



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*

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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 haptor 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 endemism 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% endemism. 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 endemism 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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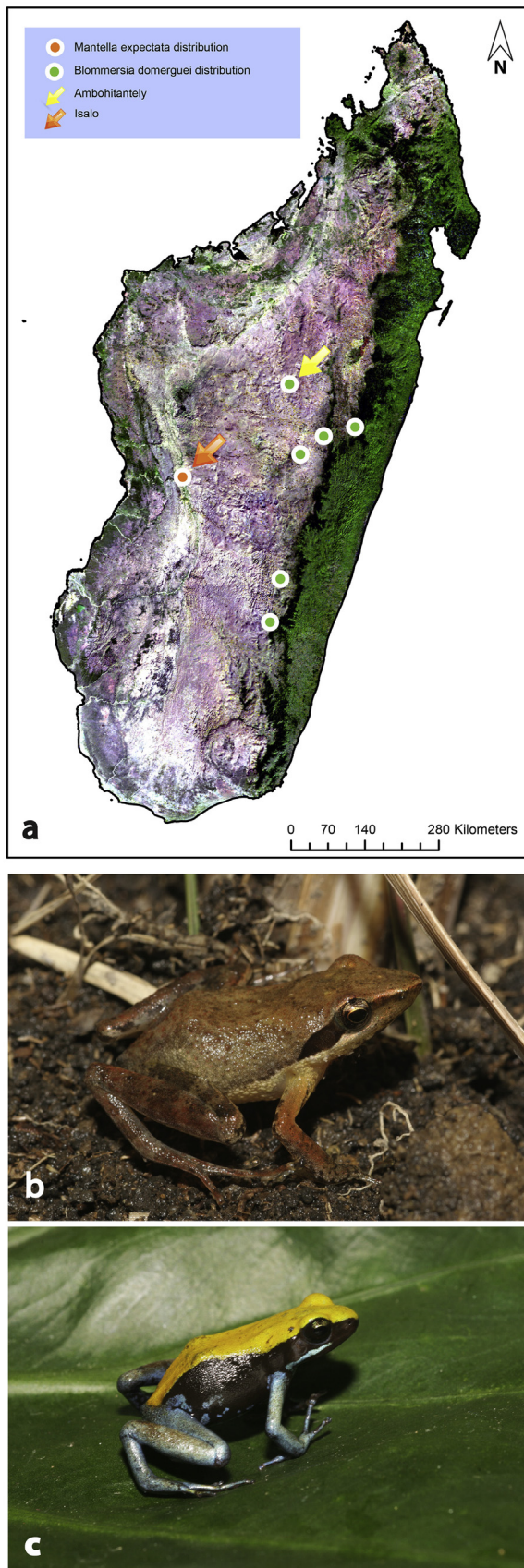


Fig. 1. a) Map of Madagascar with the distribution areas and sampling localities of the two investigated frogs; b) *Blommersia domerguei*; c) *Mantella expectata*. (Map-Library, 2007).

