG-3' that gave a PCR product of approximately 370bp. The PCR procedure involved one initial step of 5' at 95°C for long denaturation, followed by 35 cycles of 1' at 95°C for denaturation, 2' at 48°C for annealing and 2' at 72°C for extension, and one final step of 10' at 72°C for terminal elongation. PCR and DNA purification were performed according to the procedure described in Verneau *et al.* (2009). Sequencing was completed with the Forward and Reverse primers at GATC Biotech (France). DNA sequences were edited and corrected using Sequencher™ software (Gene Codes Corporation, Ann Arbor,

Michigan, USA) and aligned using Clustal W (Thompson *et al.* 1994) which is implemented in MEGA version 4 (Tamura *et al.* 2007). Maximum Likelihood (ML) phylogenetic analysis was performed on 331 characters with regions not sequenced for all taxa and ambiguous characters excluded. A search for the best ML tree was done under the GTR +  $\Gamma$  model selected by the AIC in Modeltest (Posada and Crandall, 1998), and following a heuristic procedure with the TBR branch swapping option in PAUP\* 4.0b9 (Swofford, 2002). ML bootstrap support values were calculated with the same model of sequence evolution under the NNI branch swapping option. Uncorrected pairwise divergences (p-distances) and total character differences were also estimated within each pair of polystome taxa using PAUP\* 4.0b9, because genetic divergences may give some valuable insights to assign the taxonomic status of species.

## Results

**Class: Monogenea**

**Order: Polystomatidea**

**Family: Polystomatidae Gamble, 1896**

***Madapolystoma* n. g.**

**Generic diagnosis:** Polystomatinae: Small ovoid worm with pyriform body. Haptor with three pairs of suckers, two hamuli and 16 marginal hooklets placed as for other known polystomes (hooklets 1 & 2 situated between hamuli, hooklets 3–5 at the base of the haptoral suckers and hooklets 6–8 posterior between the third pair of suckers. Mouth subterminal, surrounded by false sucker. Pharynx medium in size and muscular. Gut bifurcate; intestinal caeca with few diverticula confluent in the haptoral region, no prehaptoral anastomoses. Genito-intestinal canal present. Testis single, small, post-ovarian, intercaecal and positioned posteriorly in the body. Vas deferens extending antero-medially to genital bulb armed with 8 genital spines. Ovary small, oval, median and placed in middle of body. Uterus tubular extending pre and post genital region and fill most of body. Two vaginae, antero-lateral to ovary; left and right vaginal ducts. Advanced intra-uterine development of larvae with hamuli and up to two pairs of suckers *in utero*. Adult parasitic in urinary bladder. Type-species: *Madapolystoma biritika* n. g. & n. sp.

***Madapolystoma biritika* n. g. & n. sp.**

(Adult: Figures 1–4)

**Specimens studied.** Two sexually mature worms from *Mantella madagascariensis*; holotype (NMB P318) and one paratype (NMB P319). Two sexually mature (paratypes NMB P320 & NMB P321) and 11 immature worms from *M. baroni*. One sexually mature worm (paratype NMB P 322) and 10 immature worms from *M. milotympanum*. All types in the Parasitic Worm Collection, National Museum, Bloemfontein, South Africa.

