Two new species of polystomes (Monogenea: Polystomatidae) from the anuran host *Guibemantis liber*

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ABSTRACT

Madapolystoma ramilijaonae n. sp. and *Madapolystoma cryptica* n. sp. (Monogenea, Polystomatidae) are described from the urinary bladder of disjunct populations of the Madagascar shrub frog *Guibemantis liber*. Although only minor morphological characters distinguish the new species from the single nominal species of the genus, i.e. *Madapolystoma biritika*, their strong and concordant differentiation in a mitochondrial and a nuclear gene supplemented by phylogenetic analyses indicates that *M. ramilijaonae* n. sp. and *M. cryptica* n. sp. should be regarded as two distinct species. Because anuran polystomes are known to be host-specific, the description of two cryptic species from a single host species points to a taxonomic complex situation in *G. liber*, a widespread frog that is characterized by the presence of several deep conspecific lineages and possibly by hybridization and admixture with other species of *Guibemantis*.

1. Introduction

Madagascar's long geographic isolation has led to a unique biota and to its status of biodiversity hotspot [1]. The island was initially part of the Gondwana supercontinent from which it started separating 165–158 Myr ago [2,3]. It remained connected to India a little longer and detached around 94–84 Myr ago [2,4], and today it is situated about 400 km from the east African coast. The long period of isolation set the scene for speciation which resulted in a unique and highly endemic biota. While Gondwanan vicariance is supported in some taxa [5,6], it seems clear that at least in terrestrial vertebrates Cenozoic transoceanic colonization was the prevalent origin of the endemic clades on Madagascar [7–11]. Recent studies indicated that the endemicity observed in Madagascar and Mascarene islands is as high as 89% for plant species, 58% for birds, 59% for freshwater fishes and more than 90% for the other vertebrates [12]. Apart from the single introduced frog *Hoplobatrachus tigerinus*, 100% of the island's anuran species are endemic. Currently the number of described frog species is 271 [13],

but there is a considerable proportion of undescribed species [14]. The true number of frogs is probably at least 373 but possibly as many as 465 [15].

The undescribed proportion of biodiversity is probably much larger in Malagasy invertebrates which remain poorly explored, except for a few groups such as scorpions, butterflies and some groups of beetles [16]. Among the invertebrates, parasites rank among the less studied groups. New species have recently been described including nematodes parasitizing chameleons and frogs [17,18], and cestodes parasitizing snakes and stingrays [19,20]. Some ectoparasites have also been studied, including leeches [21] and fleas [22], but most studies were focused on lemur parasites [23–26] and human pathogens like *Plasmodium* sp. [27–29].

A group of parasites that recently received a fair amount of attention is the Polystomatidae (Monogenea, Platyhelminthes). Due to their high degree of host-specificity, these flatworms provide an opportunity to better understand the evolution and ecology of their hosts [30–34]. Polystomes are parasites of aquatic tetrapods, mainly of amphibians and freshwater chelonians, but they also have been found parasitizing the Australian lungfish [35] and the African hippopotamus [36]. At present, 24 genera are recognized in the family. While polystome genera are typically distinguished by prominent morphological characters, species discrimination within genera is often more difficult and relies on morphological, skeletal and biometric measurements [37]. Because these variables are often not discriminative enough, host species identity is

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sometimes taken into account as additional criterion to describe new species [38,39] since polystomes are considered as strictly host-specific [40–44]. Cross-infection experiments support this statement [41,45]. For instance, *Polystoma africanum* was known from several host species but in a recent revision based on strict host-specificity, Aisien and Du Preez [46] suggested that *P. africanum* consists of a complex of several species. A standardized methodology to identify polystomes from morphometric measurements of sclerites was developed by Murith [47] and further refined by Du Preez & Maritz [37]. Despite these efforts, polystome discrimination by morphology alone in some cases still remains ambiguous [37].

Until 2009, a single anuran polystome was known to occur in Madagascar, namely *Metapolystoma brygoonis* Euzet and Combes, 1964, a species parasitizing *Ptychadena mascareniensis*. Intensive field-work in 2005–2009 revealed a spectacular and previously unknown diversity of polystomes infesting Madagascar's anurans. Two endemic genera *Madapolystoma* and *Kankana* were recently described from host species belonging to the Mantellinae, a subfamily of the endemic family Mantellidae, and from the Cophylinae, an endemic subfamily of the cosmopolitan Microhylidae, respectively [48,49]. Numerous undescribed species of *Metapolystoma* were also reported from host species of the mantellid subfamilies Boophinae and Laliostominae [50]. Considering the species-rich Malagasy anuran fauna, a great number of yet undescribed polystome species can be expected, especially within the Mantellidae.

Here we explore the polystome parasites of a widespread mantellid species, *Guibemantis liber*. Whereas mantellids show pronounced micro-endemism [15,51], some species such as *G. liber* are more widespread and occur across most of the eastern and northern rainforests of the island, often containing deep conspecific lineages of strong genetic differentiation [15]. Analyzing the polystomes of such species may provide crucial information to better understand the processes of population differentiation that sometimes are followed by admixture among lineages, and in other occasions to complete independence of lineages, and thus to the formation of new species, in both the host and the parasite species.

2. Material and methods

2.1. The host G. liber

G. liber belongs to the family Mantellidae which is the most diverse endemic amphibian family in Madagascar [52]. The genus *Guibemantis* contains arboreal frogs with heterogeneous reproductive habits. After the latest species descriptions [53], 12 species are included in this genus but several new species warrants recognition [14]. Mantellids display high levels of micro-endemicity with some species known only from a single locality [54], while *G. liber* shows a wide distribution range along the eastern part of the island (see Fig. 1). In daytime *G. liber* is found in phytotelm leaf axils. Mating takes place on vertical leaves overhanging ponds where eggs are deposited. After hatching tadpoles fall into the pond where they develop and complete metamorphosis.

2.2. Host and parasite sampling

Adults of *G. liber* were collected in the course of herpetological inventories between 2005 and 2009 from various localities throughout the range of distribution of this species (see Fig. 1). Sampling localities included the National Parks of Ranomafana, Tsaratanana and Montagne d'Ambre, the Special Reserve of Ambohitantely, the Massif of Makira and the surroundings of Andasibe, Andrakata and An'Ala villages. Frogs were anesthetized and subsequently killed using MS222 (ethyl-4-aminobenzoate), and dissected using a portable field stereo microscope to check for the presence of polystomes in the urinary bladder. Parasites were fixed in 10% neutral buffered formalin for preparing whole mounts and 96% ethanol for molecular studies. The biggest

specimens were mounted for morphological examination while the smaller ones were kept for molecular analyses.

2.3. Molecular methods

Polystome specimens of *G. liber* and one from *Mantella baroni* were dried and incubated for 60 min at 55 °C in 150 μL of 10% suspension Chelex 100 sodium (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) and 20 μL proteinase K 10 mg·mL^{-1}. The reaction was stopped at 100 °C for 15 min and DNA was stored at −20 °C until use. A fragment of the mitochondrial Cytochrome c Oxydase I (COI) gene was amplified with the primers forward L-CO1p, 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ and reverse H-Cox1p2, 5′-TAAAGAAAGAACATAATGAAAATG-3′ [55], yielding a PCR product of about 440 bp. New polystome-specific primers were also designed to process samples that gave no results with the preceding primers: forward COI-FB, 5′-TGGTATAATTAGTCATATATG-3′ and reverse COI-RB, 5′-AACAACAAAYCAAGAATCATG-3′. Size of PCR product was about 390 bp. A portion of the nuclear 28S rRNA gene was amplified in two overlapping fragments of about 1 kb and 500 bp, with the primers forward LSU5′: 5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′ and reverse IR14: 5′-CATGTTAAACTCCTTGGTCCG-3′ for the 5′ terminal end and the primers forward IF15: 5′-GTCTGTGGCGTAGTGGTAGAC-3′ and reverse LSU3′: 5′-TAGAAGCTTCCTGAGGGAAACTTCGG-3′ for the 3′ terminal end, respectively. DNA amplification was conducted following the procedure described in Verneau et al. [50]. All PCR products were purified using the kit Wizard SV Gel and PCR Clean-Up System (Promega, Charbonnières-les-Bains, France) and sent to GATC Biotech (Konstanz, Germany) for sequencing with the PCR primers.