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

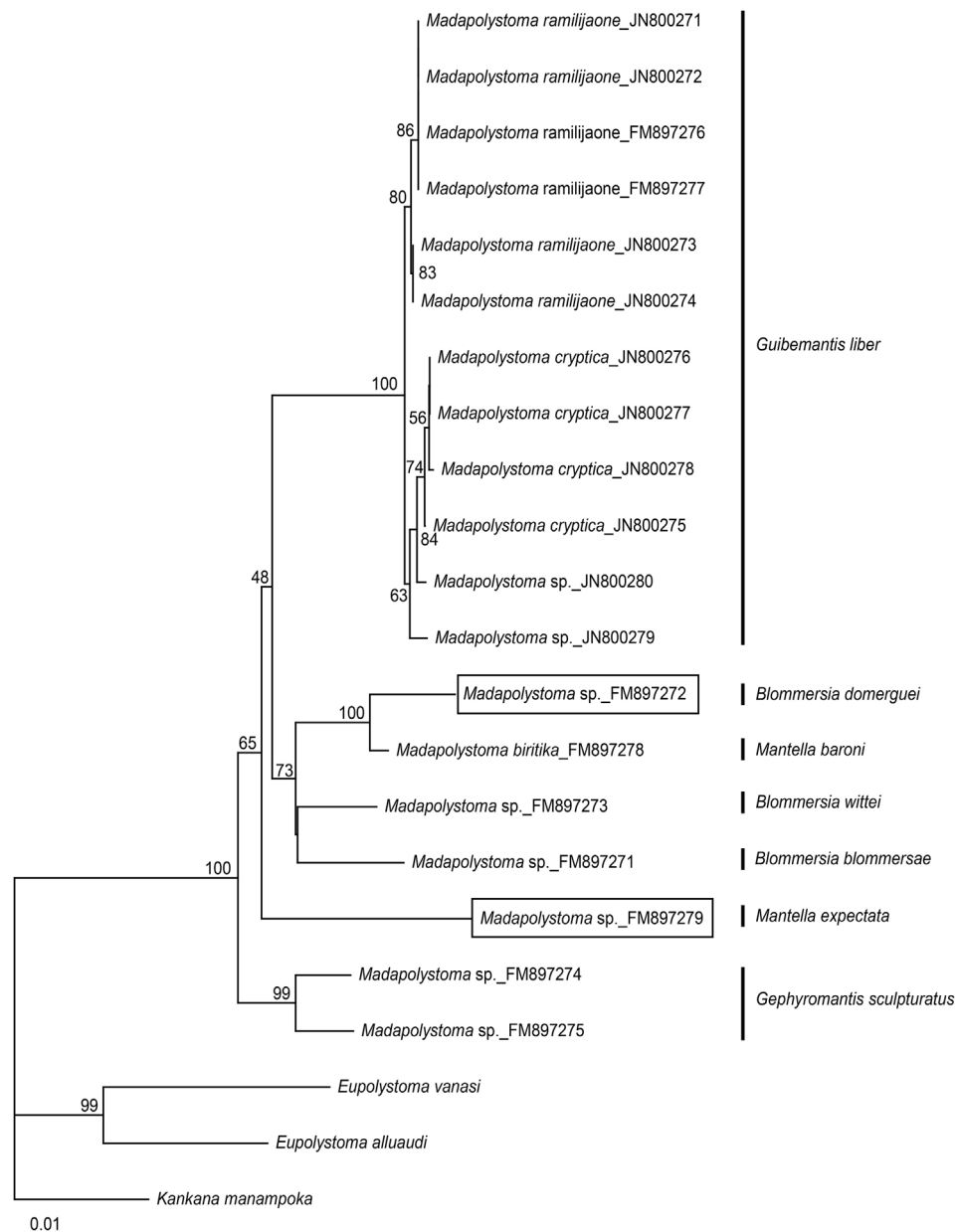


Fig. 2. Minimum Evolution tree for *Madapolystoma* spp. Numbers on nodes indicate bootstrap support values. *Madapolystoma* sp. from *B. domerguei* refers to *M. magnahami* n. sp. and *Madapolystoma* sp. from *M. expectata* refers to *M. isaloensis* n. sp.

