



## First monogenean flatworm from a microhylid frog host: *Kankana*, a new polystome genus from Madagascar

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### ABSTRACT

*Kankana manampoka* n. gen., n. sp. (Monogenea, Polystomatidae), is described from the urinary bladder of the narrow-mouthed frog *Platypelis pollicaris*. This is the first record of a polystome from the Microhylidae and the third polystome genus from Madagascar, next to *Metapolystoma* and *Madapolystoma*. The extensive uterus and presence of hamuli resemble *Metapolystoma* but the vitellarium confined to the lateral fields in *Kankana* is different. *Madapolystoma* also has an extensive uterus but contain only up to 32 advanced developed larvae. Based on the extensive uterus filling the body proper and the vitellarium confined to two lateral fields posterior in the body this new polystome resembles *Eupolystoma* known from Africa and India. However, unlike *Eupolystoma*, the gonads are in the middle of the body, vaginae are lacking and a pair of hamuli is present. A molecular phylogenetic analysis of concatenated 18S and 28S ribosomal RNA gene sequences supplemented by genetic distances inferred from 28S and COI sequences showed that this new genus is more related to *Madapolystoma*, a genus only reported from Madagascar, than to *Eupolystoma* known from Africa and India and *Metapolystoma* known from Africa and Madagascar.

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### 1. Introduction

Madagascar is known as the island of biodiversity and endemism and is ranked as one of the most important hotspots for biodiversity conservation [1]. Currently about 280 anuran species are known from Madagascar and all but one of the known species are endemic to the island [2]. This anuran species count is however a gross underestimation and the true figure could possibly be as high as 465 species [3]. This high diversity and exceptional degree of endemism is not restricted to amphibians but applies to various forms of life on the island. The rich anuran fauna is believed to be the result of several colonization events whereby some lineages dispersed directly from Africa [4,5] while other lineages are more ancient [6] and may have reached Madagascar via India [7].

As a result of their amphibious life style that allows for parasite infection in aquatic as well as terrestrial environments, frogs and toads are hosts of a great variety of parasites. For example, the Common Platanna

(*Xenopus laevis* Daudin) serves as host for 25 species representing all major parasite groups except the acanthocephalans [8]. One group of parasites that radiated with their anuran hosts is the polystomatid flatworms that comprise 23 genera. Anuran polystomes from the Ethiopian realm include five genera, namely *Polystoma* Zeder, 1800, *Protopolystoma* Bychowsky, 1957, *Eupolystoma* Kaw, 1950, *Metapolystoma* Combes, 1976 and *Madapolystoma* Du Preez, Raharivololoniaina, Verneau and Vences, 2010. *Polystoma* is a cosmopolitan and diverse genus with 33 described and several undescribed species currently known from Africa and 21 from other continents. *Protopolystoma* is known only from *Xenopus* and includes six species infecting different *Xenopus* species. *Eupolystoma* is known from three host species on the African continent and two from India. *Metapolystoma* is known from two host species in Africa and one species, *Metapolystoma brygoonis* (Euzet and Combes, 1964), from *Ptychadena mascareniensis* in Madagascar. *Madapolystoma* is restricted to Madagascar where it has been described from poison frogs in *Mantella* [9] but contains several undescribed species known from frogs in *Blommersia*, *Guibemantis* and *Gephyromantis* [10].

During a large-scale survey of polystome parasites of Malagasy frogs we identified the first polystome from a representative of the Microhylidae, i.e., *Platypelis pollicaris* (Boulenger). Here we report this discovery and provide a formal description of a new species and genus.

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**Table 1**  
Parasite species investigated, host species, geographical origin and GenBank accession numbers. Almost all sequences were reported elsewhere (Bentz et al. 2006; Du Preez et al. 2007; Verneau et al. 2009; Badets et al., 2011). \* Indicates new sequences.

Parasite species	Hosts species	Origin	Accession numbers		
			18S	28S	COI
<i>Kankana manampoka</i> n.gen. and n.sp.	<i>Platyelalis pollicaris</i>	Madagascar: Ranomafana	HM854292*	HM854293*	JF699307*
<i>Madapolystoma</i> sp.	<i>Mantella expectata</i>	Madagascar: Pet trade	FM897296	FM897279	–
<i>Madapolystoma</i> sp.	<i>Blommersia wittei</i>	Madagascar: Isalo	FM897290	FM897273	JF699308*
<i>Madapolystoma</i> sp.	<i>Blommersia blommersae</i>	Madagascar: An'Ala	FM897288	FM897271	JF699309*
<i>Madapolystoma biritika</i>	<i>Mantella baroni</i>	Madagascar	FM897295	FM897278	JF699300*
<i>Madapolystoma</i> sp.	<i>Guibemantis liber</i>	Madagascar: An'Ala	FM897293	FM897276	JF699301*
<i>Madapolystoma</i> sp.	<i>Blommersia domerguei</i>	Madagascar: Ambohitantely	FM897289	FM897272	–
<i>Madapolystoma</i> sp.	<i>Gephyromantis sculpturatus</i>	Madagascar: An'Ala	FM897292	FM897275	JF699302*
<i>Eupolystoma alluaudi</i>	<i>Bufo</i> sp.	Togo	AM051066	AM157199	FR667558
<i>Eupolystoma vanasi</i>	<i>Schismaderma carens</i>	South Africa	AM157185	AM157200	FR667559
<i>Polystoma dawiekoki</i>	<i>Ptychadena anchietae</i>	Tanzania	AM051069	AM157204	AM913856
<i>Polystoma integerrimum</i>	<i>Rana temporaria</i>	France	AM051071	AM157206	JF699306*
<i>Metapolystoma cachani</i>	<i>Ptychadena longirostris</i>	Nigeria	FM897280	FM897262	–
<i>Polystoma gallieni</i>	<i>Hyla meridionalis</i>	France	AM051070	AM157205	JF699305*
<i>Metapolystoma bryoonis</i>	<i>Ptychadena mascareniensis</i>	Madagascar: Ambatolampy	FM897287	FM897270	FM897300
<i>Polystoma claudecombesi</i>	<i>Amietia angolensis</i>	South Africa	FM897281	FM897263	–
<i>Polystoma lopezromani</i>	<i>Phrynohyas venulosa</i>	Paraguay	AM051072	AM157207	AM913863
<i>Polystoma testimagna</i>	<i>Strongylopus fasciatus</i>	South Africa	AM157194	AM157217	AM913860
<i>Polystoma cuvieri</i>	<i>Physalaemus cuvieri</i>	Paraguay	AM051068	AM157203	AM913862
<i>Polystoma nearcticum</i>	<i>Hyla versicolor</i>	USA	AM051074	AM157210	AM913863
<i>Polystoma floridana</i>	<i>Hyla cinerea</i>	USA	AM157188	AM57211	AM913870
<i>Polystoma indicum</i>	<i>Rhacophorus maximus</i>	India	AM157193	AM157216	JF699303*
<i>Diplorchis ranae</i>	<i>Rana rugosa</i>	Japan	AM157184	AM157198	JF699304*