2.4. DNA sequence analyses

Sequences were edited and examined with the software Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA) and each observed substitution was verified. COI sequences were aligned using ClustalW [56] implemented in the program MEGA v. 4.0. [57], whereas alignment of 28S rDNA sequences was done regarding to the ribosomal RNA secondary structure of polystomes defined by Badets et al. [30]. In order to explore the monophyly and relationships of polystomes parasitizing *G. liber*, COI sequences of *Madapolystoma biritika* and *Madapolystoma* spp. reported in Du Preez et al. [48] and Raharivololoniaina et al. [49], as well as one new sequence obtained for a polystome of *M. baroni* were also included in a global phylogenetic analysis (Table 1).

Phylogenetic analyses were conducted on the COI sequences following three different approaches. Bayesian inference (BI) was carried out using the software MrBayes v. 3.1 [58] under the GTR model, with five 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% majority rule consensus tree was computed on the last 9000 trees to obtain the Bayesian posterior probability for each association. Maximum parsimony (MP) was performed with PAUP* v. 4.0b10 following a heuristic search with random addition of taxa (10 replicates) and tree bisection reconnection (TBR) on all informative unweighted characters. The robustness of nodes was evaluated through a non-parametric bootstrap analysis with 1000 pseudo-replicates. For maximum likelihood (ML), the best appropriate model of sequence evolution was selected from the hierarchical likelihood ratio tests implemented in the program MODELTEST v. 3.06 [59]. The model GTR + Γ was used with empirically determined substitution rates ([A, C] = 1.6110; [A,G] = 19.3690; [A,T] = 4.8750; [C,G] = 0.0000; [C,T] = 6.7038; [G,T] = 1.0000) and nucleotide frequencies (Pi [A] = 0.2652; Pi [C] = 0.0920; Pi [G] = 0.1859; Pi [T] = 0.4569), a null proportion of invariable sites and a shape parameter of α = 0.2789. The ML analysis and ML parametric bootstrapping were performed on all characters with PAUP*, following a heuristic procedure under the TBR and NNI branch swapping options, respectively.
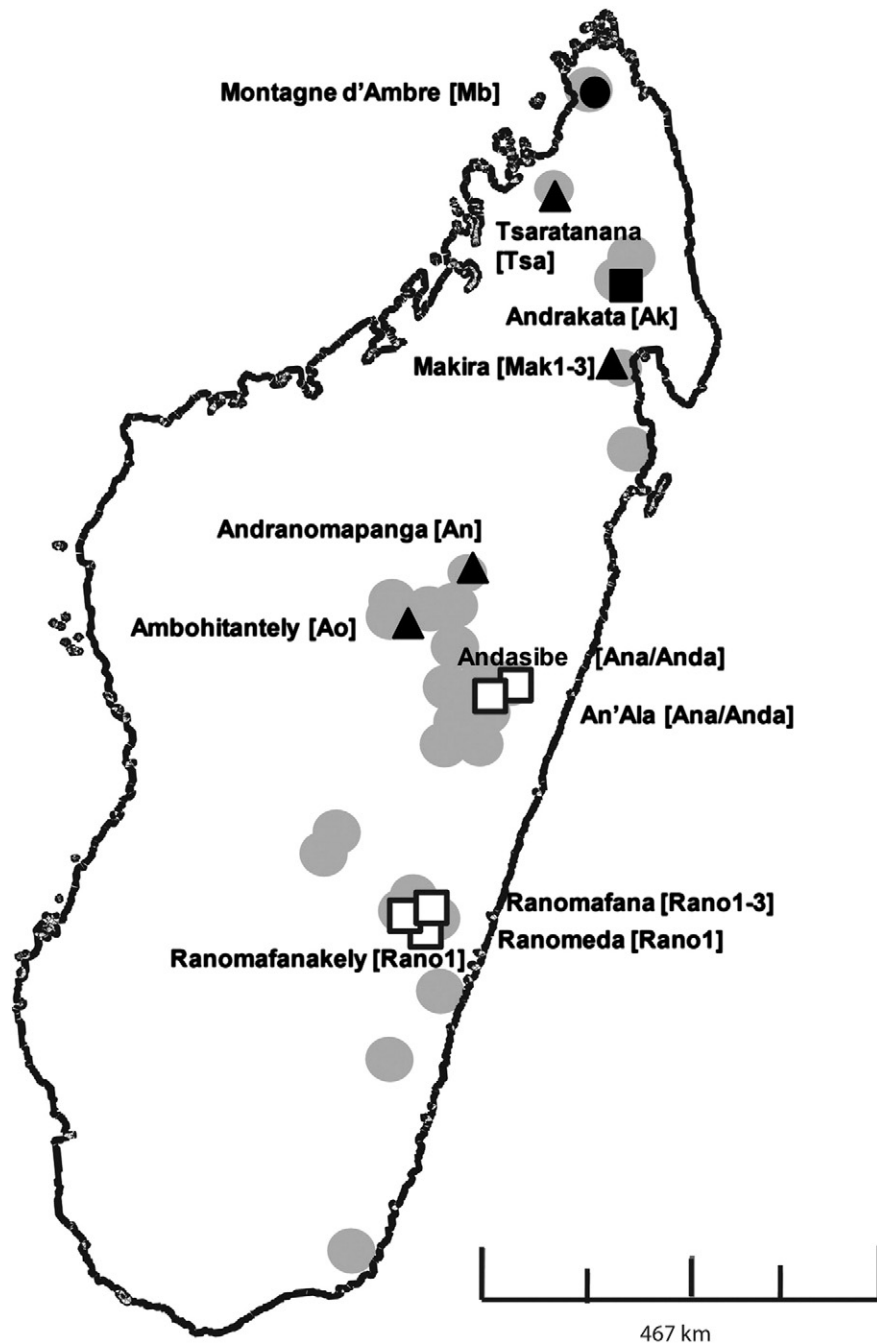
Fig. 1. Distribution of *G. liber* after Glaw and Vences [14] (gray areas). Black symbols refer to the polystomes sampling localities. ▲ refers to *Madapolystoma* sp. A; □ refers to *Madapolystoma* sp. B; ● refers to *Madapolystoma* sp. C from *G. liber* in Montagne d'Ambre and ■ refers to *Madapolystoma* sp. D from *G. liber* in Andrakata. Haplotypes detected in every locality are indicated in brackets.

Uncorrected pairwise distances (*p*-distances) were estimated independently for the whole COI data set and for the 28S data set using PAUP* v. 4.0b10 [60] to assess genetic divergences within *Madapolystoma*. Sites with ambiguous nucleotides were excluded for genetic divergence estimates. Results are discussed in the light of the COI and 28S molecular species delineation procedures defined by Du Preez et al. [61] on amphibian polystomes.

2.5. Morphology and morphometry

Polystomes were washed free of fixative and stained overnight in weak solution of acetocarmine, dehydrated, cleared in xylene and mounted in Canada balsam. Specimens were examined using a Nikon

Eclipse E800 compound microscope (Nikon, Netherlands). Body and organs were measured using the Nikon NIS elements software program. All measurements are given in micrometers. Marginal hooklets were measured using the protocol of Du Preez and Maritz [37] to discriminate species groups.

2.6. Marginal hooklet morphometrics

According to Murith et al. [62], shape and size of marginal hooklets remain constant throughout polystome development. Du Preez and Maritz [37] evaluated marginal hooklet morphology to find the combination of measurements with the highest classification potential. This was found to be the product of the total length (a in Fig. 5) and the

Table 1
List of parasites investigated including host species, geographical origin and locality, COI and 28S haplotypes in brackets with number of analyzed individuals and GenBank accession numbers.

