International Journal for Parasitology: Parasites and Wildlife 14 (2021) 161–178

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

First record of *Metapolystoma* (Monogenea: Polystomatidae) from *Boophis* tree frogs in Madagascar, with the description of five new species

Willem Landman [a,*], Olivier Verneau [a,b,c], Liliane Raharivololoniaina [d], Louis du Preez [a,e]

[a] Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, Private Bag X 6001, Potchefstroom, 2520, South Africa
[b] University of Perpignan Via Domitia Centre de Formation et de Recherche sur les Environnements Méditerranéens, UMR 5110, F-66860, Perpignan, France
[c] CNRS, Centre de Formation et de Recherche sur les Environnements Méditerranéens, UMR 5110, F-66860, Perpignan, France
[d] Department of Animal Biology, University of Antananarivo, BP 566, Antananarivo 101, Madagascar
[e] South African Institute for Aquatic Biodiversity, Private Bag 1015, Makhanda, 6140, South Africa

ARTICLE INFO

Keywords:
Boophis
Madagascar
Metapolystoma
Monogenea
Polystomes

ABSTRACT

Although Madagascar has more than 350 frog species of which all but two are endemic to the island, the known polystome (Monogenea: Polystomatidae) diversity parasitizing Malagasy frogs is low, encompassing five species of *Madapolystoma*, one species of *Kankana* and one *Metapolystoma*. Investigating the parasite diversity of frog parasites at selected Malagasy localities led to the discovery of undescribed polystomes. Five treefrogs, *Boophis albilabris*, *Boophis doulioti*, *Boophis luteus*, *Boophis madagascariensis* and *Boophis occidentalis* were found to be infected and are reported here as hosts for new *Metapolystoma* species. Morphological investigation, combining examination of body length, haptor length, genital bulb width, genital crown diameter, genital spine number, genital spine length, ovary length, egg length, hamulus length, hamulus guard length and hamulus hook length, revealed five distinct morphotypes. Phylogenetic analysis and genetic divergences obtained for three of the five morphotypes, support the distinction of new species. *Metapolystoma ansuanum* n. sp. is described from *B. luteus*, *Metapolystoma falcatum* n. sp. from *B. doulioti*, *Metapolystoma multiova* n. sp. from *B. occidentalis*, *Metapolystoma theroni* n. sp. from *B. madagascareniensis* and *Metapolystoma vencesi* n. sp. from *B. albilabris*. Finally, although the validity of *Metapolystoma* as taxon is not fully resolved yet, the phylogenetic position of the described species and their morphology provide clear evidence for new metapolystome taxa.

1. Introduction

Madagascar is well known for its high species diversity and endemicity, particularly in the cases of plants and vertebrates (Myers et al., 2000). When it comes to amphibian diversity, Madagascar is globally ranked in the top twelve (Andreone et al., 2008). Its unique herpetofauna serve as hosts for an equally unique and diverse assemblage of parasites (see Wohltmann et al., 2007; Junker et al., 2010; Rocha et al., 2012; Kuzmin et al., 2013; Landman et al., 2018). However, little is known about Madagascar's anuran polystome flatworm diversity, since only 86 of the 356 known frog species (Frost, 2020) have been screened for polystomes at a few accessible localities in the past (Verneau et al., 2009). Malagasy polystomes are currently represented by four genera encompassing a single chelonian and seven anuran species, all of these were found in the urinary bladder of their host. *Uropolystomoides chabaudi* (Euzet and Combes, 1965) from *Pelomedusa subrufa* (Bonnaterre, 1789) is the only polystome known from turtles. *Metapolystoma brygoonis* (Euzet and Combes, 1964) was the first polystome found within a Malagasy anuran host, as described from *Ptychadena mascareniensis* (Duméril and Bibron, 1841). Besides these two species, *Kankana manampoka* Raharivololoniaina et al. 2011 was described from *Cophyla pollicaris* (Boulenger, 1888). Five other species of *Madapolystoma* were reported from mantellids, namely *Madapolystoma biritika* Du Preez et al. (2010) from *Mantella madagascariensis* (Grandidier, 1872), *M. isaloensis* Landman et al. (2018) from *Mantella expectata* Busse and Böhme, 1922 and *M. magnahami* Landman et al. (2018) from *Blommersia domerguei* Guibé, 1974. *Madapolystoma cryptica* Berthier et al. 2014 and *M. ramilijaonae* Berthier et al. 2014 were conversely described from the same host species *Guibemantis liber* (Peracca, 1893).

Metapolystoma brygoonis was initially described as *Polystoma* and later elevated (Combes, 1976). This separation of *Metapolystoma* and *Polystoma* was based on morphological characters, including the large

* Corresponding author. Name
E-mail address: Willie.Landman@nwu.ac.za (W. Landman).

https://doi.org/10.1016/j.ijppaw.2021.01.012
Received 6 December 2020; Received in revised form 26 January 2021; Accepted 26 January 2021
Available online 17 February 2021
2213-2244/© 2021 The Author(s). Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Table 1
Frog species under survey with their site of collection in Madagascar.