**Type host.** *Mantella madagascariensis* (Grandidier, 1872)

**Other hosts.** *Mantella baroni* and *M. milotympanum*.

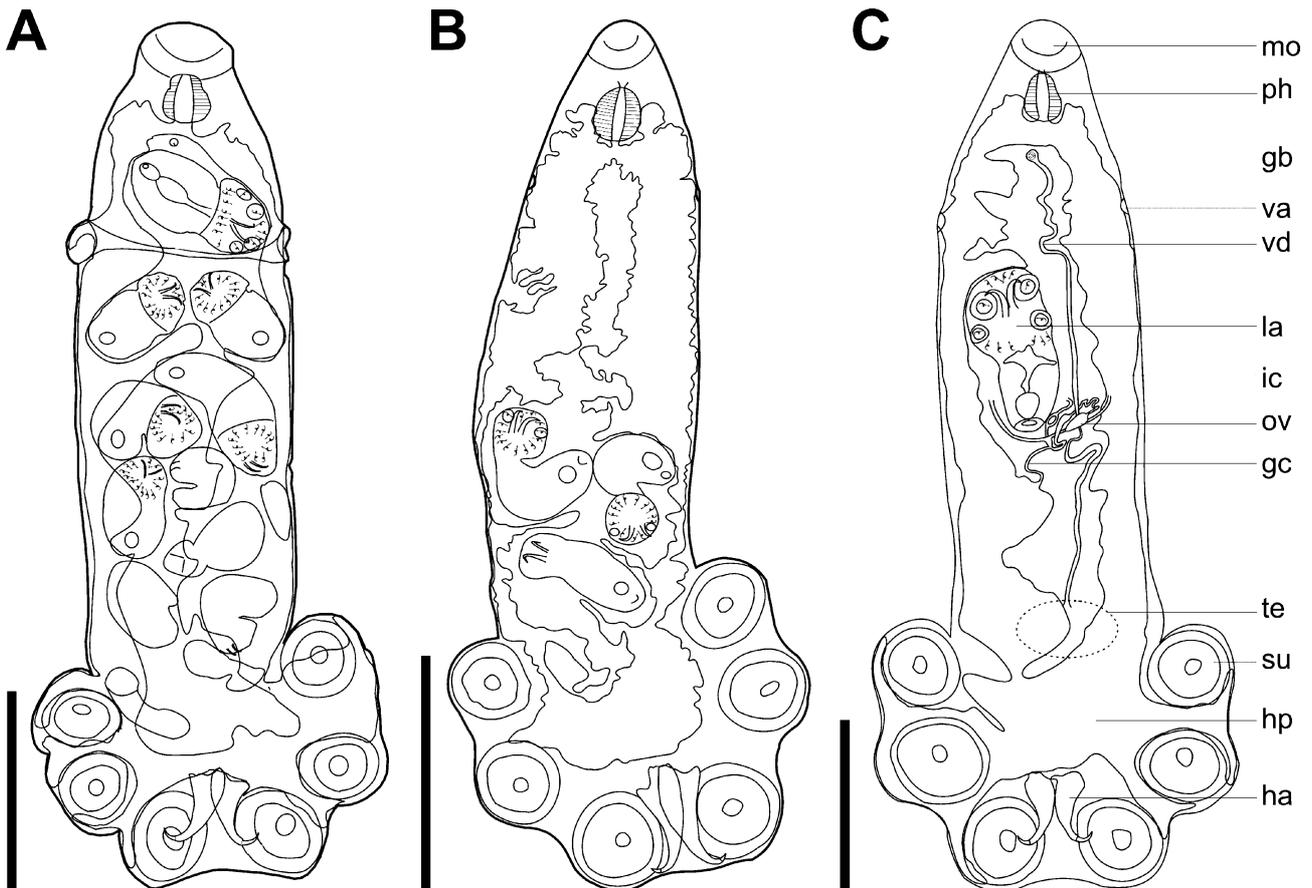
**Site.** Urinary bladder

**Locality for type host.** Exact locality unknown.

**Etymology.** The species name “biritika” is Malagasy for extremely small and refers to the small size of the parasite.

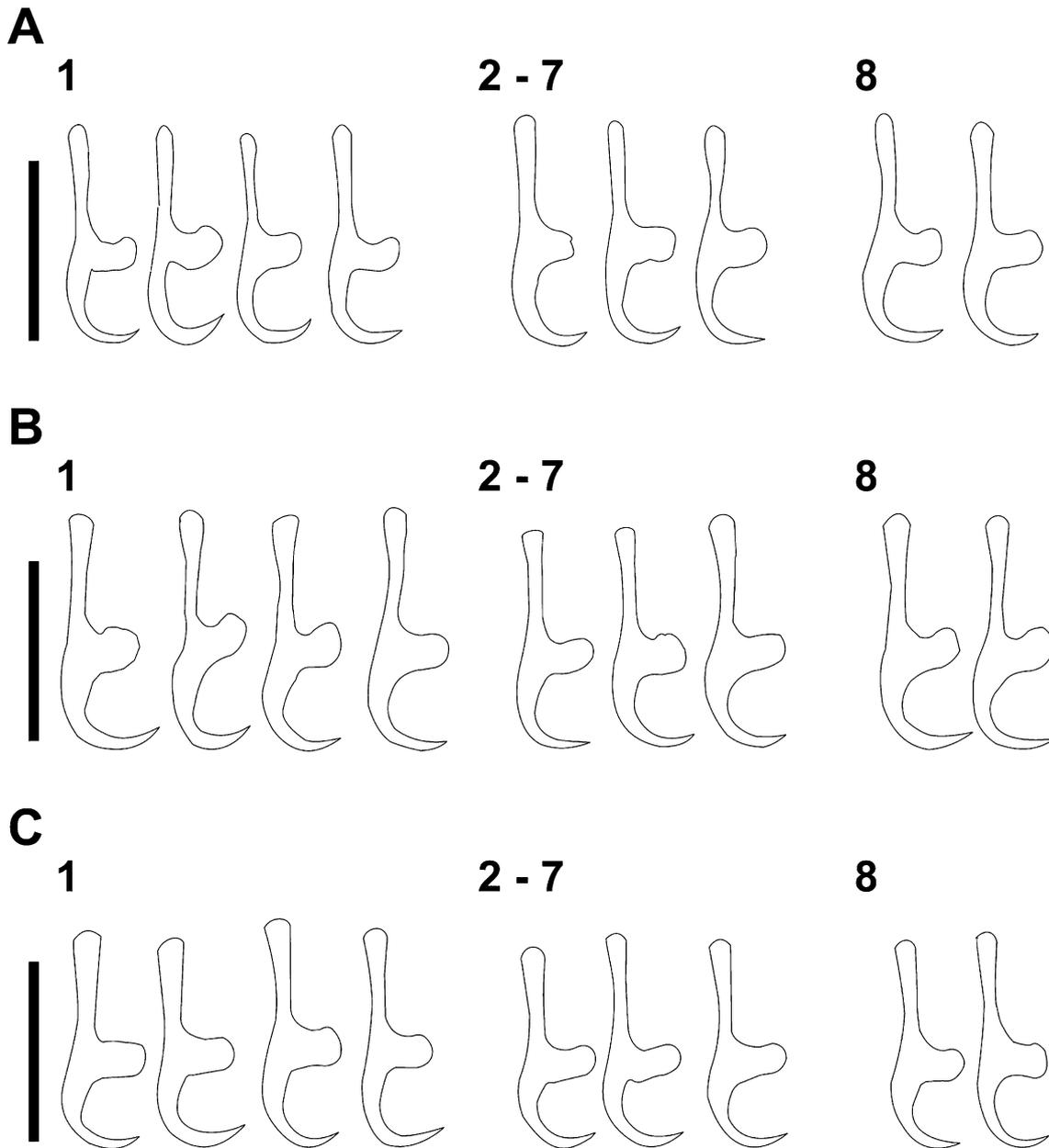
**Description.** Based on five mature specimens Table 2. Adults pyriform (Figures 1 & 2) and vary in length from 1,304 to 3,041. Eyespots not observed in adult. Mouth subterminal, ventral, surrounded by false oral sucker. Pharynx spherical. Intestine bifurcate, confluent posteriorly with a few medial diverticula, no prehaptoral anastomoses. Testis single, follicular, post-ovarian, median posteriorly in body. Vas deferens widens slightly anteriorly forming seminal vesicle, narrows at genital bulb to open at common genital opening. Genital bulb armed with 8 genital spines. Two vaginae, on lateral margins in middle of body antero-lateral to ovary; vaginal ducts

descending. Ovary in middle and median of body, oval (Figure 2C). Oviduct leaves ovary, ascends, forms ootype. Uterus tubular and coiled extending from haptor to genital bulb holding embryos in an advanced stage of development. For the parasites from *M. madagascareniensis* the uterus holds respectively 13 and 32 embryos, three and 18 respectively for polystomes from *M. baroni* and one for the parasite from *M. milotympanum*. Embryos from the *M. madagascareniensis* parasite vary in size from 109 x 95 for small embryos to 286 (234–330) x 123 (116–139) for developed embryos; hamuli already visible in small embryos reaching 46 in developed embryos, pharynx 47 x 34; for polystomes from *M. baroni* the embryos vary from 109 for small undeveloped embryos to 286 (234–330) x 123 (116–139) for developed embryos; hamuli start development in small embryos reaching 46 in developed embryos; pharynx 47 x 34; the parasite from *M. milotympanum* had a single advanced embryo in utero with mouth, pharynx, intestine, developing gonads, hamuli and two pairs of suckers visible; 457 long and 213 wide, hamuli with recurved points respectively 41 and 40, oral sucker 70 and pharynx 80 x 63.