## 2. Materials and methods

During February 2009, adult *P. pollicaris* were collected by hand during daytime in degraded primary rainforest dominated by bamboo stands at Sahamalaotra in Ranomafana National Park, Southern Central East of Madagascar. At night, specimens were collected from another bamboo-dominated stretch of less disturbed forest 1–2 km from the first site. Both localities are located in Ranomafana National Park in the Southern Central East of Madagascar. Prior to dissection frogs were anesthetized and killed with methane 3-aminomethanesulfonate (MS 222). The urinary bladder of each specimen was carefully examined using a stereo microscope, to check for the presence of polystomes. If any parasites were present, the urinary bladder was removed and placed in a Petri dish containing dechlorinated tap water and the parasites removed. Two mature parasites were fixed in 10% formol-saline under cover slip pressure, one mature parasite in 95% EtOH under cover slip pressure, one immature specimen in ammonium picrate and one small immature parasite in 95% EtOH. Flat fixed specimens were removed after 1 h using a small camel hair brush and transferred to vials containing fixative. The cover slip of the semi permanent preparation in ammonium-picrate was secured and sealed with clear nail varnish.

Frogs were preserved in 70% EtOH. They were labeled with ZCMV field numbers (Zoological Collection of Miguel Vences) and deposited in the herpetological collections of the Zoological Staatssammlung München, Germany (ZSM) and Université d'Antananarivo, Département de Biologie Animale, Madagascar (UADBA).

Parasites were washed free of fixative and stained overnight in a weak solution of acetocarmine, dehydrated, cleared in xylene and mounted in Canada balsam. Mounted specimens were examined using a Nikon Eclipse E800 compound microscope. Body and organs were measured using NIS D elements software program. All parasite measurements are in micrometers.

One parasite from *P. pollicaris* was dried and incubated at 55 °C in 150 µL of Chelex 10% and 20 µL of Proteinase K at 10 mg.mL<sup>-1</sup> for 60 min. The reaction was stopped at 100 °C for 15 min and DNA stored at –20 °C until use. The complete 18S rRNA gene was amplified in two overlapping fragments of about 1 kb each, with the primers F18: 5'-ACCTGGTTGATCCTGCCAGTAG-3' and 18RG: 5'-CTCTCTTAACCATTACTTCGG-3' for the 5' terminal end and the primers 18F3: 5'-GGACGGCATGTTACTTTGA-3' and IR5: 5'-TACGGAACTTGT-TACGAC-3' for the 3' terminal end. A portion of the 28S rRNA gene was amplified in two overlapping fragments of about 1 kb and 500 bp respectively, with primers LSU5': 5'-TAGGTCGACCCGCTGAAYT-TAAGCA-3' and IR14: 5'-CATGTTAACTCCTTGGTCCG-3' for the 5' terminal end and the primers IF15: 5'-GTCTGTGGCGTAGTGGTAGAC-3' and LSU3': 5'-TAGAAGCTTCTGAGGGAACTTCGG-3' for the 3' terminal end. About 440 bp of the mitochondrial Cytochrome c Oxidase I (COI) were also amplified with primers L-CO1p: 5'-TTTTTGGGCATCCTGAGGTTTAT-3' and H-Cox1p2: 5'-TAAAGAAAGAA-CATAATGAAAATG-3' [11] for that specimen and nine other polystomes of our DNA collection that were collected from different host species (see Table 1). PCRs for 18S, 28S and COI were conducted following procedures described in Ref. [10].

**Table 2**  
Summary of characteristics of polystomatid genera from the Ethiopian realm.

Character	Character states	<i>Kankana</i> n. gen.	<i>Eupolystoma</i>	<i>Metapolystoma</i>	<i>Madapolystoma</i>	<i>Protopolystoma</i>	<i>Polystoma</i>
Number of: pairs of hamuli		1	0	1	1	2	1
Uterus	(0 = absent, 1 = small, 2 = medium, 3 = long)	3	3	2	3	0	1
Ovary	(1 = anterior, 2 = median, 3 = posterior)	2	3	2	2	1	1
Testis	(1 = anterior, 2 = median, 3 = posterior)	2	3	2	3	2	2
Vaginae present	(0 = absent, 1 = present)	0	1	1	1	0	1
Vitellarium	(1 = throughout body, 2 = narrow posterior lateral fields)	2	1 or 2	1	2	1	1