Frog species	No. of specimens collected	Locality
B. madagascariensis	30	Andasibe (Indri Reserve)
B. albilabris	6	Ambatolahy and Andringita massif
B. doulioti	22 frogs, 10 tadpoles	Ankarafantsika
B. luteus	9	Isalo (Cascade des Nymphes Special Reserve)
B. occidentalis	5	Isalo (Cascade des Nymphes Special Reserve)

extended uterus containing many eggs that fills the largest part of the body and the posterior position of the large ovary (Combes, 1976). The validity of *Metapolystoma* as a genus has however been under dispute ever since its nested position within *Polystoma* was demonstrated at the molecular level (Bentz et al., 2001; Verneau et al., 2002; Olson and Tkach, 2005). Up to the present, the three known *Metapolystoma* species have been described from grass frogs belonging to *Ptychadena*: *M. brygoonis* from *P. mascareniensis* in Madagascar, *M. cachani* (Gallien, 1956) from *Ptychadena longirostris* (Peters, 1870) in Ivory Coast and *Metapolystoma porosissimae* du Preez and Kok 1992, from *Ptychadena porosissima* (Steindachner, 1867) in South Africa. It was therefore likely that many Madagascan polystomes awaited discovery (Landman et al., 2018).

Among the 27 anuran genera found in Madagascar (Frost, 2020), *Boophis* Tschudi, 1838 is endemic to Madagascar and the Mayotte Island in the Comoros (Frost, 2020; Glaw and Vences, 2007), hence totalling 79 currently recognised species (Frost, 2020) representing a diverse and species-rich group within the Mantellidae Günter, 1859. During herpetological surveys conducted in Madagascar in 2005, 2006 and 2007, several species of *Boophis* from different localities were examined for polystome parasites. *Boophis albilabris* (Boulenger, 1888), *Boophis doulioti* (Angel, 1934), *Boophis luteus* (Boulenger, 1882), *Boophis madagascariensis* (Peters, 1874) and *Boophis occidentalis* Glaw & Vences, 1994 were found to be infected with five distinct, unknown *Metapolystoma* species. Since the collection of additional materials was constrained by permit restrictions and administrative difficulties in Madagascar, and because preliminary molecular and morphological data converged towards the same result, our objective was to describe new parasites collected from distinct frog species despite their small sample size.

2. Material and methods

2.1. Host and parasite sampling

Annually during February–March of 2005, 2006 and 2007, several *Boophis* species were collected in Madagascar (Table 1). Following collection by hand at night, frogs were individually kept overnight in clear plastic bags containing 50 ml of tap water. After 24 h, the water in which the frogs were kept was poured through two plankton sieves with mesh sizes of 500 and 100 μm respectively. The 500 μm sieve retained course debris and most of the faeces, while the 100 μm sieve retained parasite eggs and fine debris. The content of the 100 μm sieve was then washed into a Petri dish and inspected for the presence of polystome eggs using a dissecting microscope. Infected frogs were euthanized with MS222 (Ethyl-3-aminobenzoate methanesulfonate) and dissected for parasite investigation using a Nikon SMZ-645 dissecting microscope. Where no eggs were observed, two representatives per species per locality were dissected to check for the presence of non egg-producing polystomes. The kidneys, urinary bladder and accessory bladders were removed and inspected in a Petri dish containing 0.6% Ringers solution. For the purpose of molecular studies, some juvenile polystomes were preserved in absolute ethanol. The remainder of the juvenile polystomes were mounted in ammonium picrate glycerine or preserved in 10% NBF. Adult parasites were fixed in 10% NBF under coverslip pressure.