**FIGURE 2.** *Madapolystoma biritika* n. g., n. sp. Ventral view of holotype (A) and paratypes (B&C). Abbreviations: gc, genito-intestinal canal; gb, genital bulb; ha, hamulus; hp, haptor; ic, intestinal caecum; la, larva; mo, mouth; ov, ovary; ph, pharynx; su, sucker; te, testis distribution; va, vagina; vd, vas deferens. Scale bars: 500  $\mu$ m.

Embryos encapsulated in thin membrane, non operculate. Embryos not ciliated, cilia were not observed in any developmental stage of the larvae. Sixteen marginal hooklets, placement as for other polystomes: pairs one and two posterior-most between suckers; pairs three, four and five at bases of suckers and pairs six to eight anterior in haptor between anterior-most suckers. Marginal hooklets 24.2 (21.4–26.1) for parasites from *M. madagascareniensis*; 24.6 (20.4–26.9) for parasites from *M. baroni* and 23.8 (21.2–26.1) for parasites from *M. milotympanum*. Hooklets 1 not bigger than hooklets 2–8 (Figure 3).



**FIGURE 3.** Marginal hooklets 1, 2–7 and 8 from larvae and subadult specimens of *Madapolystoma biritika* n. g., n. sp. A, hooklets for specimens from *Mantella madagascariensis*; B, hooklets for specimens from *Mantella baroni*; C, hooklets for specimens from *Mantella milotympanum*. Scale bar = 20  $\mu$ m.

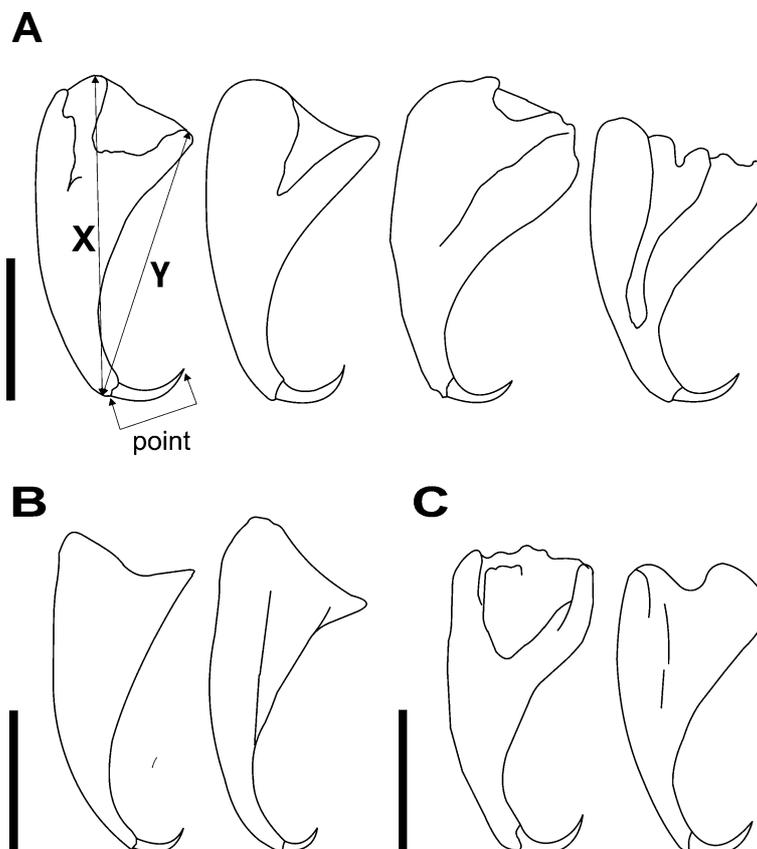
Measurements of six immature specimens collected from *M. madagascariensis* during 2008: Body length 905 (526–1,128), greatest width 236 (174–286). Haptor length 298 (175–397), haptor width 365 (205–512). False oral sucker 110 (82–143). Pharynx length 98 (75–115), pharynx width 89 (68–105). Hamulus length 122 (106–133), recurved point 46 (42–47) (Figure 4). Diameter of sucker pair one 114 (75–143), sucker pair two 89 (58–109) and sucker pair three 70 (44–90).