Polystomes species	Host species	Geographical origin/locality	COI haplotype	Accession number	28S haplotype	Accession number
Madapolystoma sp. A	*Guibemantis liber*	Madagascar/Tsaratanana	[Tsa] (7)	JN015522[a]	TSA (4)	JN800275[a]
Madapolystoma sp. A	*Guibemantis liber*	Madagascar/Andranomapanga	[An] (2)	JN015515[a]	AN/AO (2)	JN800276[a]
Madapolystoma sp. A	*Guibemantis liber*	Madagascar/Ambohitantely	[Ao] (5)	JN015511[a]	AN/AO (1)	JN800276[a]
Madapolystoma sp. A	*Guibemantis liber*	Madagascar/Makira	[Mak1] (3)	JN015519[a]	MAK (5)	JN800278[a]
Madapolystoma sp. A	*Guibemantis liber*	Madagascar/Makira	[Mak2] (2)	JN015518[a]		
Madapolystoma sp. A	*Guibemantis liber*	Madagascar/Makira	[Mak3] (1)	JN015520[a]		
Madapolystoma sp. B	*Guibemantis liber*	Madagascar/Andasibe	[Ana/Anda] (6)	JN015523[a]	ANA/ANDA (1)	JN800271[a]
Madapolystoma sp. B	*Guibemantis liber*	Madagascar/An'Ala	[Ana/Anda] (6)	JN015514[a]	ANA/ANDA (3)	JN800272[a]
Madapolystoma sp. B	*Guibemantis liber*	Madagascar/Ranomafana	[Rano1] (39)	JN015512[a]	RANO (5)	JN800273[a]
Madapolystoma sp. B	*Guibemantis liber*	Madagascar/Ranomafana	[Rano2] (10)	JN015516[a]		
Madapolystoma sp. B	*Guibemantis liber*	Madagascar/Ranomafana	[Rano3] (4)	JN015517[a]		
Madapolystoma sp. B	*Guibemantis liber*	Madagascar/Ranomafanakely	[Rano1] (10)	JN015525[a]		
Madapolystoma sp. B	*Guibemantis liber*	Madagascar/Ranomeda	[Rano1] (6)	JN015524[a]		
Madapolystoma sp. C	*Guibemantis liber*	Madagascar/Montagne d'Ambre	[Mb] (12)	JN015513[a]	MB (2)	JN800280[a]
Madapolystoma sp. D	*Guibemantis liber*	Madagascar/Andrakata	[Ak] (2)	JN015521[a]	AK (1)	JN800279[a]
Madapolystoma biritika	*Mantella baroni*	Madagascar/Vohiparara	[a] (1)	FR667564		
Madapolystoma biritika	*Mantella baroni*	Madagascar	[b] (1)	FR667565	[B] (1)	FM897278
Madapolystoma biritika	*Mantella baroni*	Madagascar	[c] (1)	FR667566		
Madapolystoma biritika	*Mantella baroni*	Madagascar/Sahasoratra	[d] (1)	FR667567		
Madapolystoma biritika	*Mantella baroni*	Madagascar/Ranomafana	[e] (1)	FR667568		
Madapolystoma biritika	*Mantella baroni*	Madagascar/Ranomeda	[j] (1)	JN015510[a]		
Madapolystoma biritika	*Mantella madagascareniensis*	Madagascar	[f] (1)	FR667569		
Madapolystoma biritika	*Mantella madagascareniensis*	Madagascar	[g] (1)	FR667570		
Madapolystoma biritika	*Mantella madagascareniensis*	Madagascar	[h] (1)	FR667571		
Madapolystoma biritika	*Mantella mylotympanum*	Madagascar	[i] (1)	FR667572		
Madapolystoma sp.	*Blommersia wittei*	Madagascar/Isalo	(1)	FR667561	(1)	FM897273
Madapolystoma sp.	*Blommersia blommersae*	Madagascar/An'Ala	[a] (1)	FR667560		
Madapolystoma sp.	*Blommersia blommersae*	Madagascar/An'Ala	[b] (1)	JF699309	[A] (1)	FM897271
Madapolystoma sp.	*Gephyromantis sculpturatus*	Madagascar/An'Ala	(1)	FR667562	(1)	FM897274
Eupolystoma alluaudi	*Bufo* sp.	Togo	(1)	FR667558	(1)	AM157199
Eupolystoma vanasi	*Schismaderma carens*	South Africa	(1)	FR667559	(1)	AM157200

[a] Indicates new sequences obtained in this study.

width at the level of the guard (c in Fig. 5) versus the product of the total length and the length of a tangent between the tip of the blade to the guard (b in Fig. 5) of marginal hooklet 1. A scatterplot was done for some *Madapolystoma* specimens of *G. liber* sampled in Tsaratanana, An'Ala, Andasibe, Ranomafana and Montagne d'Ambre.

3. Results

### 3.1. Molecular analyses

Newly determined sequences were deposited in GenBank (Table 1). The COI molecular sampling included 129 polystome specimens in total, among which 115 from *G. liber* and 14 from six other mantellid host species and the 28S molecular sampling included 24 polystome specimens from *G. liber* and four from other mantellid host species (Table 1). Two other sequences from *Eupolystoma* were used for rooting the tree according to Verneau et al. [50]. In the dataset used for phylogenetic analysis, each recovered haplotype was represented only once. The topology of the Bayesian consensus tree based on the COI sequences is very similar to the best ML (tree score = 1721.02464) and 50% majority rule MP consensus trees. Consequently, we depict only the phylogenetic relationships of polystomes inferred from the Bayesian analysis with Bayesian posterior probabilities and bootstrap proportions assessed from both ML and MP analyses (Fig. 2). The phylogenetic analyses show with high confidence values the monophyly of *G. liber* polystomes and confirm the monophyly of *M. biritika*.

The COI sequences inferred from polystomes parasitizing *G. liber* revealed 12 haplotypes. These formed four different lineages which for convenience we will here provisionally name *Madapolystoma* sp. A–D. Of these four lineages, *Madapolystoma* sp. A contained six closely related haplotypes from four different northern localities (Table 2),

Tsaratanana, Andranomapanga, Ambohitantely and Makira (Table 1; Fig. 1). *Madapolystoma* sp. B included four haplotypes from Ranomafana and from the An'Ala/Andasibe area. The two other lineages, *Madapolystoma* sp. C (sister to *M.* sp. A) and *Madapolystoma* sp. D (sister to *M.* sp. B), were represented by single haplotypes found at Montagne d'Ambre and Andrakata, respectively. 28S sequences revealed the existence of seven haplotypes as listed in Table 3. Three of these, 'MAK', from Makira, 'TSA', from Tsaratanana and 'AO/AN', from Ambohitantely and Andranomapanga, were determined from specimens and populations that belonged to the COI lineage *Madapolystoma* sp. A. Two haplotypes, 'RANO', from Ranomafana and 'ANA/ANDA', from An'Ala and Andasibe, fit *Madapolystoma* sp. B. Finally haplotypes 'MB' from Montagne d'Ambre and 'AK' from Andrakata (Table 3) fit with *Madapolystoma* sp. C and D, respectively.