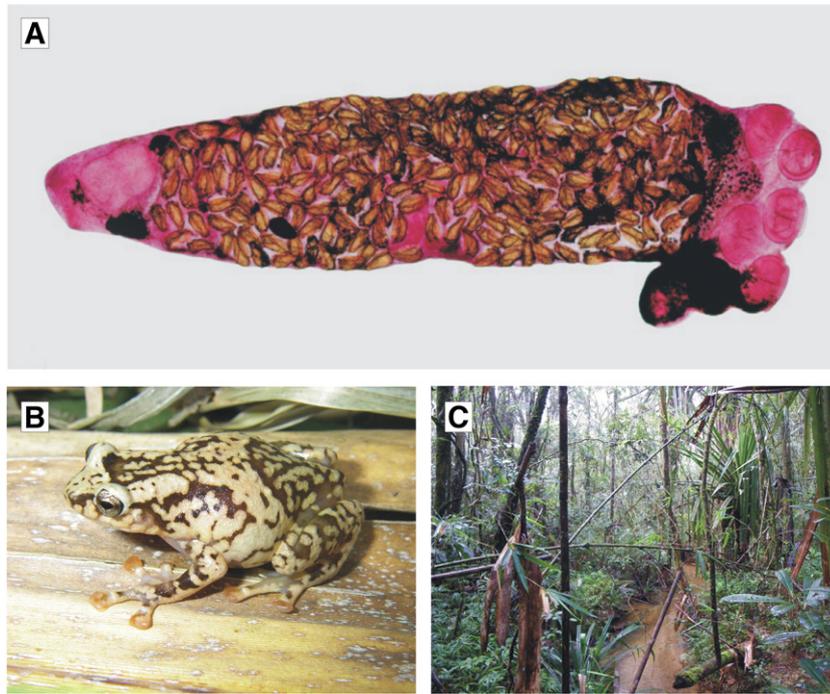


Fig. 1. A, Holotype of *Kankana manampoka* n. gen, n. sp.; B, The host *Platypelis pollicaris*; C, Type locality in the Sahamalaotra forest, Ranomafana National Park.

PCR products were purified using the kit Wizard SV Gel and PCR Clean-Up System of Promega and sent to GATC Biotech (Konstanz, Germany) for sequencing. The F18/18RG PCR portion was sequenced with F18, 18RG and an internal primer 18RC: 5'-TACGAGCTTTTAACTGCAG-3' while the 18F3/IR5 portion was sequenced with 18F3, IR5 and an internal primer S1: 5'-ATTCCGATAACGAACGAGACT-3'. The LSU5'/IR14 PCR fragment was sequenced with primers IR13: 5'-GTCGTGGCTTACACCCTGAGG-3' and IR14 while the IF15/LSU3' fragment was sequenced with the IF15 primer. COI was sequenced with both PCR primers L-CO1p and H-Cox1p2. Newly determined sequences were deposited in GenBank (see Table 1).

All new sequences were edited with the software Sequencher 4.5 of Gene Codes Corporation. The whole 18S and 28S sequences (see Table 1) were aligned following a polystome alignment defined by Badets et al. (2011) [12] that considers the secondary structure of rRNAs (stems and loops). The phylogenetic position of *Kankana manampoka* n. gen., n. sp. among the Polystomatidae was deduced from a Bayesian analysis on the complete data set following a doublet model. Bayesian Inferences were run with the software MrBayes v. 3.1 [13], with four chains running for a million generations, sampling each 100 cycles. The first 1000 trees were removed as the burn-in phase upon empirical evaluation. The 50 percent majority rule consensus tree was computed on the last 9000 trees to obtain the Bayesian posterior probabilities for each association. It was rooted with *Diplorchis ranae*, according to Refs. [10,12].

COI sequences were aligned using Clustal W implemented in the program MEGA v. 4.0. [14]. Uncorrected pairwise divergences ( $p$ -distances) were calculated from partial 28S and COI sequences, respectively, using PAUP\* v.4.0b9 [15]. After excluding gaps and ambiguous aligned regions, 28S and COI distances were based on 1349 and 313 characters, respectively.

### 3. Results

#### 3.1. *Kankana* n. gen.

##### 3.1.1. Diagnosis

Polystomatidae, Polystomatinae. Large elongate ovoid body, about 4 mm long. Intestinal caeca blind, with small diverticula, no prehaptoran anastomoses. Haptor with 3 pairs of suckers, one pair of

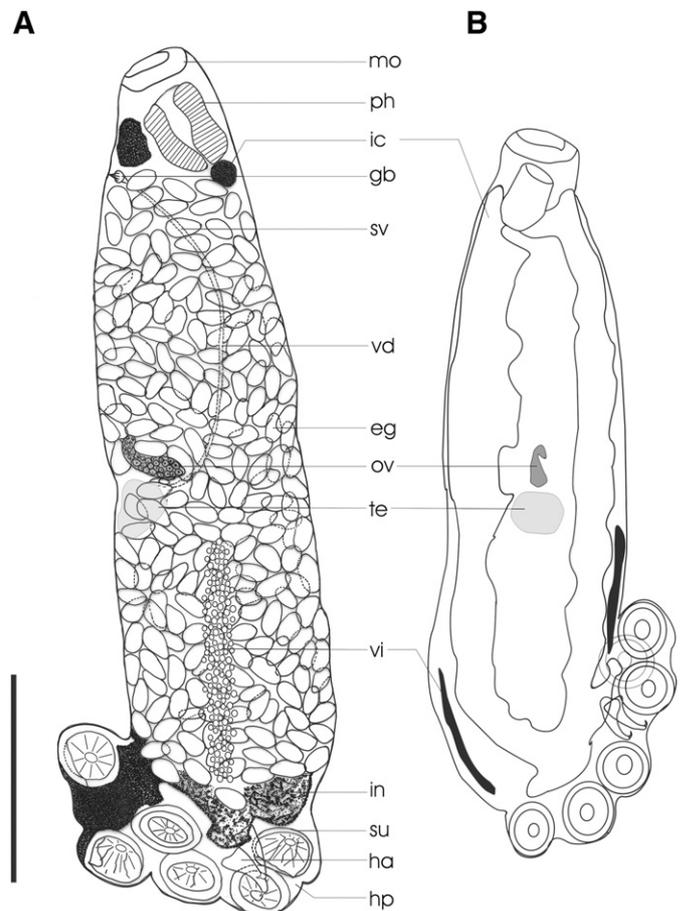


Fig. 2. *Kankana manampoka* n. gen, n. sp. A, Lateral view of holotype; B, Ventral view of paratype; Abbreviations: eg, egg; gb, genital bulb; ha, hamulus; hp, haptor; in, intestine; mo, mouth; ov, ovary; ph, pharynx; su, sucker; sv, seminal vesicle; te, testis; vd, vas deferens; vi, vitellarium. Scale-bar: 1 mm.

hamuli and 16 marginal hooklets: hooklets I and II situated between hamuli, with hooklets III, IV and V at base of suckers 1, 2 and 3, respectively, and hooklets VI, VII and VIII at anterior margin of haptor. Testis single, small, diffuse, post-ovarian confined to intracaecal region. Vas deferens extends antero-medially to copulatory organ (genital bulb), armed with 8 spines, opens into common genital pore. Ovary small, comma shaped, medial. Uterus extensive and packed with eggs filling the body proper posterior to pharynx. Vitellarium in two lateral fields confined to posterior part of body proper. Vaginae absent. Egg operculate. Adult parasitic in urinary bladder of Malagasy microhylid frogs. Type-species *K. manampoka* n. gen., n. sp.