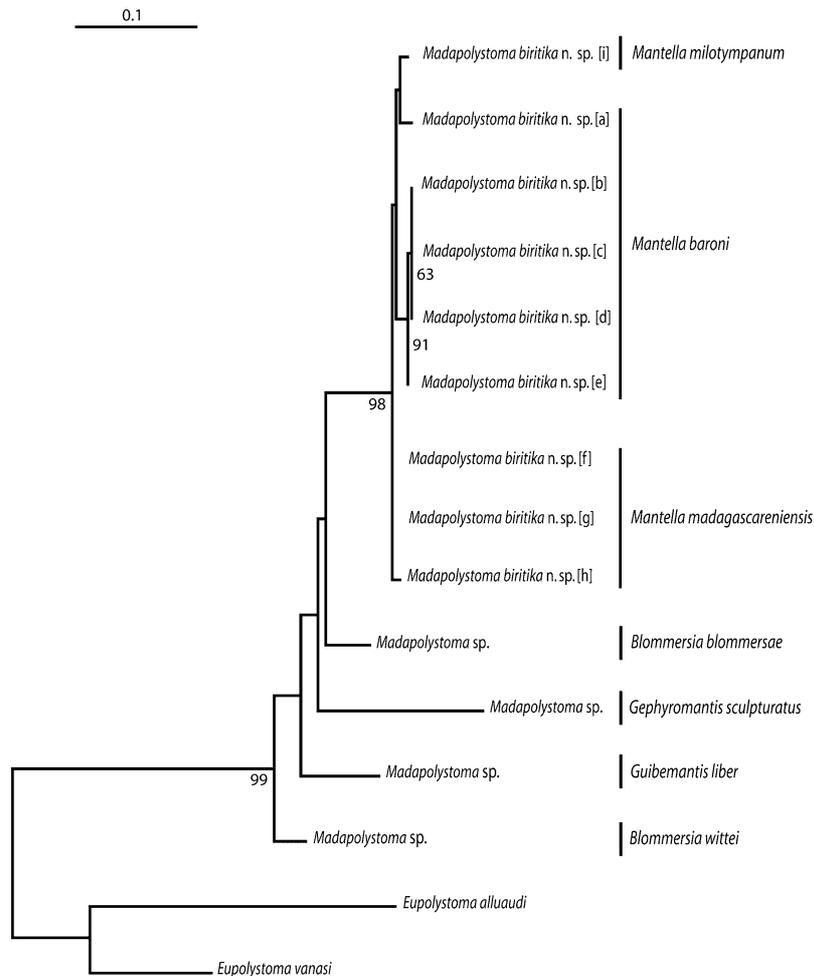
Measurements of 11 immature specimens collected from *M. baroni*: Body length 841 (520–1,120), greatest width 251 (197–290). Haptor length 286 (190–374), haptor width 354 (250–426). Hamulus length 116 (65–139), recurved point 44 (42–45). Diameter of sucker pair one 113 (79–139), sucker pair two 87 (48–108) and sucker pair three 74 (60–94).

Measurements of 10 immature specimens collected from *M. milotympanum*: Body length 764 (646–967), greatest width 260 (238–296). Haptor length 247 (200–325), haptor width 325 (165–445). Hamulus length 112 (99–129), recurved point 46 (44–48). Diameter of sucker pair one 98 (78–135), sucker pair two 74 (49–100) and sucker pair three 49 (31–75).

**TABLE 1.** List of parasites investigated, their host species, geographical origin, field and GenBank accession numbers.

Parasite species	Host species	Origin	Field numbers for polystomes	Accession numbers for COI
<i>Eupolystoma alluaudi</i>	<i>Bufo</i> sp.	Togo		FR667558
<i>Eupolystoma vanasi</i>	<i>Schismaderma carens</i>	South Africa		FR667559
<i>Madapolystoma</i> sp.	<i>Blommersia blommersae</i>	Madagascar: An'Ala	PL060210B	FR667560
<i>Madapolystoma</i> sp.	<i>Blommersia wittei</i>	Madagascar: Isalo	PL070221D	FR667561
<i>Madapolystoma</i> sp.	<i>Gephyromantis sculpturatus</i>	Madagascar: An'Ala	PL060209G	FR667562
<i>Madapolystoma</i> sp.	<i>Guibemantis liber</i>	Madagascar: An'Ala	PL060211I	FR667563
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [a]	<i>Mantella baroni</i>	Madagascar: Vohiparara	PL060220K	FR667564
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [b]	<i>Mantella baroni</i>	Madagascar	PL050123J1	FR667565
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [c]	<i>Mantella baroni</i>	Madagascar	PL050123J2	FR667566
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [d]	<i>Mantella baroni</i>	Madagascar: Sahasoratra	PL060214E	FR667567
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [e]	<i>Mantella baroni</i>	Madagascar: Ranomafana	P15Mb06	FR667568
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [f]	<i>Mantella madagascariensis</i>	Madagascar	PL011126B1	FR667569
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [g]	<i>Mantella madagascariensis</i>	Madagascar	PL011126B2	FR667570
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [h]	<i>Mantella madagascariensis</i>	Madagascar	PL080204A	FR667571
<i>Madapolystoma biritika</i> <b>n. g., n. sp.</b> [i]	<i>Mantella milotymanum</i>	Madagascar	PL050114E	FR667572

**FIGURE 4.** Hamulus of *Madapolystoma biritika* **n. g., n. sp.** A, hamuli from mature specimens from the host *Mantella madagascariensis*; B, hamuli from mature specimens from the host *Mantella baroni*; C, hamuli from mature specimens from the host *Mantella milotymanum*. Scale bar = 100  $\mu$ m.



**FIGURE 5.** Maximum Likelihood tree inferred from an analysis of 331 nucleotides of DNA sequences of the mitochondrial COI gene. Values along branches correspond to bootstrap proportions after 1,000 replicates. Letters [a] to [h] indicate different individuals of the same polystome candidate species.

### Molecular analyses

The genetic divergences (uncorrected pairwise distances) in the amplified COI fragment among parasites of *M. baroni* range from 0 to 2.41% (Table 3). Molecular divergences of polystomes of *M. baroni*, to those of *M. milotympanum* and *M. madagascariensis* range from 1.51% to 2.11% and from 1.21% to 1.81%, respectively (Table 3). Similarly, polystomes of *M. milotympanum* and *M. madagascariensis* differ by 0.90% to 1.20%. According to Du Preez *et al.* (2007), the molecular threshold typically found between species of polystomes amounts approximately to about 2.0% in the COI gene. Therefore, at this stage molecular data do not support considering polystomes of *M. baroni*, *M. milotympanum* and *M. madagascariensis* as separate polystome species. On the other hand, the molecular divergences estimated between polystomes of these three host species and parasites of *Gephyromantis sculpturatus*, *Guibemantis liber*, *Blommersia blommersae* and *B. wittei* range from 6.94% to 13.89%. Similarly, polystomes of *Gephyromantis*, *Guibemantis* and *Blommersia* diverge from each other by at least 7.55%. According to these results and the phylogenetic relationships within mantellid polystomes (Figure 5), we can suspect at least five new species infecting mantellid frogs in Madagascar. A more extensive sampling of parasites for molecular studies as well as for morphometric analyses are needed to assess whether the species we describe herein may be a composite of more cryptic species. With the limited material available at this stage and the relatively low molecular divergence between specimens we here opt for a more conservative approach and assign all parasites infecting *Mantella* species to a single polystome species. Finally, regarding the

molecular divergences between *Eupolystoma* and *Madapolystoma* n. g. that range from 19.69% to 24.16% (Table 3), we consider that the polystomes surveyed here from mantellid frogs in Madagascar are best assigned to a new genus, as also indicated by various characters of their morphology and reproductive mode, phylogenetic evidence based on combined analysis of the 18S and 28S ribosomal genes (Verneau *et al.*, 2009), and 28S molecular divergences (Table 4) inferred from a larger polystome sampling (see Verneau *et al.* 2009).

