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 haptor 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 of the island over an extended period of time (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) Ur- polystomoides Tinsley and Tinsley, 2016 with a single species Ur- polystomoides chabaudi (Euzet and Combes, 1965) from the chelonian host Pelomedusa subrufa (Lacépède, 1788); (ii) Metapolystoma Combes, 1976, with a single species Metapolystoma brygonis (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 ramiiljonae Berthier et al., 2014, from the same host Guibemantis liber (Peracca, 1893); (iv) Kankana Raharivololoniaia et al., 2011, with a single species Kankana manampoka Raharivololoniaia et al., 2011 from the

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) Ur- polystomoides Tinsley and Tinsley, 2016 with a single species Ur- polystomoides chabaudi (Euzet and Combes, 1965) from the chelonian host Pelomedusa subrufa (Lacépède, 1788); (ii) Metapolystoma Combes, 1976, with a single species Metapolystoma brygonis (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 ramiiljonae Berthier et al., 2014, from the same host Guibemantis liber (Peracca, 1893); (iv) Kankana Raharivololoniaia et al., 2011, with a single species Kankana manampoka Raharivololoniaia et al., 2011 from the
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. 

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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).
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

| Polystome species | Host species | Location | Genbank Accession Number |
|-------------------|--------------|----------|--------------------------|
| *Madapolystoma*   |              |          |                          |
| *ramilijaonae*    | *Guibemantis liber* | Madagascar: | JN800271 |
| *ramilijaonae*    | *Guibemantis liber* | Madagascar: | JN800272 |
| *ramilijaonae*    | *Guibemantis liber* | An’Ala | JN800273 |
| *ramilijaonae*    | *Guibemantis liber* | Ranomafana | JN800274 |
| *ramilijaonae*    | *Guibemantis liber* | Madagascar: | FM897276 |
| *ramilijaonae*    | *Guibemantis liber* | An’Ala | FM897277 |
| *cryptica*        | *Guibemantis liber* | Madagascar: | JN800275 |
| *cryptica*        | *Guibemantis liber* | Tsiaratanana | JN800276 |
| *cryptica*        | *Guibemantis liber* | Madagascar: | JN800277 |
| *cryptica*        | *Guibemantis liber* | Madagascar: | JN800278 |
| *cryptica*        | *Guibemantis liber* | Madagascar: | JN800279 |
| *cryptica*        | *Guibemantis liber* | Madagascar: | JN800280 |
| *sp.*              | *Guibemantis liber* | Madagascar: | FM897278 |
| *biritika*        | *Mantella baroni* | Madagascar: | FM897273 |
| *sp.*              | *Blommeria wittei* | Madagascar: | FM897274 |
| *sp.*              | *Gephyromantis sculpturus* | Madagascar: | FM897275 |
| *sp.*              | *Gephyromantis sculpturus* | An’Ala | FM897271 |
| *sp.*              | *Blommeria* | Madagascar: | FM897272 |
| *sp.*              | *Blommeria domerguei* | Madagascar: | FM897279 |
| *sp.*              | *Mantella expectata* | Madagascar: | HM854293 |
| *Kankana manampoka* | *Cophyla pollicaris* | Madagascar: | AM157200 |
| *Eupolystoma vanasi* | *Schismaderma carens* | Madagascar: | AM157199 |
| *Eupolystoma alluaudi* | *Bufo* | Togo | AM157199 |
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

*Blommeria domerguei* (Mantellidae).

#### 3.2.3. Type locality

Ambositantarantely 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  | 0.0000   | 0.016    | 0.016    | 0.0000   | 0.0000   | 0.0055   | 0.0062   | 0.0062   | 0.0070   | 0.0062   |
| Madapolystoma ramilijaonae  | 0        | 0.016    | 0.016    | 0.0000   | 0.0000   | 0.0055   | 0.0062   | 0.0062   | 0.0070   | 0.0062   |
| Madapolystoma ramilijaonae  | 2        | 2        | 0        | 0.016    | 0.016    | 0.0055   | 0.0062   | 0.0062   | 0.0070   | 0.0062   |
| Madapolystoma ramilijaonae  | 0        | 0        | 2        | 2        | 0.0000   | 0.0055   | 0.0062   | 0.0062   | 0.0070   | 0.0062   |
| Madapolystoma cryptica      | 7        | 7        | 7        | 7        | 7        | 0.0008   | 0.0008   | 0.0016   | 0.0055   |
| Madapolystoma sp.           | 8        | 8        | 8        | 8        | 8        | 1        | 0.0000   | 0.0008   | 0.0062   |
| Madapolystoma sp.           | 9        | 9        | 9        | 9        | 9        | 2        | 1        | 1        | 0.0070   |
| Madapolystoma sp.           | 8        | 8        | 6        | 6        | 8        | 8        | 7        | 8        | 8        | 9        |
| Madapolystoma sp.           | 7        | 7        | 7        | 7        | 7        | 4        | 5        | 5        | 4        | 7        |
| Madapolystoma               | 55       | 55       | 53       | 53       | 55       | 55       | 55       | 56       | 56       | 57       | 56       |
| Madapolystoma sp.           | 52       | 52       | 52       | 52       | 52       | 52       | 52       | 53       | 53       | 54       | 55       |
| Madapolystoma sp.           | 61       | 61       | 61       | 61       | 61       | 61       | 61       | 62       | 62       | 63       | 62       |
| Madapolystoma sp.           | 58       | 58       | 60       | 60       | 58       | 58       | 58       | 60       | 61       | 62       | 61       |
| Madapolystoma sp.           | 59       | 59       | 57       | 57       | 59       | 59       | 59       | 60       | 60       | 61       | 60       |
| Madapolystoma sp.           | 68       | 68       | 67       | 67       | 68       | 68       | 68       | 69       | 70       | 70       | 71       |
| Madapolystoma sp.           | 76       | 76       | 74       | 74       | 76       | 76       | 77       | 78       | 78       | 78       | 78       |
| Kankana manampoka            | 110      | 110      | 108      | 108      | 110      | 110      | 110      | 110      | 110      | 109      | 111      |
| Eupolystoma vanasi AM157200  | 145      | 145      | 143      | 143      | 145      | 145      | 144      | 145      | 144      | 145      | 146      |
| Eupolystoma albacali AM157199| 134      | 134      | 132      | 132      | 134      | 134      | 134      | 135      | 135      | 136      | 134      |