### 3.1.2. Remarks

The newly proposed genus *Kankana* n. gen. shares various morphological characteristics with several other polystomes, however the combination of character is unique. A comparison of the major characteristics of polystomes known from the Ethiopian realm is given in Table 2. At first glance this new parasite with its extensive uterus and lack of prehaptoran anastomoses resembles *Madapolystoma*, *Metapolystoma* and *Eupolystoma*. However, unlike *Metapolystoma* the vitellarium is confined to the lateral fields, a characteristic it shares with *Madapolystoma* and *Eupolystoma*. Unlike *Eupolystoma* it has a pair of hamuli, a character it shares with *Madapolystoma*. Both *Eupolystoma* and *Madapolystoma* have vaginae while *Kankana* n. gen. has none. The position of the testis is midbody for *Kankana* n. gen. while it is posteriormost for both *Eupolystoma* and *Madapolystoma*.

### 3.2. *K. manampoka* n. gen., n. sp.

Class: Monogenea

Order: Polystomatidea

Family: Polystomatidae Gamble, 1896

#### 3.2.1. Type host

*P. pollicaris*; sexually mature female (ZCMV 8563) (Fig. 1). Herpetology collection of the *Département de Biologie Animale, Université d'Antananarivo* (UADBA 6138).

#### 3.2.2. Type locality

Rainforest at Sahamalaotra, Ranomafana National Park, Madagascar (21°14.113 S, 47°23.767' E) (Fig. 1). Two host specimens were found on leaves of guava trees 1.5–2 m above the ground along a stream. The remaining 13 host specimens were collected 1–2 km away, in primary rainforest with frequent bamboo stands, along a nearby stream. Additional host specimens from the same population are catalogued as ZSM 527/2009 - 531/2009.

#### 3.2.3. Type-specimens

Holotype (NMB P323) and three paratypes (NMB P324 -326) deposited in the Parasitic Worm Collection, National Museum, Aliwal street, Bloemfontein 9300, South Africa; one paratype (2010.8.27.3) in the Parasitic Worms Collection, Natural History Museum, London.

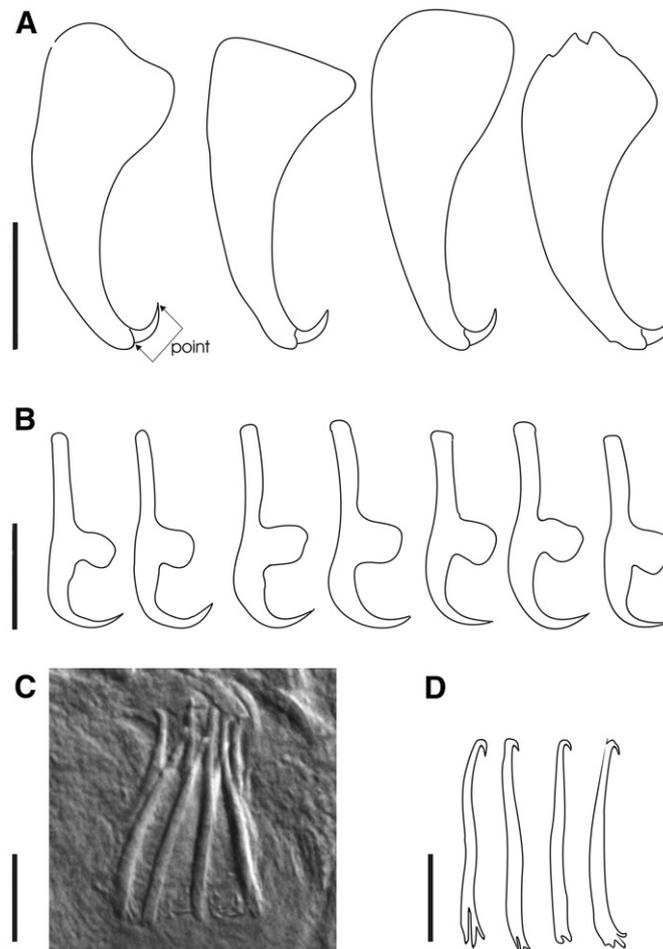


Fig. 3. *Kankana manampoka* n. sp. A, hamuli (First one from holotype and remainder from paratypes); B, random selection of marginal hooklets 1, posteriormost; C, micrograph of crown of genital spines; D, Genital spines drawn from paratypes. Scale-bars: A 100  $\mu$ m, B–D 10  $\mu$ m.

3.2.4. Specimens studied

Three sexually mature parasites, one subadult parasite, one immature parasite and two eggs containing fully developed larvae. Of the two *P. pollicaris* collected at Sahamalaotra one was infected with a single mature parasite and of the 13 host specimens collected in the nearby bamboo forest, three were infected. One specimen had a mature as well as an immature parasite probably indicating an auto-reinfection or a secondary infection of the postmetamorphic frog. For the total host sample, the prevalence was 27% and the mean intensity 1.25.

3.2.5. Site

Urinary bladder.