Among the COI sequences, the uncorrected *p*-distance estimates range from 0.26 to 1.33% within *Madapolystoma* sp. A and from 0.25 to 1.76% within *Madapolystoma* sp. B. Divergences between *Madapolystoma* sp. A and *Madapolystoma* sp. B range from 5.00% to 6.82%, largely exceeding the threshold of about 2% proposed by Du Preez et al. [61] to be indicative of species-level differentiation. Moreover, *Madapolystoma* spp. C and D show divergences to their sister lineages higher than 2%, that is 2.46% ± 0.28 between *Madapolystoma* sp. C and haplotypes of *M.* sp. A, and 3.91% ± 0.14 between *Madapolystoma* sp. C and *M.* sp. B. This suggests that these two lineages may also belong to undescribed *Madapolystoma* species. Finally, all COI haplotypes recovered from polystomes of *G. liber* diverge from haplotypes of *M. biritika* by at least 8.08% (Table 2). *P*-distance estimated among the 28S sequences reinforces these results (see Table 3). Divergence between *Madapolystoma* sp. A and *M.* sp. B haplotypes ranges from 0.67% ± 0.09, with divergences of 0.15% within *Madapolystoma* sp. B and 0.15% ± 0.05 within *Madapolystoma* sp. A. Moreover, *Madapolystoma* sp. C, only found in
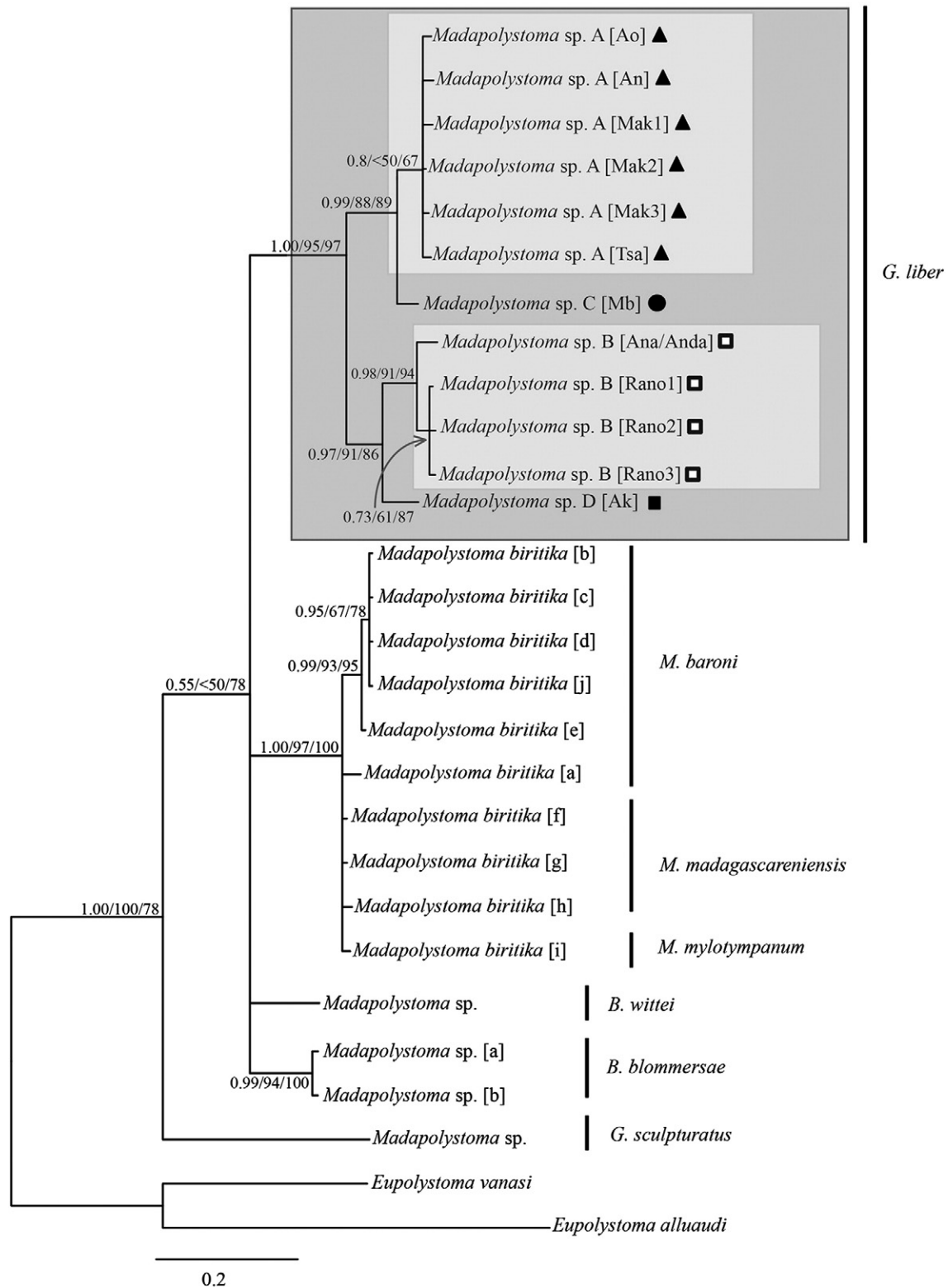
Fig. 2. Bayesian maximum likelihood tree inferred from analysis of COI sequences. Haplotypes in brackets and symbols refer to geographical localities of Malagasy polystomes (see Fig. 1 and Table 1). Values indicate respectively Bayesian posterior probabilities, ML and MP bootstrap proportions after 1000 replicates. Letters a to j after *M. biritika* and a to b after *Madapolystoma* sp. refer to the COI haplotype of the distinct specimens (see Table 1).

Montagne d'Ambre, shows a minimum divergence of 0.31% ± 0.05 to other haplotypes, and *Madapolystoma* sp. D from Andrakata shows a minimum of 0.54% ± 0.08 divergence (in both cases to *M.* sp. A). Finally, all 28S haplotypes recovered from polystomes of *G. liber* diverge from haplotypes of *M. biritika* by at least 3.6%. These 28S distances again surpass the species-level threshold of 0.07% proposed for this gene by Du Preez et al. [61] and confirm the existence of four distinct genetic entities among the *Madapolystoma* parasitizing *G. liber*.

Table 2
Mean character differences (above diagonal) and total character differences (below diagonal) inferred from comparisons of COI sequences.

		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1)	*M.* sp. A [Ao] (*G. liber*)	–	0.00798	0.00505	0.01010	0.00765	0.01010	0.02273	0.05808	0.05303	0.05303	0.05556	0.05808	0.09848	0.09848
(2)	*M.* sp. A [An] (*G. liber*)	3	–	0.00801	0.01333	0.01084	0.00797	0.02112	0.05555	0.05027	0.05029	0.05290	0.04760	0.09244	0.09244
(3)	*M.* sp. A [Mak1] (*G. liber*)	2	3	–	0.00505	0.00255	0.00505	0.02273	0.06313	0.05808	0.05808	0.05556	0.05808	0.10101	0.10101
(4)	*M.* sp. A [Mak2] (*G. liber*)	4	5	2	–	0.00770	0.01010	0.02778	0.06818	0.06313	0.06313	0.06061	0.06313	0.09596	0.09596
(5)	*M.* sp. A [Mak3] (*G. liber*)	3	4	1	3	–	0.00765	0.02576	0.06471	0.05951	0.05943	0.05695	0.05951	0.09813	0.09813
(6)	*M.* sp. A [Tsa] (*G. liber*)	4	3	2	4	3	–	0.02778	0.06313	0.05808	0.05808	0.05556	0.05808	0.09596	0.09596
(7)	*M.* sp. C [Mb] (*G. liber*)	9	8	9	11	10	11	–	0.05556	0.05556	0.05556	0.05808	0.04545	0.10101	0.10101
(8)	*M.* sp. B [Ana/Anda] (*G. liber*)	23	21	25	27	25	25	22	–	0.01515	0.01768	0.01768	0.04040	0.10101	0.10101
(9)	*M.* sp. B [Rano1] (*G. liber*)	21	19	23	25	23	23	22	6	–	0.00253	0.00253	0.03788	0.09596	0.09596
(10)	*M.* sp. B. [Rano2] (*G. liber*)	21	19	23	25	23	23	22	7	1	–	0.00505	0.03788	0.09848	0.09848
(11)	*M.* sp. B [Rano3] (*G. liber*)	22	20	22	24	22	22	23	7	1	2	–	0.04040	0.09596	0.09596
(12)	*M.* sp. D [Ak] (*G. liber*)	23	18	23	25	23	23	18	16	15	15	16	–	0.11364	0.11364
(13)	*M. biritika* [a] (*M. baroni*)	39	35	40	38	38	38	40	40	38	39	38	45	–	0.00000
(14)	*M. biritika* [b] (*M. baroni*)	39	35	40	38	38	38	40	40	38	39	38	45	0	–
(15)	*M. biritika* [c] (*M. baroni*)	39	35	40	38	38	38	40	40	38	39	38	45	0	0
(16)	*M. biritika* [d] (*M. baroni*)	39	35	40	38	38	38	40	40	38	39	38	45	0	0
(17)	*M. biritika* [e] (*M. baroni*)	32	31	33	31	33	38	35	31	30	31	30	37	1	1
(18)	*M. biritika* [j] (*M. baroni*)	37	33	38	38	36	38	39	37	36	37	36	42	11	11
(19)	*M. biritika* [f] (*M. madagascariensis*)	29	28	30	30	30	38	32	29	28	27	28	34	5	5
(20)	*M. biritika* [g] (*M. madagascariensis*)	29	28	30	30	30	38	32	29	28	27	28	34	5	5
(21)	*M. biritika* [h] (*M. madagascariensis*)	36	32	37	37	35	38	37	37	35	35	35	40	7	7
(22)	*M. biritika* [i] (*M. mylotympanum*)	36	32	37	37	35	38	37	36	35	36	35	40	9	9
(23)	*M.* sp. (*B. wittei*)	36	32	37	39	35	38	38	34	33	33	33	34	36	36
(24)	*M.* sp. [a] (*B. blommersae*)	28	27	29	31	29	38	33	29	31	30	31	36	29	29
(25)	*M.* sp. [b] (*B. blommersae*)	46	45	47	49	45	38	45	47	43	43	43	46	49	49
(26)	*M.* sp. (*G. sculpturatus*)	33	29	34	36	32	38	36	32	34	35	34	39	29	29
(27)	*Eupolystoma vanasi*	77	73	76	76	76	38	76	78	76	76	76	75	83	83
(28)	*Eupolystoma alluaudi*	86	85	87	85	87	38	84	85	83	83	83	83	81	81