|              | JN800271 | FM897278 | FM897273 | FM897274 | FM897275 | FM897271 | FM897272 | FM897279 | HM854293 | AM157200 | AM157199 |
|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Madapolystoma ramilijaonae  | 0.0055   | 0.0429   | 0.0406   | 0.0476   | 0.0453   | 0.0461   | 0.0531   | 0.0593   | 0.0859   | 0.1132   | 0.1046   |
| Madapolystoma ramilijaonae  | 0.0055   | 0.0429   | 0.0406   | 0.0476   | 0.0453   | 0.0461   | 0.0531   | 0.0593   | 0.0859   | 0.1132   | 0.1046   |
| Madapolystoma ramilijaonae  | 0.0055   | 0.0414   | 0.0406   | 0.0476   | 0.0468   | 0.0445   | 0.0523   | 0.0578   | 0.0843   | 0.1116   | 0.1030   |
| Madapolystoma ramilijaonae  | 0.0055   | 0.0414   | 0.0406   | 0.0476   | 0.0468   | 0.0445   | 0.0523   | 0.0578   | 0.0843   | 0.1116   | 0.1030   |
| Madapolystoma ramilijaonae  | 0.0055   | 0.0429   | 0.0406   | 0.0476   | 0.0453   | 0.0461   | 0.0531   | 0.0593   | 0.0859   | 0.1132   | 0.1046   |
| Madapolystoma ramilijaonae  | 0.0055   | 0.0429   | 0.0406   | 0.0476   | 0.0453   | 0.0461   | 0.0531   | 0.0593   | 0.0859   | 0.1132   | 0.1046   |
| Madapolystoma cryptica      | 0.0031   | 0.0429   | 0.0406   | 0.0476   | 0.0468   | 0.0461   | 0.0539   | 0.0601   | 0.0851   | 0.1124   | 0.1046   |
| Madapolystoma cryptica      | 0.0039   | 0.0437   | 0.0414   | 0.0484   | 0.0476   | 0.0468   | 0.0546   | 0.0609   | 0.0859   | 0.1132   | 0.1054   |
| Madapolystoma cryptica      | 0.0039   | 0.0437   | 0.0414   | 0.0484   | 0.0476   | 0.0468   | 0.0546   | 0.0609   | 0.0859   | 0.1132   | 0.1054   |
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 vaginæ, 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 conical seminal vesicle. Uterus sac-like holds four 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). Narrower 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.
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 poly-

3.3.5. Type material

Morphological description are based on one mature and 19 im-

![Image of a parasite](image_url)

(Fig. 3d).

(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 poly-

3.3.5. Type material

Morphological description are based on one mature and 19 im-

![Image of a parasite](image_url)

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

3.7. Zoobank
The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:4C10D3CF-44C2-4DB4-90B9-648CF1FD0CE1. The LSID for the new name Madapolystoma isaloensis n. sp. is urn:lsid:zoobank.org:act:744DAD34-F102-4946-9135-813DD528118A.

3.8. Etymology
The species epithet refers to the type locality, Isalo.

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 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 parasites found among Malagasy frogs clearly indicated they could be separated from each other and from the three other species 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. of M. expectata, molecular results thus supported the morphological description of two new species, i.e. M. magnahami n. sp. and M. isaloensis n. sp. 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 of size of genital spines (Table 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 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...
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 unflassen 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, Euprostylestoma, Kankana, Neodiplorchis, Parapolystoma, Pseudodiplorchis and Sundapolystoma all have extended uteri (Du Preez et al., 2003; Raharivololonaaina 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 inoperculum strategy. All the known hosts for Madapolystoma are 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 Gyrodactylus (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, Guihemantis and Mantella deposit their egg clutches terrestrially or semi-terrestrially. While species of Blommersia and Guihemantis 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), it is in Pseudodiplostomium 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. isoaloeensis 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 polostomatids 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 polostomatids, 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. isoaloeensis n. sp.

Conflicts of interest

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