3.2.6. Description

Description and measurements based on three adult, egg-producing parasites. Holotype and one paratype were accidentally laterally flattened when fixed under cover slip pressure. The average measurement in micrometers is followed by the range in parenthesis. Measurements of marginal hooklets based on adult parasites, one

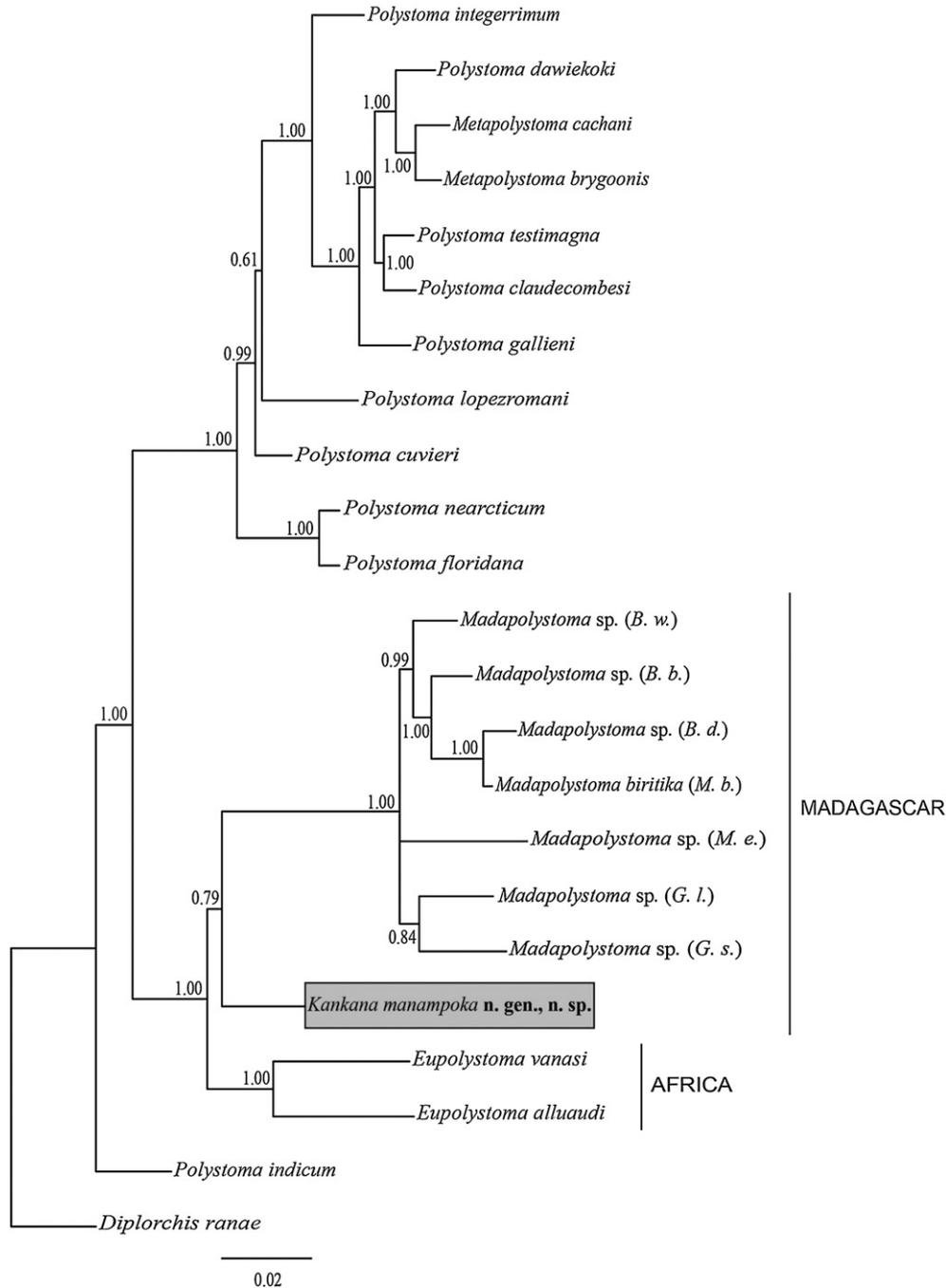


Fig. 4. Bayesian tree inferred from the analysis of concatenated complete 18S and partial 28S rRNA gene sequences. Abbreviations in bracket refer to host species, from top to bottom: B. w. = *Blommersia wittei*; B. b. = *Blommersia blommersae*; B. d. = *Blommersia domerguei*; M. b. = *Mantella baroni*; M. e. = *Mantella expectata*; G. l. = *Guibemantis liber*; G. s. = *Gephyromantis sculpturatus*. Values at nodes indicate Bayesian posterior probabilities.

**Table 3**  
Mean character differences (above diagonal) and total character differences (below diagonal) inferred from comparisons of 28S rDNA sequences (1337 characters). Abbreviations: *M. e.* = *Mantella expectata*; *B. w.* = *Blommersia wittei*; *G. l.* = *Guibemantis liber*; *B. b.* = *Blommersia blommersae*; *M. b.* = *Mantella baroni*; *B. d.* = *Blommersia domerguei*; *G. s.* = *Gephyromantis sculpturatus*.

		1	2	3	4	5	6	7	8	9	10	11
1	<i>Kankana manampoka n. g. and n. sp.</i>	–	0.06153	0.08006	0.03188	0.01038	0.08747	0.07932	0.04893	0.07116	0.07858	0.07191
2	<i>Madapolystoma sp. (M. e.)</i>	83	–	0.06523	0.05411	0.05930	0.06227	0.06153	0.06968	0.04299	0.05782	0.05115
3	<i>Madapolystoma sp. (B. w.)</i>	108	88	–	0.08006	0.07709	0.08525	0.02372	0.09044	0.07042	0.06079	0.03262
4	<i>Madapolystoma sp. (G. l.)</i>	43	73	108	–	0.02669	0.08377	0.07932	0.04744	0.06375	0.07042	0.07191
5	<i>Madapolystoma sp. (B. b.)</i>	14	80	104	36	–	0.08451	0.07561	0.04374	0.06894	0.07413	0.06820
6	<i>Madapolystoma biritika (M. b.)</i>	118	84	115	113	114	–	0.07932	0.08895	0.04744	0.08154	0.07635
7	<i>Madapolystoma sp. (B. d.)</i>	107	83	32	107	102	107	–	0.08599	0.06672	0.05930	0.02520
8	<i>Madapolystoma sp. (G. s.)</i>	66	94	122	64	59	120	116	–	0.07709	0.08821	0.08154
9	<i>E. alluaudi</i>	96	58	95	86	93	64	90	104	–	0.06597	0.06004
10	<i>E. vanasi</i>	106	78	82	95	100	110	80	119	89	–	0.05115
11	<i>P. integerrimum</i>	97	69	44	97	92	103	34	110	81	69	–
12	<i>P. dawiekoki</i>	99	78	27	101	94	102	25	113	85	78	35
13	<i>M. cachani</i>	58	74	105	51	54	113	101	68	89	98	86
14	<i>M. brygoonis</i>	38	70	105	27	33	109	99	60	84	97	88
15	<i>P. gallieni</i>	105	78	38	106	102	110	27	118	85	79	34
16	<i>P. claudecombesi</i>	51	82	115	48	46	122	109	71	98	108	96
17	<i>P. testimagina</i>	102	75	29	102	97	103	17	112	80	76	31
18	<i>P. lopezromani</i>	104	75	61	101	99	106	50	114	82	78	38
19	<i>P. cuvieri</i>	93	60	44	90	90	90	35	105	66	68	21
20	<i>P. nearcticum</i>	102	73	55	101	97	104	44	110	81	77	35
21	<i>P. floridana</i>	102	72	54	101	97	103	43	110	80	76	32
22	<i>P. indicum</i>	97	62	67	95	92	94	66	111	69	59	53
23	<i>D. ranae</i>	106	78	82	95	100	110	80	119	89	0	69