		(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)
(1)	*M.* sp. A [Ao] (*G. liber*)	0.09848	0.09848	0.09654	0.09343	0.08729	0.08729	0.09091	0.09091	0.09091	0.07639	0.08333	0.12154	0.20249	0.23674
(2)	*M.* sp. A [An] (*G. liber*)	0.09244	0.09244	0.09374	0.08742	0.08446	0.08446	0.08450	0.08452	0.08463	0.07387	0.07660	0.11922	0.20212	0.23466
(3)	*M.* sp. A [Mak1] (*G. liber*)	0.10101	0.10101	0.09974	0.09596	0.09047	0.09047	0.09343	0.09343	0.09343	0.07933	0.08586	0.12425	0.20000	0.23976
(4)	*M.* sp. A [Mak2] (*G. liber*)	0.09596	0.09596	0.09371	0.09596	0.09057	0.09057	0.09343	0.09343	0.09848	0.08478	0.09091	0.12955	0.19998	0.23423
(5)	*M.* sp. A [Mak3] (*G. liber*)	0.09813	0.09813	0.09982	0.09290	0.09053	0.09053	0.09028	0.09038	0.09053	0.07953	0.08257	0.12190	0.20027	0.24299
(6)	*M.* sp. A [Tsa] (*G. liber*)	0.09596	0.09596	0.09361	0.09091	0.08437	0.08437	0.08838	0.09091	0.09343	0.07919	0.08586	0.12420	0.20520	0.23411
(7)	*M.* sp. C [Mb] (*G. liber*)	0.10101	0.10101	0.10571	0.09984	0.09642	0.09642	0.09343	0.09343	0.09596	0.09002	0.09091	0.11870	0.19991	0.23159
(8)	*M.* sp. B [Ana/Anda] (*G. liber*)	0.10101	0.10101	0.09339	0.09343	0.08711	0.08711	0.09343	0.09091	0.08586	0.07960	0.08081	0.12433	0.20495	0.23450
(9)	*M.* sp. B [Rano1] (*G. liber*)	0.09596	0.09596	0.09007	0.09091	0.08388	0.08388	0.08838	0.08838	0.08333	0.08497	0.08586	0.11376	0.19894	0.22887
(10)	*M.* sp. B [Rano2] (*G. liber*)	0.09848	0.09848	0.09295	0.09343	0.08079	0.08079	0.08838	0.09091	0.08333	0.08221	0.08838	0.11377	0.19875	0.22875
(11)	*M.* sp. B [Rano3] (*G. liber*)	0.09596	0.09596	0.09025	0.09091	0.08405	0.08405	0.08838	0.08838	0.08333	0.08503	0.08586	0.11375	0.19912	0.22899
(12)	*M.* sp. D [Ak] (*G. liber*)	0.11364	0.11364	0.11133	0.10606	0.10205	0.10205	0.10101	0.10101	0.08586	0.09851	0.09848	0.12148	0.19661	0.22913
(13)	*M. biritika* [a] (*M. baroni*)	0.00000	0.00000	0.00287	0.02778	0.01518	0.01518	0.01768	0.02273	0.09091	0.07928	0.07323	0.12888	0.21769	0.22302
(14)	*M. biritika* [b] (*M. baroni*)	0.00000	0.00000	0.00287	0.02778	0.01518	0.01518	0.01768	0.02273	0.09091	0.07928	0.07323	0.12888	0.21769	0.22302
(15)	*M. biritika* [c] (*M. baroni*)	–	0.00000	0.00287	0.02778	0.01518	0.01518	0.01768	0.02273	0.09091	0.07928	0.07323	0.12888	0.21769	0.22302
(16)	*M. biritika* [d] (*M. baroni*)	0	–	0.00287	0.02778	0.01518	0.01518	0.01768	0.02273	0.09091	0.07928	0.07323	0.12888	0.21769	0.22302
(17)	*M. biritika* [e] (*M. baroni*)	1	1	–	0.02139	0.01212	0.01212	0.01518	0.01820	0.09664	0.07890	0.07551	0.12710	0.22207	0.22785
(18)	*M. biritika* [j] (*M. baroni*)	11	11	7	–	0.01535	0.01535	0.02020	0.01515	0.08586	0.07679	0.07323	0.12921	0.21285	0.22623
(19)	*M. biritika* [f] (*M. madagascariensis*)	5	5	4	5	–	0.00000	0.00611	0.01212	0.09638	0.06965	0.07224	0.12955	0.22412	0.22699
(20)	*M. biritika* [g] (*M. madagascariensis*)	5	5	4	5	0	–	0.00611	0.01212	0.09638	0.06965	0.07224	0.12955	0.22412	0.22699
(21)	*M. biritika* [h] (*M. madagascariensis*)	7	7	5	8	2	2	–	0.01010	0.08586	0.07110	0.06818	0.12362	0.21208	0.21800
(22)	*M. biritika* [i] (*M. mylotympanum*)	9	9	6	6	4	4	4	–	0.08586	0.07115	0.06566	0.12365	0.21294	0.22912
(23)	*M.* sp. (*B. wittei*)	36	36	32	34	32	32	34	34	–	0.06871	0.06818	0.10555	0.18680	0.22075
(24)	*M.* sp. [a] (*B. blommersae*)	29	29	26	28	23	23	26	26	25	–	0.00279	0.12319	0.19529	0.23063
(25)	*M.* sp. [b] (*B. blommersae*)	49	49	42	49	43	43	47	47	40	1	–	0.12104	0.19171	0.22864
(26)	*M.* sp. (*G. sculpturatus*)	29	29	25	29	24	24	27	26	27	45	46	–	0.20491	0.23435
(27)	*Eupolystoma vanasi*	83	83	73	81	74	74	81	81	71	70	74	73	–	0.18327
(28)	*Eupolystoma alluaudi*	81	81	75	82	75	75	79	83	80	82	85	83	64	–

The reciprocally monophyletic grouping into mitochondrial lineages, absence of haplotype sharing in a nuclear gene, and differentiation in both a mitochondrial and a nuclear gene above the distance thresholds usually characterizing distinct polystome species suggests that *Madapolystoma* sp. A and *M.* sp. B correspond to two independent evolutionary lineages which we will in the following describe as *Madapolystoma cryptica* n. sp. and *Madapolystoma ramilijaonae* n. sp. The status of *Madapolystoma* sp. C and D needs further study due to their lower genetic differentiation to their respective sister species and the smaller amount of material available.

Table 3
Mean character differences (above diagonal) and total character differences (below diagonal) inferred from comparisons of 28S sequences.