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

Ambositantely 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
<i>Madapolystoma ramilijaonae</i> _JN800271		0.0000	0.0016	0.0016	0.0000	0.0000	0.0055	0.0062	0.0062	0.0070	0.0062
<i>Madapolystoma ramilijaonae</i> _JN800272	0		0.0016	0.0016	0.0000	0.0000	0.0055	0.0062	0.0062	0.0070	0.0062
<i>Madapolystoma ramilijaonae</i> _JN800273	2	2		0.0000	0.0016	0.0016	0.0055	0.0062	0.0062	0.0070	0.0047
<i>Madapolystoma ramilijaonae</i> _JN800274	2	2	0		0.0016	0.0016	0.0055	0.0062	0.0062	0.0070	0.0047
<i>Madapolystoma ramilijaonae</i> _FM897276	0	0	2	2		0.0000	0.0055	0.0062	0.0062	0.0070	0.0062
<i>Madapolystoma ramilijaonae</i> _FM897277	0	0	2	2	0		0.0055	0.0062	0.0062	0.0070	0.0062
<i>Madapolystoma cryptica</i> _JN800275	7	7	7	7	7	7		0.0008	0.0008	0.0016	0.0055
<i>Madapolystoma cryptica</i> _JN800276	8	8	8	8	8	8	1		0.0000	0.0008	0.0062
<i>Madapolystoma cryptica</i> _JN800277	8	8	8	8	8	8	1	0		0.0008	0.0062
<i>Madapolystoma cryptica</i> _JN800278	9	9	9	9	9	9	2	1	1		0.0070
<i>Madapolystoma sp.</i> _JN800279	8	8	6	6	8	8	7	8	8	9	
<i>Madapolystoma sp.</i> _JN800280	7	7	7	7	7	7	4	5	5	4	7
<i>Madapolystoma britika</i> _FM897278	55	55	53	53	55	55	55	56	56	57	56
<i>Madapolystoma sp.</i> _FM897273	52	52	52	52	52	52	52	53	53	54	55
<i>Madapolystoma sp.</i> _FM897274	61	61	61	61	61	61	61	62	62	63	62
<i>Madapolystoma sp.</i> _FM897275	58	58	60	60	58	58	60	61	61	62	61
<i>Madapolystoma sp.</i> _FM897271	59	59	57	57	59	59	59	60	60	61	60
<i>Madapolystoma sp.</i> _FM897272	68	68	67	67	68	68	69	70	70	71	71
<i>Madapolystoma sp.</i> _FM897279	76	76	74	74	76	76	77	78	78	78	78
<i>Kankana niananipoka</i> _HM854293	110	110	108	108	110	110	109	110	110	109	111
<i>Eupolystoma vanasi</i> _AM157200	145	145	143	143	145	145	144	145	145	144	146
<i>Eupolystoma alluaudi</i> _AM157199	134	134	132	132	134	134	134	135	135	136	134
<i>Madapolystoma ramilijaonae</i> _JN800271		0.0055	0.0429	0.0406	0.0476	0.0461	0.0531	0.0593	0.0859	0.1132	0.1046
<i>Madapolystoma ramilijaonae</i> _JN800272	0.0055		0.0429	0.0406	0.0476	0.0461	0.0531	0.0593	0.0859	0.1132	0.1046
<i>Madapolystoma ramilijaonae</i> _JN800273	0.0055	0.0414		0.0406	0.0476	0.0445	0.0523	0.0578	0.0843	0.1116	0.1030
<i>Madapolystoma ramilijaonae</i> _JN800274	0.0055	0.0414	0.0406		0.0476	0.0445	0.0523	0.0578	0.0843	0.1116	0.1030
<i>Madapolystoma ramilijaonae</i> _FM897276	0.0055	0.0429	0.0406	0.0406		0.0461	0.0531	0.0593	0.0859	0.1132	0.1046
<i>Madapolystoma ramilijaonae</i> _FM897277	0.0055	0.0429	0.0406	0.0406	0.0476		0.0531	0.0593	0.0859	0.1132	0.1046
<i>Madapolystoma cryptica</i> _JN800275	0.0031	0.0429	0.0406	0.0406	0.0476	0.0461		0.0601	0.0851	0.1124	0.1046
<i>Madapolystoma cryptica</i> _JN800276	0.0039	0.0437	0.0414	0.0484	0.0476	0.0468	0.0546		0.0859	0.1132	0.1054
<i>Madapolystoma cryptica</i> _JN800277	0.0039	0.0437	0.0414	0.0484	0.0476	0.0468	0.0546	0.0609		0.1132	0.1054

Table 2 (continued)

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

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 μ m; C, 100 μ m; D, 25 μ m. 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 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 3Body measurements in micrometres for all known *Madapolystoma* spp.

	<i>Madapolystoma magnahami</i> n. sp.	<i>Madapolystoma isaloensis</i> n. sp.	<i>Madapolystoma cryptica</i>	<i>Madapolystoma biritika</i>	<i>Madapolystoma ramilijaonae</i>
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 <i>in utero</i>	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 pre-haptoral 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 saciform, 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).