immature parasite and two eggs containing fully developed larvae released by the holotype.

**3.2.6.1. Adult.** General characteristics of mature, egg-producing parasite typical of polystomatids (Fig. 2). Body elongate, total length 4092 (3672–4488); greatest width 1035 (1002–1074). Haptor length 1479 (1342–1670); haptor width 883 (735–999). Haptor suckers 6; mean diameter 289 (247–338); 2 hamuli, smooth surface with no incision and thus no inner and outer roots (Fig. 3; hamulus length 260 (251–268); hamulus point 34 (31–36). Mouth terminal; false oral sucker 310 (305–315) wide; pharynx elongate, pharynx length 389 (372–398), pharynx width 294 (270–310). Intestine bifurcate with small medial diverticula and no prehaptor anastomoses. Intestinal caeca extending into anterior region of haptor where it forms haptor anastomosis (Fig. 2).

Ovary situated in middle of body (Fig. 2); ovary length 286 (260–313); ovary width 112 (95–128). Vaginae absent. Testis follicular, ventral, confined to small medial area directly posterior to ovary (Fig. 2). Vas deferens runs from testis medially in anterior direction and widens slightly to form seminal vesicle which extends to antero-ventral genital bulb. Vitellarium restricted to discrete narrow strip of follicles along lateral fields of posterior part of body (Fig. 2). Genital bulb 47 (46–48) armed with 8 genital spines 22 (20–23) long (Fig. 3). Uterus tubular, extensive, filling most of body proper, may contain up to 231 yellow eggs. Eggs operculated, length 157 (146–164), width 83 (81–86); intra-uterine development was noticed in a few eggs. Marginal hooklets small, more or less of same length and shape, 18 (16–19) (Fig. 3B).

### 3.3. Molecular phylogenetic analysis and genetic divergences

Phylogenetic relationships among polystomes support the position of *K. manampoka* as sister group to *Madapolystoma* (although with a very low Bayesian posterior probability = 0.79, see Fig. 4), with *Eupolystoma* as the sister group to this clade (Bayesian posterior probability = 1.00, see Fig. 4). The mean molecular divergences estimated are  $6.93\% \pm 0.018$  for 28S and  $9.52\% \pm 0.021$  for COI among the most divergent *Madapolystoma* spp., and  $6.60\%$  for 28S and  $17.89\%$  for COI between the two species of *Eupolystoma* (see Tables 3 and 4). Besides, the mean molecular divergence estimated between *K.*

*manampoka* and the most divergent *Madapolystoma* spp. is of  $5.71\% \pm 0.028$  for 28S and of  $15.34\% \pm 0.01$  for COI, and is of  $7.49\% \pm 0.005$  for 28S and of  $19.97\% \pm 0.002$  for COI between *K. manampoka* and *Eupolystoma* spp. According to Ref. [16], the molecular level of divergence at which polystomes are likely to belong to distinct species is approximately 0.07% for 28S and 1.91% for COI. Considering the high levels of 28S and COI genetic divergences between *K. manampoka* and species of *Eupolystoma* and *Madapolystoma*, and the phylogenetic position of *K. manampoka* within Madagascan polystomes, we may therefore consider this parasite as a separate species. Similarly, though the mean 28S genetic divergence between *K. manampoka* and *Madapolystoma* spp. is less than between the most divergent species of *Madapolystoma*, which is likely due to slow evolutionary rates of rRNA genes in *K. manampoka* (Fig. 4), the high levels of COI genetic divergences estimated between this new species and species of *Eupolystoma* and *Madapolystoma* provide molecular support to assign this species to a new genus, in addition to its unique combination of morphological character states.

## 4. Discussion

The phylogeny presented in Fig. 4 showed *Kankana* to be a sister group of *Madapolystoma*. Although both genera share characteristics like hamuli, extensive uterus, ovary in the midbody and a vitellarium confined to the lateral fields, there are some distinct differences. *Kankana* is bigger and the uterus may contain more than 200 eggs. The uterus of *Madapolystoma* on the other hand contains only a few non operculated eggs with very advanced developed non-ciliated larvae [9]. Unlike *Madapolystoma* which has delicate thin non-operculate transparent egg capsules, the eggs of *Kankana* are yellow tan and operculated as for most other polystomes. Furthermore the testis is situated in the middle of the body as for *Metapolystoma* compared to the posteriormost position in *Madapolystoma* [9].

Sclerites including the hamuli, marginal hooklets and genital spines have proved to be very useful as taxonomic characteristics [17]. The marginal hooklets of *Kankana* are all of the same size and shape, a characteristic that it shares with *Eupolystoma* (see Ref. [18]) and *Madapolystoma* (see Ref. [9]). This is in contrast with the usual situation in polystomes where the most posterior pair of marginal