		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
(1)	*M.* sp. A [AO/AN] (*G. liber*)	–	0.00078	0.00155	0.00388	0.00698	0.00698	0.00698	0.03721	0.03333	0.03798	0.03953	0.10233	0.08605
(2)	*M.* sp. A [MAK] (*G. liber*)	1	–	0.00233	0.00310	0.00775	0.00775	0.00775	0.03798	0.03411	0.03876	0.04031	0.10310	0.08682
(3)	*M.* sp. A [TSA] (*G. liber*)	2	3	–	0.00233	0.00543	0.00543	0.00543	0.03566	0.03178	0.03643	0.03798	0.10078	0.08450
(4)	*M.* sp. C [MB] (*G. liber*)	5	4	3	–	0.00620	0.00620	0.00620	0.03798	0.03411	0.03876	0.04031	0.10310	0.08682
(5)	*M.* sp. B [ANA/ANDA] (*G. liber*)	9	10	7	8	–	0.00155	0.00620	0.03721	0.03333	0.03798	0.03953	0.10078	0.08527
(6)	*M.* sp. B [RANO] (*G. liber*)	9	10	7	8	2	–	0.00465	0.03566	0.03333	0.03643	0.03953	0.09922	0.08372
(7)	*M.* sp. D [AK] (*G. liber*)	9	10	7	8	8	6	–	0.03798	0.03566	0.03876	0.04031	0.10310	0.08527
(8)	*M. biritika* [B] (*M. baroni*)	48	49	46	49	48	46	49	–	0.02636	0.02868	0.03953	0.10000	0.08682
(9)	*M.* sp. (*B. wittei*)	43	44	41	44	43	43	46	34	–	0.02713	0.03411	0.09922	0.08450
(10)	*M.* sp. [A] (*B. blommersae*)	49	50	47	50	49	47	50	37	35	–	0.03566	0.10078	0.08295
(11)	*M.* sp. (*G. sculpturatus*)	51	52	49	52	51	51	52	51	44	46	–	0.09070	0.07442
(12)	*Eupolystoma vanasi*	132	133	130	133	130	128	133	129	128	130	117	–	0.05736
(13)	*Eupolystoma alluaudi*	111	112	109	112	110	108	110	112	109	107	96	74	–
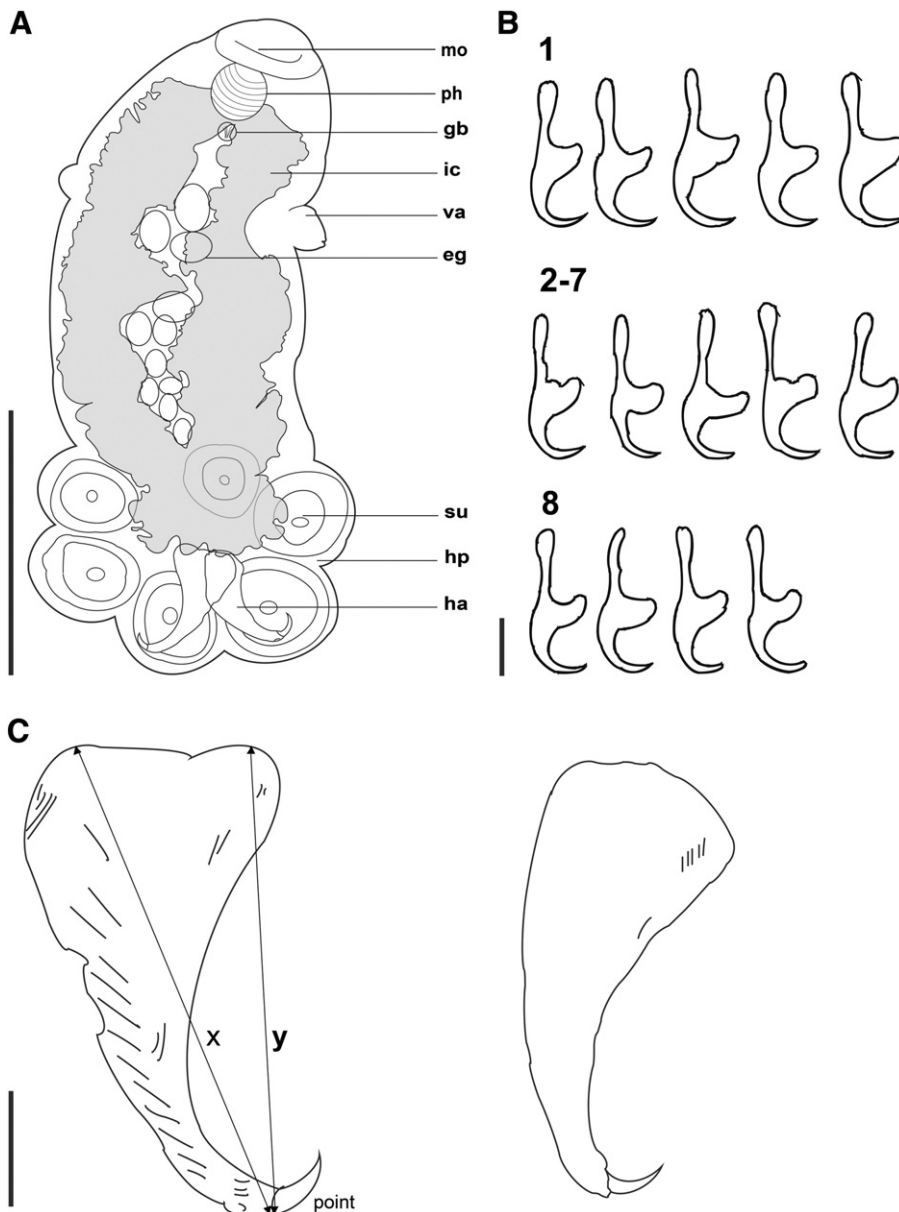
Fig. 3. (A) Ventral view of *M. cryptica* n. sp. holotype, (B) marginal hooklets 1, 2–7 and 8 from adult and subadult specimens and (C) hamuli from mature specimens. Scale bars: A, 500 µm; B, 10 µm; C, 50 µm. Abbreviations: eg, egg in uterus; gb, genital bulb; ha, hamulus; hp, haptor; ic, intestinal cecum; mo, mouth; ph, pharynx; su, sucker; va, vagina.

3.2. Species descriptions

3.2.1. M. cryptica n. sp. (Fig. 3)

Class: Monogenea Carus, 1863
Order: Polystomatidea Lebedev, 1988
Family: Polystomatidae Gamble, 1896

3.2.1.1. *Specimens studied.* Three sexually mature specimens (holotype NMB P 327 and two paratypes NMB P 328–329) and 6 immatures from a single locality, i. e., Tsaratanana. Types are deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein 9301, South Africa; remaining specimens are in the collection of LDP.

Type host: G. liber (Peracca, 1893)
Type locality: Tsaratanana, Madagascar.
Site: Urinary bladder
Etymology: The species epithet is a Latin adjective referring to the cryptic morphology of this species that has mainly been delimited on the basis of its molecular divergence.

3.2.1.2. *Description.* Measurements for mature and immature parasites are given in Tables 4 and 5, respectively. Body is pyriform, with a length of 1027 to 1239. The mouth is subterminal and surrounded by a false oral sucker. Spherical pharynx. No eyespots observed in adults. Intestine bifurcates and converges posteriorly with some medial diverticula, and no prehaptoral anastomoses (Fig. 3). Haptor with three pairs of suckers, two hamuli and 16 marginal hooklets placed as for other known polystomes (marginal hooklets 1 and 2 situated posteriormost between hamuli, marginal hooklets 3–5 at the base of the haptoral suckers and marginal hooklets 6–8 between the third pair of suckers. Two vaginae, on lateral margins, one third from the anterior end. Testis and ovary positions were unclear and obscured by intestine. Uterus is tubular and coiled extending full length of the body, holding several eggs in early stages of development ranging from 44 μm in length posterior in the uterus to 67 μm anterior in the uterus.

3.2.2. M. ramilijaonae n. sp. (Fig. 4)

Class: Monogenea Carus, 1863
Order: Polystomatidea Lebedev, 1988
Family: Polystomatidae Gamble, 1896

Table 4
Body measurements for sexually mature specimens of *Madapolystoma cryptica* n. sp. (3 specimens) and *Madapolystoma ramilijaonae* n. sp. (4 specimens). All measurements in micrometers.