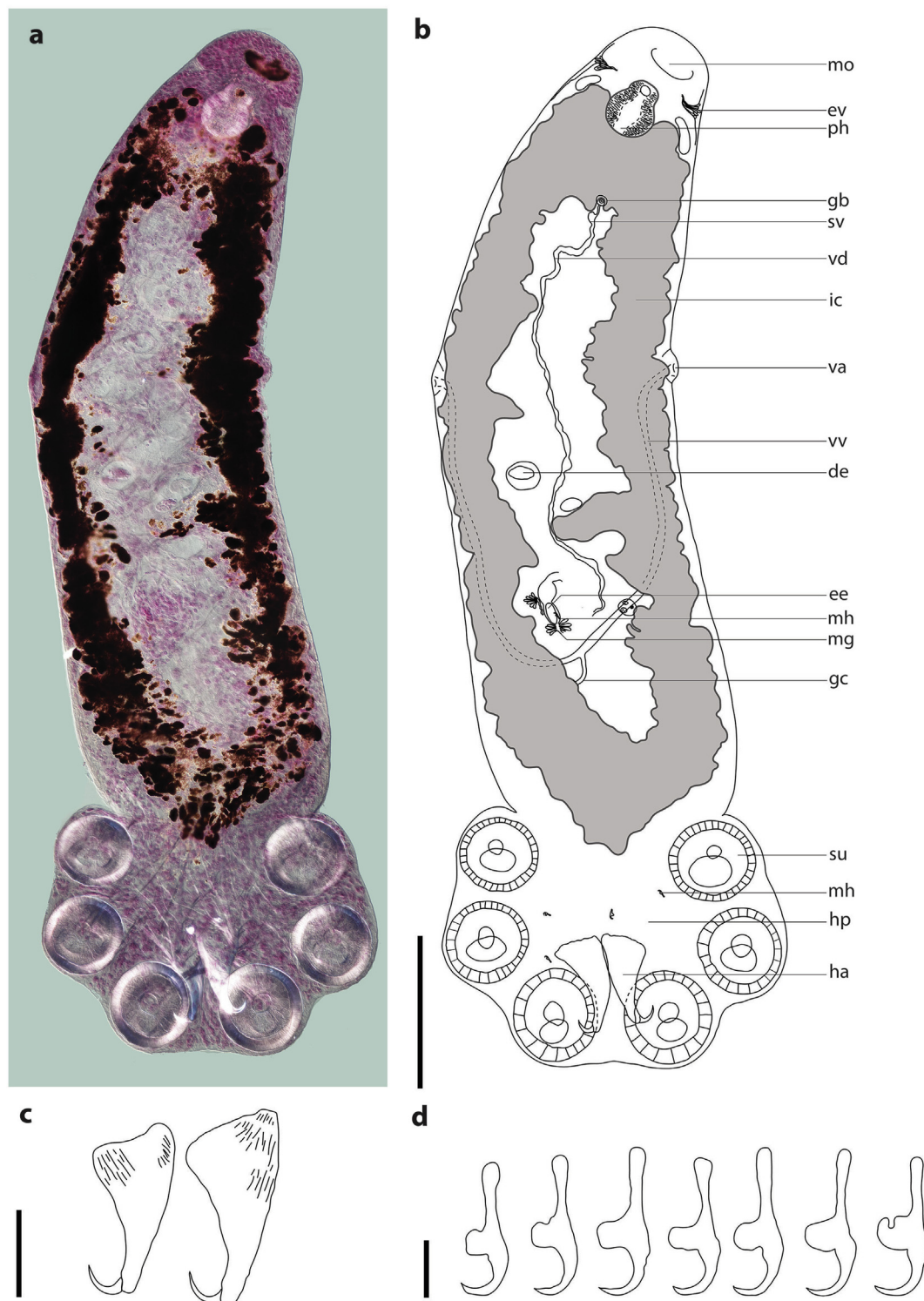


Fig. 4. a–b) Ventral view of *M. isaloensis* n. sp. holotype. (c) Hamuli from mature specimens and (d) Marginal hooklets 1–8. Scale bars: B, 200 μ m; C, 100 μ m; D, 20 μ m. Abbreviations: de, developing embryo; ee, early embryo; ev, excretory vessel; gb, genital bulb; gc, genito-intestinal canal; ha, 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

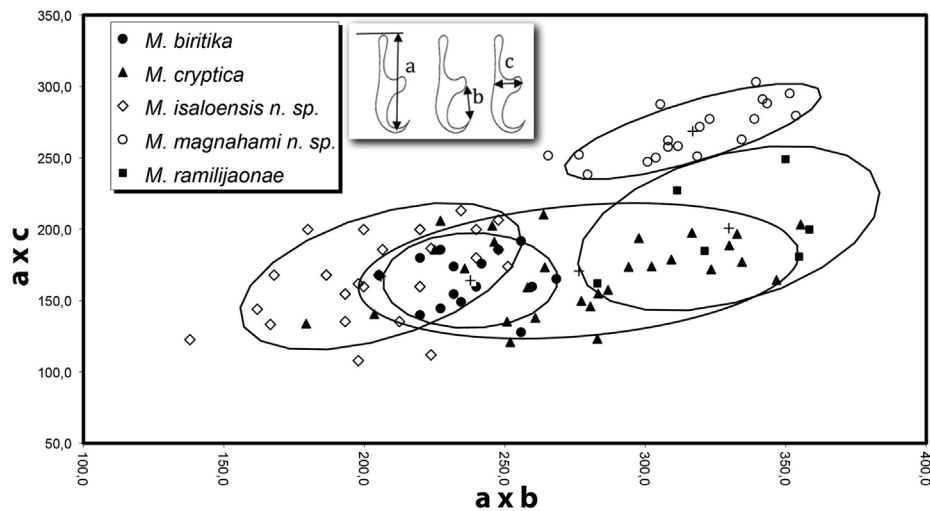


Fig. 5. Scatter diagram of $a \times c$ plotted against $b \times c$ for all known *Madapolystoma* spp., *M. magnahami* n. sp. and *M. isaloensis* n. sp. The ellipses represent 95% of the confidence interval about the mean.

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 haptor 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 observed. 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 *Pseudodiplorchis 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 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.

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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