12	13	14	15	16	17	18	19	20	21	22	23
0.07339	0.04299	0.02817	0.07784	0.03781	0.07561	0.07717	0.06894	0.07561	0.07561	0.07191	0.07858
0.05782	0.05486	0.05189	0.05782	0.06079	0.05560	0.05565	0.04448	0.05411	0.05337	0.04596	0.05782
0.02001	0.07784	0.07784	0.02817	0.08525	0.02150	0.04527	0.03262	0.04077	0.04003	0.04967	0.06079
0.07487	0.03781	0.02001	0.07858	0.03558	0.07561	0.07495	0.06672	0.07487	0.07487	0.07042	0.07042
0.06968	0.04003	0.02446	0.07561	0.03410	0.07191	0.07346	0.06672	0.07191	0.07191	0.06820	0.07413
0.07561	0.08377	0.08080	0.08154	0.09044	0.07635	0.07867	0.06672	0.07709	0.07635	0.06968	0.08154
0.01853	0.07487	0.07339	0.02001	0.08080	0.01260	0.03711	0.02595	0.03262	0.03188	0.04893	0.05930
0.08377	0.05041	0.04448	0.08747	0.05263	0.08302	0.08458	0.07784	0.08154	0.08154	0.08228	0.08821
0.06301	0.06597	0.06227	0.06301	0.07265	0.05930	0.06085	0.04893	0.06004	0.05930	0.05115	0.06597
0.05782	0.07265	0.07191	0.05856	0.08006	0.05634	0.05788	0.05041	0.05708	0.05634	0.04374	0.00000
0.02595	0.06375	0.06523	0.02520	0.07116	0.02298	0.02821	0.01557	0.02595	0.02372	0.03929	0.05115
–	0.07339	0.07116	0.02372	0.07858	0.01631	0.03934	0.02817	0.03484	0.03410	0.04374	0.05782
99	–	0.03410	0.07339	0.03484	0.07191	0.06899	0.06079	0.07116	0.06968	0.06894	0.07265
96	46	–	0.07413	0.03113	0.07116	0.07124	0.06227	0.06894	0.06894	0.06523	0.07191
32	99	100	–	0.08006	0.01779	0.03413	0.02446	0.03262	0.03188	0.04596	0.05856
106	47	42	108	–	0.07858	0.07790	0.06820	0.07339	0.07265	0.07191	0.08006
22	97	96	24	106	–	0.03414	0.02372	0.02891	0.02817	0.04374	0.05634
53	93	96	46	105	46	–	0.01930	0.02301	0.02524	0.04675	0.05788
38	82	84	33	92	32	26	–	0.01779	0.01705	0.03336	0.05041
47	96	93	44	99	39	31	24	–	0.00371	0.04374	0.05708
46	94	93	43	98	38	34	23	5	–	0.04151	0.05634
59	93	88	62	97	59	63	45	59	56	–	0.04374
78	98	97	79	108	76	78	68	77	76	59	–

hooklets is significantly enlarged and more robust as reported for *Polystoma* (see Refs. [19–21]), *Protopolystoma* (see Ref. [22]) and *Metapolystoma* (see Ref. [23]).

Unlike most other polystome genera, the blade of the hamuli in *Kankana* does not have a deep cut dividing it into an inner and outer root resembling a developing hamulus. Furthermore the hamuli do not have deep grooves that are usually present on polystome hamuli. The morphology of hamuli has been shown to be of key taxonomic significance in for example *Gyrodactylus* (see Refs. [24–26]). In a paper dealing with the evolution of hamuli, Timoveeva [27] speculated that hamuli originated as adult attachment organs of protomonogeneans inhabiting the gills of early vertebrates. The original lateral pair hamuli were pre-haptor but the fundamental direction in the evolution of monogeneans was the concentration of all attachment structures on the haptor and in the course of this evolutionary process, the hamuli migrated to the haptor. An in depth study on the morphology, embryology, functioning and evolution of polystome hamuli is long overdue.

Polystomes are characterized by very low prevalence and intensity of infection and as a rule, fewer than five worms are present in an infected frog [28]. *Eupolystoma* is an exception in this regard as they may be found in numbers as many as 2000 worms per host [18]. The expanded uterus and intrauterine development observed in *Kankana* pose the same opportunity of an internal cycle as is the case for *Eupolystoma*. The adult and subadult worms found in one *Platypelis* specimen indicate that in the case of *Kankana* we have either an internal cycle or larvae can infect postmetamorphic frogs. The known hosts for *Eupolystoma* are quite large toads with huge urinary bladders rich in capillary blood vessels that can support large parasite populations. *Platypelis* is a small frog with a body size of about 25 mm. It is thus unlikely that they will be able to support large numbers of parasites. In the present study the maximum number of parasites per infected host was two.

Of great significance for the understanding of the evolution and biology of these parasites is the fact that *K. manampoka* is a polystome to be described from a microhylid host. The Microhylidae is a complex anuran group with more than 70 genera and 450 species of almost cosmopolitan tropical distribution [7]. Many microhylids have explosive reproduction in lentic waters with specialized filter-feeding

tadpoles (e.g., [29]). Other microhylid subclades have nidicolous tadpoles, that is, non-feeding larvae that develop only on the yolk reserves of the egg. One such group is the microhylid subfamily Cophylinae, endemic to Madagascar, to which the arboreal *Platypelis* belongs [30,31].

Very little is known on the breeding behavior of *P. pollicaris* from Ranomafana and it is uncertain whether data from populations in the Northern Central East (reported by Ref. [32]) fully apply also to populations from Ranomafana which according to our own, unpublished data are genetically and bioacoustically divergent. Regarding the reproduction of the host population of *Kankana*, we thus have, to a large extent, to extrapolate from what we know from other populations and species of *Platypelis*, and the related *Cophyla*. All *Platypelis* are arboreal rain forest species that breed in water that accumulates at either the base of *Pandanus* screw pine leaves, in tree holes, or in bamboo stems [33]. In all *Platypelis* where larvae have been found, these were non-feeding tadpoles swimming freely in the phytotelmic water. This tadpole stage allows for polystome transmission in an aquatic environment. Usually, a single male occupies one tree hole and guards the eggs and tadpoles. Since in several species of *Platypelis*, *Cophyla*, and other arboreal cophylines it has been observed that in the same tree hole there can be tadpoles/embryos in different developmental stages, usually in 2–3 well delimited clusters of stages, we can assume that various females can sequentially (at random time intervals) approach the same breeding site and deposit eggs therein that will be fertilized and guarded by the same male. Because occurrence of *P. pollicaris* in Ranomafana appears to be largely linked to bamboo, we postulate that it mostly reproduces in water-filled open bamboo stems. These water holding bamboo stems are most likely holding the secrets to the life cycle of *K. manampoka*.