Measurement	*Madapolystoma cryptica* n. sp.	*Madapolystoma ramilijaonae* n. sp.
Body length	1151 (1027–1239)	2948 (1493–3481)
Maximum width	429 (411–439)	705 (602–857)
Haptor length	387 (337–424)	661 (567–771)
Haptor width	475 (431–550)	922 (765–1013)
Hamulus length X (Figs.3 & 4)	227 (215–239) 179 (163–195)	
Hamulus length Y (Figs.3 & 4)	208 (197–219) 178 (154–193)	
Hamulus point length	36 (31–39)	39 (31–47)
False oral sucker	71 (58–83)	123 (90–156)
Pharynx length	94	175 (164–184)
Pharynx width	58	154 (151–156)
Genital bulb diameter	31	33 (30–38)
Number of genital spines	7	5–8
Genital spine length	14	15 (14–16)
Sucker diameter	160 (122–186)	216 (188–244)

Table 5
Body measurements for immature specimens of *Madapolystoma cryptica* n. sp. (1 specimen), *Madapolystoma ramilijaonae* n. sp. (50 specimens) and *Madapolystoma* sp. C from Montagne d'Ambre (5 specimens). All measurements in micrometers.

Measurement	*Madapolystoma cryptica* n. sp.	*Madapolystoma ramilijaonae* n. sp.	*Madapolystoma* sp. C (Montagne d'Ambre)
Body length	756	934 (642–1365)	850 (795–920)
Maximum width	268	310 (197–722)	263 (230–285)
Haptor length	317	327 (245–475)	287 (269–316)
Haptor width	370	417 (323–716)	378 (353–393)
Hamulus length X	147	136 (87–232)	151 (143–155)
Hamulus length Y	–	128 (96–214)	–
Hamulus point length	36	37 (19–44)	41 (33–45)
False oral sucker	–	85 (32–132)	–
Pharynx length	–	108 (84–188)	–
Pharynx width	–	83 (67–101)	–
Genital bulb diameter	–	24 (20–28)	–
No. of genital spine	9	5, 7 or 8	–
Genital spine length	14 (13–15)	12 (10–14)	–
Sucker diameter	–	109 (64–223)	117 (88–151)

3.2.2.1. *Specimens studied.* Four sexually mature (holotype NMB P330 and paratypes NMB P 331–333) and 50 immature parasites from three different localities, i.e., An'Ala, Andasibe and Ranomafana. Types are deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein 9301, South Africa; remaining specimens are in the collection of LDP.

Type host: G. liber (Peracca, 1893)
Localities: An'Ala, Andasibe, Ranomafana, Ranomafanakely, Ranomeda in Madagascar.
Site: Urinary bladder
Etymology: The species epithet is a tribute to the late professor Olga Ramilijaona Ravoahangimalala, who headed the Département de Biologie Animale at the University of Antananarivo, Madagascar at the time of this study.

3.2.2.2. *Description.* Measurements for mature and immature parasites are given in Tables 4 and 5, respectively. Based on general body features the new species can be assigned to *Madapolystoma*. Body is pyriform, with a length of 1493 to 3481. Eyespots were not observed in adults. The mouth is subterminal and ventral, and surrounded by false oral sucker. Pharynx is spherical. Intestine bifurcates and converges posteriorly with some medial diverticula, no prehaptoral anastomoses. Testis and ovary were obscured by intestine and were not observed. Genital bulb armed with 7 genital spines in specimens from An'Ala and Ranomafana, and 5, 7 or 8 genital spines in specimens from Andasibe. Two vaginae, on lateral margins of the body. Uterus is tubular and coiled extending from genital bulb to haptor holding up to 21 eggs in various stages of development. Eggs in the holotype vary from a 52 μm egg posteriorly in the uterus and showing no development to a egg containing a fully developed embryo of 379 μm anterior in the uterus; two embryos in advanced stage of development with hamuli were already visible and were observed in the holotype (Fig. 4); developing embryo was encapsulated in a thin transparent membrane. Haptor with three pairs of suckers, two hamuli and 16 marginal hooklets were organized as for other known polystomes (marginal hooklets 1 and 2 posteriormost between hamuli, hooklets 3–5 at the base of the haptoral suckers and hooklets 6–8 posterior between the third pair of suckers).

Although *M. cryptica* n. sp. and *M. ramilijaonae* n. sp. are morphologically very similar, they differ in total body length that ranges from 1027 to 1239 in *M. cryptica* n. sp. compared to 1493–3481 in *M. ramilijaonae* n. sp. Also the hamuli, false oral sucker, pharynx and haptoral suckers of *M. ramilijaonae* n. sp. are bigger showing no overlap with *M. cryptica* n. sp.
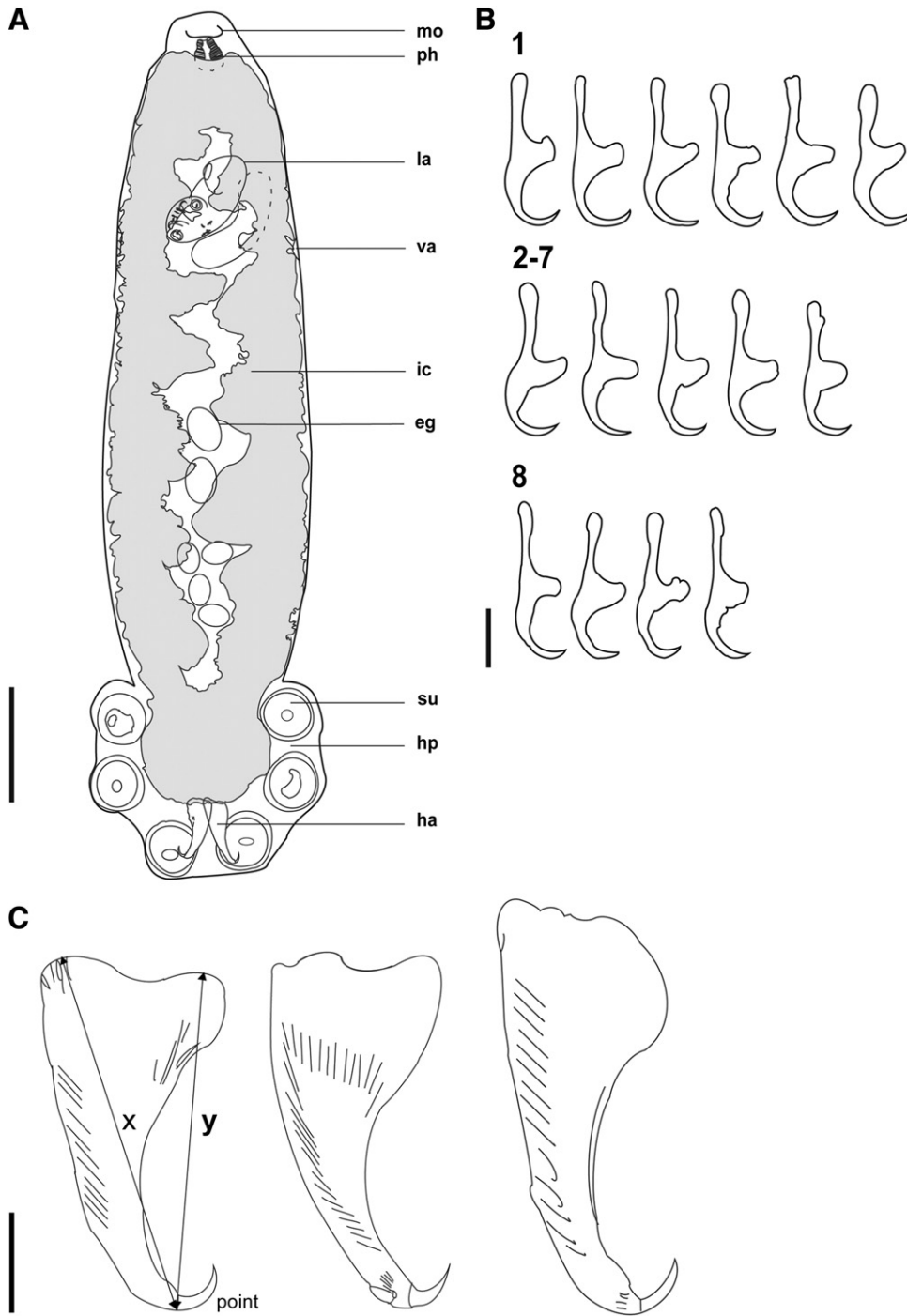
Fig. 4. (A) Ventral view of *M. ramilijaonae* n. sp. holotype, (B) marginal hooklets 1, 2–7 and 8 from adult and subadult specimens and (C) hamuli from mature specimens. Scale bars: A, 500 μm; B, 10 μm; C, 50 μm. Abbreviations: eg, egg; ha, hamulus; hp, haptor; ic, intestinal cecum; la, larvae; mo, mouth; ph, pharynx; su, sucker; va, vagina.