**TABLE 2.** Body measurements for *Madapolystoma biritika* n.g., n. sp. based on five mature specimens. All measurements in micrometers.

Measurement	Combined sample	From <i>M. baroni</i>	From <i>M. madagascareniensis</i>	From <i>M. milotympanum</i>
Body Length	2,041 (1,304 – 3,041)	1,506 (1,304 – 1,786)	2,604 (2,168 – 3, 041)	2,518
Greatest width	512 (420 – 597)	428 (420 – 435)	554 (545 – 562)	597
Haptor length	679 (594 – 788)	594	667 (626 – 707)	788
Haptor width	882 (707 – 1,046)	707	887 (862 – 912)	1,046
Width at vagina	481 (369 – 548)	369	503 (460 – 546)	548
Hamulus length X	215 (190 – 238)	221 (190 – 238)	208 (205 – 210)	214 (212 – 217)
Hamulus guard length Y	199 (175 – 223)	206 (201 – 216)	193 (175 – 223)	199 (199 – 199)
Hamulus point length	45 (34 – 50)	38 (34 – 42)	49 (47 – 50)	50 (49 – 50)
False oral sucker	160 (95 – 206)	95	204 (202 – 206)	137
Pharynx length	136 (117 – 164)	117	143 (122 – 164)	140
Pharynx width	119 (105 – 125)	105	125 (124 – 125)	120
Ovary length	61 (36 – 85)	36		85
Ovary width	26 (18 – 34)	18		34
Genital bulb diameter	20 (18 – 24)	20	19 (18 – 19)	24
No of genital spine	8	8	8	8
Genital spine length	10.7 (10.5 – 10.9)	10.9 & 10.5		
Sucker diameter	200 (160 – 255)	181 (174 – 192)	197 (168 – 243)	236 (215 – 255)

Haptor with three pairs of laterally located suckers, diameter of sucker pair one (posteriormost) 228 (213–243), sucker pair two 186 (168–193) and sucker pair three 178 (168–194), two hamuli, robust, outer length (X in Figure 4A) 215 (190–238), inner length (Y in Figure 4A) 199 (175–223), recurved point (Figure 4A) 45 (34–50). Sixteen marginal hooklets, placement as for other polystomes: pairs one and two posterior-most between suckers; pairs three, four and five at bases of suckers and pairs six to eight anterior in haptor between anterior-most suckers. Marginal hooklets 24 (21–26); hooklets 1 not bigger than hooklets 2–8 (Figure 3).

## Discussion

The fauna and flora of the Madagascar “microcontinent” evolved in isolation for more than 80 million years and today it is known as centre of diversity and endemism of numerous plant and animal groups. The Gondwanan roots of the island's fauna are well documented in the fossil fauna from the Latest Cretaceous (Krause *et al.* 1997) and in the phylogenetic relationships of various extant lineages (e.g., Noonan & Chippindale 2006), but evidence points to a biotic change in deep time (Krause *et al.* 1997). The origins of the current fauna and flora are probably a composite of a limited number of old Gondwanan relictual groups and numerous lineages that originated by possibly old Cenozoic dispersal from Asia and by well-documented more recent dispersal from Africa (e.g., Poux

**TABLE 3.** Mean character differences (uncorrected p-distances; above diagonal) and total character differences (below diagonal) inferred from comparisons of DNA sequences of the COI gene (331 aligned positions).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 <i>Madapolystoma biritika</i> [d]	-	0.00000	0.00000	0.00303	0.02417	0.02115	0.01813	0.01511	0.01511	0.08157	0.09970	0.09668	0.13293	0.22727	0.23263
2 <i>Madapolystoma biritika</i> [c]	0	-	0.00000	0.00303	0.02417	0.02115	0.01813	0.01511	0.01511	0.08157	0.09970	0.09668	0.13293	0.22727	0.23263
3 <i>Madapolystoma biritika</i> [b]	0	0	-	0.00303	0.02417	0.02115	0.01813	0.01511	0.01511	0.08157	0.09970	0.09668	0.13293	0.22727	0.23263
4 <i>Madapolystoma biritika</i> [e]	1	1	1	-	0.02121	0.01818	0.01515	0.01212	0.01212	0.07879	0.09697	0.09394	0.12727	0.22188	0.22727
5 <i>Madapolystoma biritika</i> [a]	8	8	8	7	-	0.01511	0.01813	0.01511	0.01511	0.08459	0.09970	0.09366	0.13897	0.22424	0.23263
6 <i>Madapolystoma biritika</i> [i]	7	7	7	6	5	-	0.00906	0.01208	0.01208	0.07855	0.09970	0.09063	0.13293	0.22424	0.23565
7 <i>Madapolystoma biritika</i> [h]	6	6	6	5	6	3	-	0.00604	0.00604	0.07553	0.09668	0.09063	0.12991	0.22424	0.22659
8 <i>Madapolystoma biritika</i> [g]	5	5	5	4	5	4	2	-	0.00000	0.06949	0.09668	0.08761	0.12991	0.22424	0.22659
9 <i>Madapolystoma biritika</i> [f]	5	5	5	4	5	4	2	0	-	0.06949	0.09668	0.08761	0.12991	0.22424	0.22659
10 <i>Madapolystoma</i> sp. [B.b.]	27	27	27	26	28	26	25	23	23	-	0.07553	0.08157	0.12689	0.20000	0.23565
11 <i>Madapolystoma</i> sp. [B.w.]	33	33	33	32	33	33	32	32	32	25	-	0.08459	0.11178	0.19697	0.22659
12 <i>Madapolystoma</i> sp. [G.l.]	32	32	32	31	31	30	30	29	29	27	28	-	0.12991	0.21212	0.23867
13 <i>Madapolystoma</i> sp. [G.s.]	44	44	44	42	46	44	43	43	43	42	37	43	-	0.21515	0.24169
14 <i>Eupolystoma vanasi</i>	75	75	75	73	74	74	74	74	74	66	65	70	71	-	0.19091
15 <i>Eupolystoma alluaud</i>	77	77	77	75	77	78	75	75	75	78	75	79	80	63	-