Based on this hypothesized reproductive behavior of *P. pollicaris* in Ranomafana, we can also develop a testable hypothesis of the reproductive cycle of *Kankana*. The relatively small ovary and testis and extended uterus of *Kankana* can be seen as an adaptation to a host with an opportunistic breeding strategy. Such morphological adaptations to the breeding behavior of a host have been documented for *Eupolystoma* [see Ref. 18] and *Pseudodiplorchis* [see Ref. 34]. Whenever the frog finds a suitable breeding site and spawns, the parasite has a number of eggs ready to release into the urinary bladder of the frog. When the frog enters the phytotelmata it hydrates and water

**Table 4**  
Mean character differences (above diagonal) and total character differences (below diagonal) inferred from comparisons of COI sequences (313 characters). Abbreviations: B. w. = *Blommersia wittei*; G. l. = *Guibemantis liber*; B. b. = *Blommersia blommersae*; M. b. = *Mantella baronii*; G. s. = *Gephyromantis sculpturatus*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1 <i>Kankana manampoka</i> n. g. and n. sp.	-	0.15335	0.14377	0.14377	0.14377	0.15974	0.16613	0.20128	0.19808	0.22364	0.19808	0.18850	0.19169	0.17572	0.15335	0.19808	0.19489	0.18530	0.16933	0.18211
2 <i>Madapolystoma</i> sp. (B. w.)	48	-	0.07987	0.07029	0.09265	0.10543	0.20767	0.17572	0.19808	0.22364	0.16613	0.15974	0.20128	0.16613	0.17572	0.19808	0.19489	0.18850	0.19169	0.22045
3 <i>Madapolystoma</i> sp. (G. l.)	45	25	-	0.07668	0.08626	0.12780	0.22364	0.19808	0.22045	0.18211	0.19169	0.19808	0.16613	0.17572	0.17891	0.19489	0.18530	0.18211	0.19169	0.21725
4 <i>Madapolystoma</i> sp. (B. b.)	45	22	24	-	0.07348	0.11821	0.21406	0.18530	0.20447	0.17572	0.18530	0.19169	0.15655	0.17252	0.21406	0.20767	0.19808	0.18211	0.19169	0.23642
5 <i>Madapolystoma hirtika</i> (M. b.)	50	29	27	23	-	0.12141	0.21725	0.20767	0.24281	0.18211	0.21086	0.20767	0.18850	0.19808	0.22045	0.22364	0.22045	0.19169	0.19169	0.2684
6 <i>Madapolystoma</i> sp. (G. s.)	52	33	40	37	38	-	0.21725	0.19489	0.20447	0.17891	0.18530	0.18850	0.16933	0.16933	0.20447	0.20767	0.19808	0.18530	0.18530	0.22364
7 <i>E. altaudi</i>	63	65	70	67	68	68	-	0.17891	0.22364	0.19169	0.20447	0.22045	0.22045	0.19489	0.22364	0.20128	0.20767	0.22045	0.22045	0.21725
8 <i>E. vanasi</i>	62	55	62	64	65	61	56	-	0.21086	0.18211	0.18211	0.20447	0.20447	0.18850	0.16294	0.19489	0.19169	0.19169	0.19169	0.19808
9 <i>P. integerrimum</i>	70	67	69	64	76	64	70	66	-	0.18211	0.17252	0.17252	0.15974	0.15655	0.14377	0.15016	0.16294	0.19489	0.19489	0.18850
10 <i>P. dawiekoki</i>	62	52	57	55	57	56	60	55	57	-	0.13099	0.13738	0.13419	0.14696	0.16613	0.15974	0.16933	0.16613	0.16613	0.20447
11 <i>M. bryoonis</i>	59	50	60	58	66	58	64	57	54	41	-	0.12780	0.12780	0.14058	0.15016	0.15335	0.16294	0.18211	0.18850	
12 <i>P. gallieni</i>	60	63	62	60	65	59	69	64	54	43	40	-	0.16613	0.12780	0.14058	0.14377	0.17891	0.18211	0.18211	
13 <i>P. testimagna</i>	55	52	52	49	59	53	63	59	50	42	40	52	-	0.12780	0.13738	0.15655	0.17252	0.17891	0.20447	
14 <i>P. lopezromani</i>	48	55	55	54	62	53	61	51	49	46	44	49	40	-	0.14696	0.15016	0.15016	0.18211	0.19808	
15 <i>P. cuvieri</i>	62	62	56	67	69	64	70	61	45	52	47	44	43	46	-	0.15974	0.15974	0.17891	0.17891	
16 <i>P. nearcticum</i>	61	61	60	65	70	65	63	60	47	50	48	48	49	47	47	-	0.04153	0.18211	0.19808	
17 <i>P. floridana</i>	58	59	61	62	69	62	65	58	51	53	51	45	54	47	50	13	-	0.19169	0.17891	
18 <i>P. indicum</i>	53	60	58	57	60	58	69	60	61	52	57	56	56	57	56	57	60	-	0.18850	
19 <i>D. ranee</i>	57	69	68	74	71	70	68	62	59	64	59	57	64	62	56	62	56	59	-	

accumulates in the urinary bladder. This signal to the parasite to release eggs which are then flushed out when the frog urinates in the water.

In *Kankana* specimens collected, we observed that a few eggs showed signs of ovoviviparity. The release of developed eggs together with undeveloped eggs provides a strategy whereby older tadpoles that may already be present in the breeding site could be infected immediately while the other parasite eggs develop and hatch at a time when the newly laid frog eggs hatched and the tadpoles are available for infection but this remains to be investigated.

Breeding in phytotelmata has an additional advantage to the polystome parasite in that the small body of water increases the likelihood for a free swimming oncomiracidium to make contact with a suitable host tadpole significantly. One would expect that the prevalence of polystomes that infect phytotelmic breeders could be quite high. This is supported by Ref. [9] who reported in Madagascar an unusual high prevalence for *Madapolystoma* sp. from *Mantella laevigata*, a poison frog species that breeds in phytotelmata.

Further studies and especially studies on *Platypelis* tadpoles are needed to shed more light on the life history strategies of this parasite. With anurans being the primary host for polystomes and Madagascar having such a rich anuran fauna it is thus not surprising to find new polystomes in Madagascar and many more await discovery.

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