3.3. Marginal hooklet morphometrics

Morphometric measurements of the marginal hooklets show no separation between *M. cryptica* n. sp. and *M. ramilijaonae* n. sp. which overlaps in the scatterplot (Fig. 5), indicating that this character does not serve to distinguish these two species. The ellipse for *Madapolystoma* sp. C is however distinct from the other two (Fig. 5) which along with the molecular data supports that this is also a separate species.

4. Discussion

4.1. Polystome systematics: from morphology to DNA taxonomy

Taxonomy based on morphological characters requires good knowledge of taxonomic groups, which poses some problems when specialists retire. In order to conserve the capacity of species discovery and recognition, a DNA-based approach was proposed by Tautz et al. [63], and further developed by Hebert et al. [64] under the term DNA barcoding.
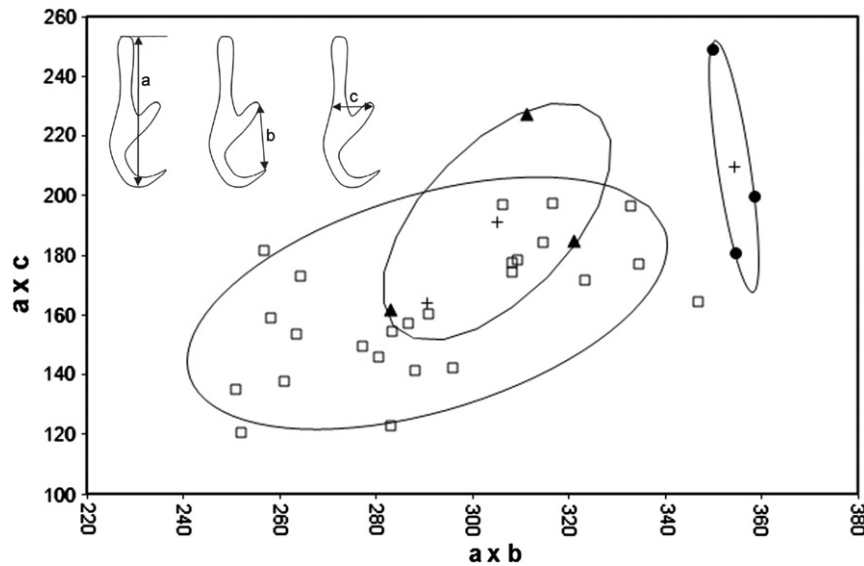
Fig. 5. Scatter diagram of 'axb' plotted against 'axc' of marginal hooklets for species groups. *M. cryptica* n. sp. (▲), *M. ramilijaonae* n. sp. (□) and *Madapolystoma* sp. C from *G. liber* of Montagne d'Ambre (●).

In this concept a DNA fragment from a standard marker is used to identify and distinguish species. The mitochondrial COI gene has been established as the core of this global bioidentification system [64]. Such molecular characters also can be of help to delimit new species when morphology does not permit distinguishing them [65] but static threshold values in a single marker can only serve to propose preliminary candidate species hypotheses which then require further integrative testing [66].

Within helminth parasites, molecular species description aided by DNA sequences is still in their infancy [67]. Over the past decade molecular ITS1 and COI data have been used progressively more and more to support morphological and morphometrical descriptions of polystomatids [31,48,49,61,68]. Du Preez et al. [61] identified a COI pairwise distance threshold that usually corresponds to species-level differentiation in amphibian polystomes to 2.0%, which is close to the threshold proposed for chelonian polystomes (about 1.5%–2.0%) by Verneau et al. [69].

Due to limited available mature parasites, high intraspecies variation and limited interspecies variation, polystomes retrieved from *G. liber* are morphologically almost indistinguishable except for apparent dimensional differences. In this study divergence estimates inferred from COI and 28S sequence comparisons were of high relevance to discriminate cryptic polystome species infecting *G. liber*, but besides the high genetic distances in both genes, a crucial argument is the concordant lack of allele (haplotype) sharing in both which indicates reduced or absent gene flow and thus evolutionary independence of the lineages herein described as new species.

4.2. Polystome diversity in Madagascar

With the advancement of molecular tools, the discovery of cryptic species among parasitic invertebrates [70–74] and even among vertebrates [15,75] escalated over the past decade. To date only three polystome species belonging to three distinct genera were described from anuran hosts in Madagascar [49]. Whereas *M. brygoonis* was reported on the Malagasy host *P. mascareniensis* [76], it has never been observed in Africa besides the occurrence of the host species. Indications are that *P. mascareniensis* on the African continent belongs to a different species [77]. Following intensive fieldwork in Madagascar a variety of polystomes were discovered among a large diversity of mantellids [50]. Through phylogenetic analyses, they showed that polystomes of the subfamilies Laliostominae and Boophinae were closely related to

M. brygoonis, suggesting the existence of at least five undescribed *Metapolystoma* species. Beside *Metapolystoma*, the genus *Madapolystoma* was reported on various host species of the subfamily Mantellinae [48,50]. *M. biritika* has originally been described from *Mantella madagascariensis* but also infects *M. baroni* and *M. milotympanum* [48]. Considering the two new polystome species reported here from *G. liber* and other putative parasite species within mantellids, we may now consider at least ten species within *Madapolystoma*. Finally *Kankana manampoka* is globally the only polystome known from a microhylid host. Molecular results placed this genus as a sister group of *Madapolystoma* [49], while *Metapolystoma* is nested within *Polystoma* [50]. If we take into account molecular data, the polystome diversity in Madagascar is probably much larger than expected from a morphological point of view. This was predicted by Verneau et al. [50] who showed that two colonization events may explain the origin of Madagascan polystomes.

4.3. Polystomes as evolutionary prints of amphibian speciation and diversification

It is well known that multiple infections are common in freshwater turtles that may harbor up to three different polystome species in distinct microhabitats. These include the urinary bladder, the conjunctival sacs and/or the pharyngeal cavity, each species being highly host and site specific [78–82]. Conversely, amphibian hosts are usually infected by one single polystome species which is located either in the urinary bladder of adult specimens, or in the branchial cavity of tadpoles for *Polystoma* species. Though a few reports have mentioned the occurrence of several polystome species within the same host species [83–86], the majority of these cases were reported in allopatric populations of these hosts. Instances of sympatric occurrence of different polystome species in single host individuals have been reported for the hosts *Ptychadena porosissima* [84], *P. pumilio* [83] and *P. tournieri* in Benin (Ibikounle, Sakiti and Verneau, unpublished observations).

The description of two (and possible existence of two additional) polystome species parasitizing a single host, namely *G. liber* in Madagascar, provides another such example. As discussed above, polystomes are known to be host- and site-specific, and to a few exceptions one parasite species is found per host species. Therefore the occurrence of *M. cryptica* n. sp. and *M. ramilijaonae* n. sp. and two further lineages of unclarified status, in allopatric *G. liber* populations, raises

questions about the modes of polystome diversification. Microendemic species are characteristic of Madagascar biodiversity [54], and this also applies to many mantellids which sometimes are only known from a single site [15,51]. *G. liber* is one of the two *Guibemantis* species with the widest distribution in Madagascar. It shows a strong polymorphism in coloration and bioacoustic signals [14] and high levels of mitochondrial divergence have been observed in the species (see [87] and Vences, unpublished data). The distribution area of this species is continuous, nuclear genes are not separated into clearly distinct clusters, and admixture among haplotype lineages occurs at some sites (Vences, unpublished data), indicating a situation that at present is best reflected by accepting a single species *G. liber* which however in the future might be partitioned into several species as new data become available. Polystomes are usually characterized by a free swimming larval stage to complete their development [34]. The absence of free swimming larvae in the reproductive cycle of *M. biritika* [48] implies physical contact between hosts for parasite dissemination. Thus, polystomes depend entirely on host ecology for reproduction, dispersal and ultimately evolution. A comparison of the phylogeographic and phylogenetic patterns of *G. liber* and the *Madapolystoma* parasites infecting it might thus be informative to better understand the diversification mechanisms in these animals.

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