Abbreviations used: B.b. = *Blommerisia blommersae*; B.w. = *Blommerisia wittei*; G.l. = *Guibemantis liber*; G.s. = *Gephyromantis sculpturatus*. [a], [b], [c], [d] and [e] are from *Mantella baroni*, [f], [g] and [h] from *Mantella madagascariensis*, and [i] from *Mantella milolympianum*.

**TABLE 4.** Mean character differences (uncorrected p-distances) inferred from comparisons of 28S DNA sequences retrieved in Verneau et al. (2009) (1384 aligned positions).

	1	2	3	4	5	6	7	8	9	10	11	12
1 <i>P. dawiekoki</i>	-											
2 <i>P. occipitalis</i>	0.00506	-										
3 <i>M. sp.4 [B. d.]</i>	0.02168	0.02095	-									
4 <i>M. sp.1 [A. m.]</i>	0.02312	0.02240	0.00289	-								
5 <i>M. brygoonis</i>	0.02023	0.01951	0.00867	0.01012	-							
6 <i>M. sp.2 [B. m.]</i>	0.01806	0.01734	0.00723	0.00867	0.00578	-						
7 <i>M. sp.3 [B. o.]</i>	0.01662	0.01590	0.00578	0.00723	0.00434	0.00145	-					
8 <i>M. cachani</i>	0.02457	0.02312	0.01445	0.01734	0.01301	0.01301	0.01156	-				
9 <i>P. testimagna</i>	0.02384	0.02529	0.02601	0.02601	0.02601	0.02384	0.02240	0.03035	-			
10 <i>P. umthakati</i>	0.02312	0.02457	0.02529	0.02529	0.02529	0.02312	0.02168	0.02962	0.00072	-		
11 <i>P. marmorati</i>	0.02601	0.02746	0.02673	0.02673	0.02529	0.02457	0.02312	0.03107	0.01662	0.01590	-	
12 <i>P. claudecombesi</i>	0.02457	0.02601	0.02673	0.02673	0.02384	0.02312	0.02168	0.02962	0.00361	0.01590	0.01590	-
13 <i>P. gallieni</i>	0.03179	0.03035	0.03324	0.03324	0.03179	0.03107	0.02962	0.03685	0.02601	0.02529	0.02673	0.02529
14 <i>P. integririmum</i>	0.03829	0.03829	0.03902	0.03902	0.03757	0.03540	0.03540	0.04335	0.03468	0.03396	0.03540	0.03540
15 <i>P. nearcticum</i>	0.04552	0.04335	0.04841	0.04986	0.04697	0.04408	0.04335	0.05058	0.04118	0.04046	0.04191	0.04335
16 <i>P. floridana</i>	0.04480	0.04408	0.04913	0.05058	0.04769	0.04480	0.04408	0.05130	0.04118	0.04046	0.04191	0.04335
17 <i>P. naevius</i>	0.04118	0.04046	0.04697	0.04841	0.04552	0.04191	0.04191	0.04913	0.03974	0.03902	0.04191	0.04335
18 <i>W. almae</i>	0.04191	0.03974	0.04408	0.04408	0.04263	0.03757	0.03902	0.04480	0.03829	0.03757	0.03902	0.03757
19 <i>P. cuvieri</i>	0.03685	0.03468	0.03902	0.04046	0.03757	0.03540	0.03396	0.03974	0.03613	0.03540	0.03685	0.03540
20 <i>P. sp. [L. m.]</i>	0.03685	0.03468	0.03902	0.04046	0.03757	0.03540	0.03396	0.03974	0.03613	0.03540	0.03685	0.03540
21 <i>P. lopezromani</i>	0.04913	0.04697	0.05275	0.05275	0.05130	0.04769	0.04769	0.05491	0.04624	0.04552	0.04697	0.04769
22 <i>E. alluaudi</i>	0.07587	0.07514	0.08020	0.08165	0.08165	0.07876	0.07803	0.08454	0.07659	0.07587	0.07731	0.08237
23 <i>E. vanasi</i>	0.08815	0.08671	0.09321	0.09465	0.09465	0.09176	0.09104	0.09754	0.08960	0.08887	0.09032	0.09176
24 <i>Ma. sp.1 [B. b.]</i>	0.08960	0.08743	0.09249	0.09032	0.09249	0.08815	0.08887	0.09321	0.09249	0.09176	0.09321	0.09321
25 <i>Ma. sp.7 [M. b.]</i>	0.08382	0.08454	0.08960	0.08743	0.08960	0.08526	0.08598	0.09032	0.08598	0.08526	0.08671	0.08815
26 <i>Ma. sp.2 [B. d.]</i>	0.08815	0.08960	0.09465	0.09538	0.09610	0.09249	0.09249	0.09465	0.09176	0.09104	0.09393	0.09538
27 <i>Ma. sp.3 [B. w.]</i>	0.08598	0.08454	0.09176	0.09104	0.09176	0.08671	0.08743	0.09249	0.08671	0.08598	0.08743	0.08743
28 <i>Ma. sp.6 [Gu. l.]</i>	0.09032	0.08960	0.09538	0.09538	0.09321	0.09032	0.09104	0.09610	0.09104	0.09032	0.09176	0.09176
29 <i>Ma. sp.6 [Gu. l.]</i>	0.09032	0.08960	0.09538	0.09538	0.09321	0.09032	0.09104	0.09610	0.09104	0.09032	0.09176	0.09176
30 <i>Ma. sp.4 [G. s.]</i>	0.08092	0.07948	0.08526	0.08526	0.08598	0.08165	0.08237	0.08526	0.07948	0.07876	0.08020	0.08237
31 <i>Ma. sp.5 [G. s.]</i>	0.08454	0.08309	0.08815	0.08815	0.08815	0.08382	0.08454	0.08743	0.08237	0.08165	0.08309	0.08309
32 <i>Ma. sp.8 [M. e.]</i>	0.09393	0.09249	0.10116	0.10043	0.10043	0.09610	0.09682	0.10116	0.09321	0.09249	0.09538	0.09465
33 <i>P. indicum</i>	0.05347	0.05202	0.05780	0.05925	0.05925	0.05708	0.05564	0.05997	0.05564	0.05491	0.05780	0.05997
34 <i>P. sp. [R. v.]</i>	0.05419	0.05275	0.05853	0.05997	0.05997	0.05636	0.05636	0.06069	0.05491	0.05419	0.05708	0.05925
35 <i>P. sp. [R. o.]</i>	0.05419	0.05275	0.05853	0.05997	0.05997	0.05780	0.05636	0.06069	0.05491	0.05419	0.05708	0.05925
36 <i>Pa. bulliense</i>	0.06936	0.06792	0.06936	0.07081	0.07081	0.06720	0.06720	0.07442	0.07153	0.07081	0.07298	0.07298
37 <i>D. ranae</i>	0.06792	0.06720	0.07153	0.07298	0.07153	0.06936	0.06792	0.07370	0.07009	0.07009	0.07298	0.07225

	13	14	15	16	17	18	19	20	21	22	23	24
13	<i>P. gallieni</i>	-										
14	<i>P. integerrimum</i>	0.03540	-									
15	<i>P. nearcticum</i>	0.04408	0.03540	-								
16	<i>P. floridana</i>	0.04408	0.03324	0.00650	-							
17	<i>P. naevius</i>	0.04263	0.03613	0.02457	0.02384	-						
18	<i>W. almae</i>	0.03902	0.02529	0.02529	0.02746	0.02673	-					
19	<i>P. cuvieri</i>	0.03468	0.02312	0.02457	0.02529	0.02384	0.01590	-				
20	<i>P. sp. [L. m.]</i>	0.03396	0.02312	0.02529	0.02601	0.02312	0.01590	0.00072	-			
21	<i>P. lopezromani</i>	0.04480	0.03468	0.03324	0.03685	0.03107	0.02818	0.02384	0.02312	-		
22	<i>E. alluaudi</i>	0.07587	0.07370	0.07153	0.07081	0.06792	0.06792	0.06069	0.06069	0.07225	-	
23	<i>E. vanasi</i>	0.09321	0.08887	0.08743	0.08815	0.08237	0.08382	0.07731	0.07731	0.08960	0.05419	-
24	<i>Ma. sp.1 [B. b.]</i>	0.09465	0.08237	0.08815	0.08960	0.08237	0.07876	0.07659	0.07659	0.08382	0.08092	0.09465
25	<i>Ma. sp.7 [M. b.]</i>	0.08887	0.08020	0.08671	0.08743	0.07803	0.07876	0.07731	0.07731	0.08382	0.08092	0.09176
26	<i>Ma. sp.2 [B. d.]</i>	0.09465	0.08598	0.09321	0.09321	0.08309	0.08454	0.08237	0.08237	0.09104	0.08598	0.09899
27	<i>Ma. sp.3 [B. w.]</i>	0.09032	0.07948	0.08454	0.08526	0.07803	0.07587	0.07442	0.07514	0.08165	0.08092	0.09321
28	<i>Ma. sp.6 [Gu. l.]</i>	0.08960	0.08309	0.08526	0.08526	0.08092	0.08092	0.07731	0.07659	0.08671	0.08526	0.09682
29	<i>Ma. sp.6 [Gu. l.]</i>	0.08960	0.08309	0.08526	0.08526	0.08092	0.08092	0.07731	0.07659	0.08671	0.08526	0.09682
30	<i>Ma. sp.4 [G. s.]</i>	0.08092	0.07153	0.07948	0.07876	0.07370	0.07081	0.06936	0.06864	0.07587	0.07225	0.08382
31	<i>Ma. sp.5 [G. s.]</i>	0.08092	0.07081	0.08020	0.07948	0.07587	0.07081	0.06864	0.06792	0.07442	0.07803	0.08960
32	<i>Ma. sp.8 [M. e.]</i>	0.09899	0.09321	0.09249	0.09393	0.08671	0.09104	0.08815	0.08887	0.09465	0.08887	0.09682
33	<i>P. indicum</i>	0.05636	0.05058	0.05347	0.05130	0.05058	0.04913	0.03974	0.04046	0.05564	0.05997	0.07659
34	<i>P. sp. [R. v.]</i>	0.05419	0.04986	0.05202	0.04986	0.04913	0.04697	0.03902	0.03974	0.05202	0.05925	0.07731
35	<i>P. sp. [R. o.]</i>	0.05419	0.05130	0.05275	0.05058	0.04986	0.04841	0.03902	0.03974	0.05347	0.05925	0.07731
36	<i>Pa. bulliense</i>	0.06864	0.05636	0.06720	0.06575	0.06358	0.06358	0.05564	0.05636	0.06142	0.07153	0.08454
37	<i>D. ranae</i>	0.07009	0.06214	0.06936	0.06792	0.06792	0.06358	0.05853	0.05780	0.06575	0.07514	0.08382
25	<i>Ma. sp.7 [M. b.]</i>	-										
26	<i>Ma. sp.2 [B. d.]</i>	0.01445	-									
27	<i>Ma. sp.3 [B. w.]</i>	0.02312	0.03107	-								
28	<i>Ma. sp.6 [Gu. l.]</i>	0.03685	0.04335	0.03540	-							
29	<i>Ma. sp.6 [Gu. l.]</i>	0.03685	0.04335	0.03540	0.00000	-						
30	<i>Ma. sp.4 [G. s.]</i>	0.03757	0.04335	0.03396	0.04263	0.04263	-					
31	<i>Ma. sp.5 [G. s.]</i>	0.04046	0.04624	0.03829	0.04263	0.03902	0.01301	-				
32	<i>Ma. sp.8 [M. e.]</i>	0.05130	0.05997	0.04841	0.05853	0.05853	0.05347	0.05564	-			
33	<i>P. indicum</i>	0.07876	0.08454	0.07876	0.08020	0.08020	0.07370	0.07803	0.08743	-		
34	<i>P. sp. [R. v.]</i>	0.07659	0.08309	0.07659	0.07731	0.07731	0.07153	0.07514	0.08526	0.00506	-	
35	<i>P. sp. [R. o.]</i>	0.07803	0.08382	0.07803	0.07876	0.07876	0.07298	0.07659	0.08671	0.00506	0.00289	-
36	<i>Pa. bulliense</i>	0.08382	0.09393	0.08092	0.08815	0.08815	0.07876	0.07876	0.09249	0.04191	0.04118	0.04263
37	<i>D. ranae</i>	0.08526	0.09176	0.08454	0.09176	0.09176	0.08020	0.08237	0.09538	0.04624	0.04697	0.02529

Abbreviations used: P = *Polystoma*; M = *Metapolytoma*; W = *Wetapolytoma*; E = *Eupolytoma*; Pa = *Parapolytoma*; D = *Diplorhysis*. Abbreviations in brackets refer to host species, from top to bottom: [B. d.] = *Boophis doulioti*; [A. m.] = *Aglyptodactylus madagascariensis*; [B. m.] = *Boophis madagascariensis*; [B. o.] = *Boophis occidentalis*; [L. m.] = *Leptodactylus mystaceus*; [B. b.] = *Blommerisia blommersae*; [M. b.] = *Mantiella baroni*; [B. d.] = *Blommerisia domerguei*; [B. w.] = *Blommerisia wittei*; [Gu. l.] = *Guibematis libber*; [G. s.] = *Gephyromantis sculpturatus* [M. e.] = *Mantiella expectata*; [R. v.] = *Rhacophorus viridis*; [R. o.] = *Rhacophorus omeimontis*.

*et al.* 2005; Yoder & Nowak 2006; Van der Meijden *et al.* 2007). The frogs of Madagascar display an exceptional high level of endemism with all but one of the known species being endemic to the island. Considering species descriptions currently in press, there are more than 265 scientifically described anuran species on the island plus as great a number of undescribed species (Vieites *et al.* 2009). A particular aspect of Madagascar's biota, affecting amphibians in particular, is a sometimes extreme microendemism (Vences *et al.* 2009): Many frog species appear not only endemic to the island but to tiny areas within Madagascar. In fact, a very large fraction of Malagasy frog species are known from a single site only. While this may partly be due to insufficient survey work, in numerous cases it probably reflects a true situation of small distribution ranges. The family Mantellidae (with 12 genera, among which *Mantella*) is the most species-rich radiation among Malagasy amphibians (Glaw & Vences 2006). As it seems, parasites belonging to *Madapolystoma* n. g. are only found in one large subgroup of mantellids, the subfamily Mantellinae (see Verneau *et al.* 2009). Molecular evidence points to the presence of these parasites in the genera *Blommersia*, *Gephyromantis*, *Guibemantis*, and *Mantella* (see Verneau *et al.* 2009 and Figure 5 herein), predicting that *Madapolystoma* n. g. will turn out to contain a vast and species rich radiation as well.

The extended uterus and intra uterine development of larvae as seen in *Madapolystoma* n. g. resembles the situation in *Eupolystoma* which is known only from Africa and India. However the mid body placement of the ovary and testis as well as the presence of hamuli clearly separates it from *Eupolystoma*. The absence of a ciliated free swimming larval stage and *intra uterine* development as seen in *Madapolystoma* n. g. where developing larvae are not encapsulated in eggs and progressively develop to an advance state of development with prominent hamuli and up to two pairs of suckers is unique among the known polystomes. For most polystomes the posteriormost marginal hooklet 1 is bigger than the remainder of the marginal hooklets. However, as is the case for *Eupolystoma*, the marginal hooklets are of equal length in *Madapolystoma* n. g.. According to both morphological and COI molecular data, as well as from levels of 28S molecular divergences that range from 7.22% to 9.85% between the sister taxa *Eupolystoma* and *Madapolystoma* n. g. (Table 4), we can consider *Madapolystoma* n. g. as a valid new genus.

A free swimming oncomiracidium has been reported to be a diagnostic feature of the family Polystomatidae. The absence of an oncomiracidium in *Madapolystoma* thus requires an amendment of the family characteristics. Vitellaria were not observed in any of the *Madapolystoma* specimens. The ovary is probably a germovitelarium as seen in some fish monogeneans such as *Gyrodactylus*. Small gonads and wide range of development among the *intra uterine* larvae suggests reproduction over a prolonged period of time indicating that the host is not an explosive breeder.

The mode of transfer of parasites to novel uninfected hosts remains unclear and speculative. However, the absence of a free-swimming larva and level of intra uterine development indicates a direct or indirect transfer to other post-metamorphic individuals. This would require physical contact between animals or visits to specific sites such as egg clutches. In general, mantellids of the subfamily Mantellinae which appear to be the sole *Madapolystoma* hosts are characterized by absence of strong mating amplexus and deposition of eggs at different sites but never directly into the water. *Mantella* species have been documented to display a stereotyped, highly tactile courtship and in one species, *M. laevigata*, a complex parental care (Glaw *et al.* 2000; Heying 2001). In general mantellines have a mating behaviour that involves numerous contacts between male and female, sometimes lasting several minutes, during which the male uses his femoral glands at the underside of his thighs to touch the female's dorsum. It is appealing to hypothesize that this particular mating behaviour without strong mating amplexus (see Blommers-Schlösser 1975) and the terrestrial egg deposition of mantellines are related to the lack of an aquatic larval stage also of their parasitic polystomes.

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