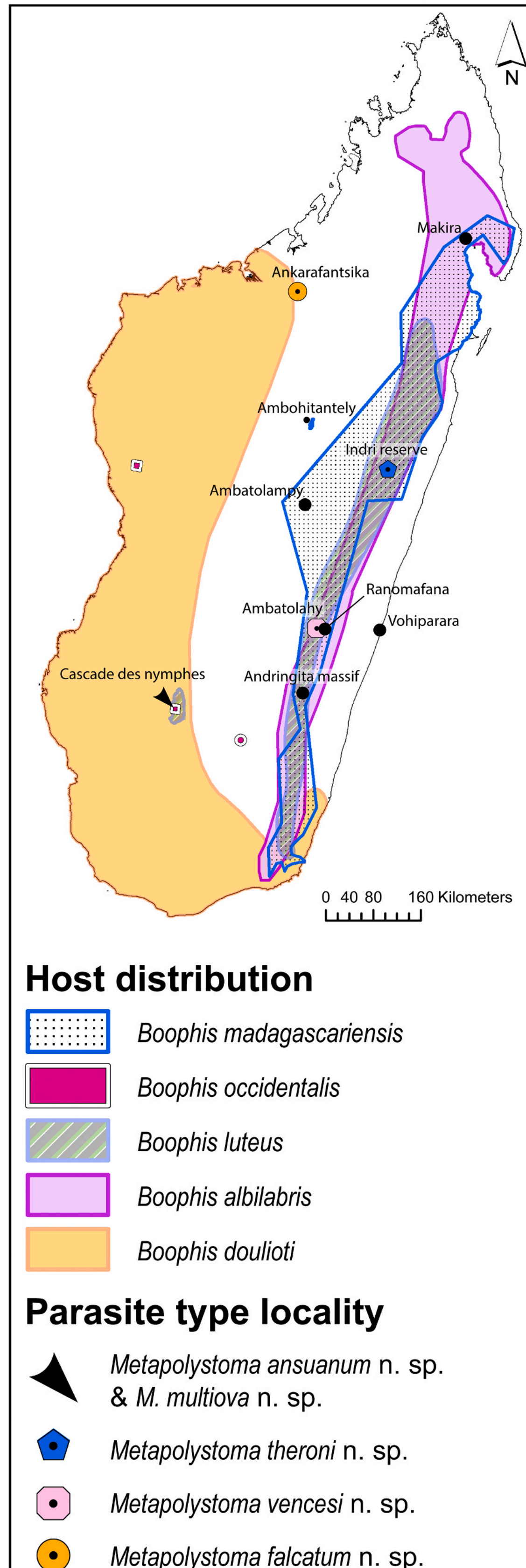

Fig. 1. Map of Madagascar showing parasite type localities and distribution areas of hosts.

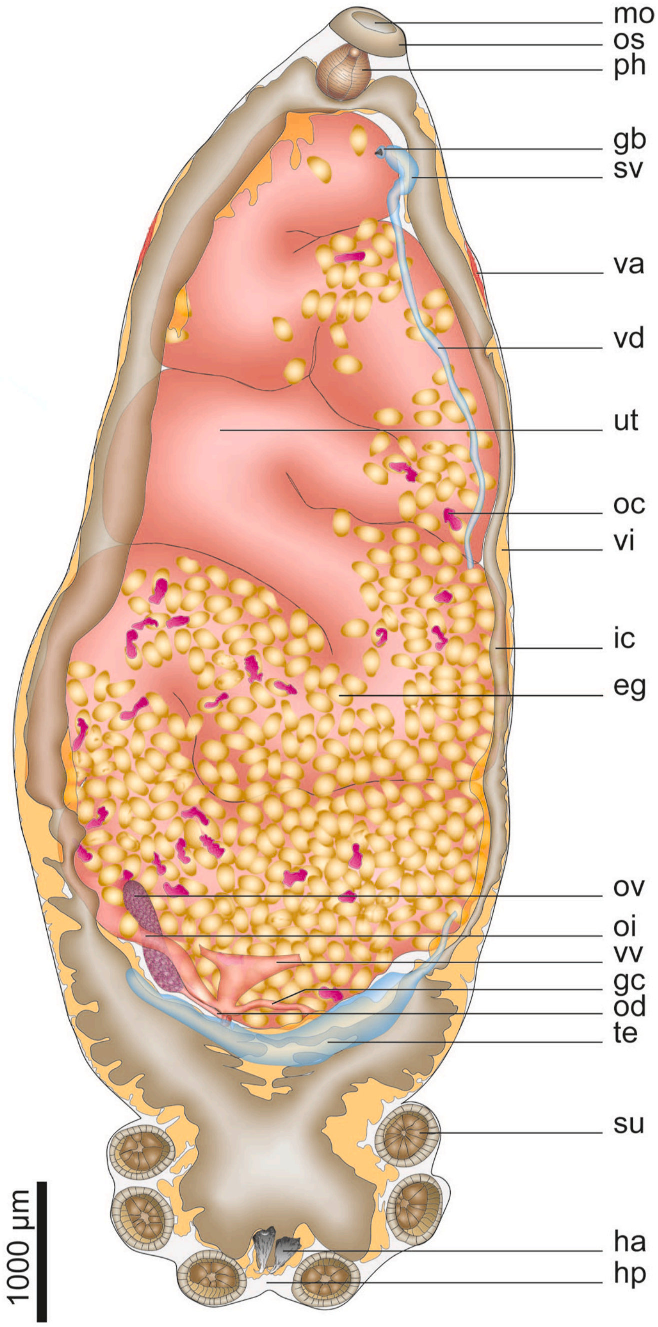

Fig. 2. Ventral view of *Metapolystoma vencesi* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vd, vas deferens; vi, vitelline; vv, vitello–vaginal canal.

Specimens were rinsed in water, stained with acetocarmine, dehydrated, cleared in xylene and mounted in Canada balsam. Five additional, fixed specimens of *B. albilabris* collected in 1972 by Mr. Blane at Andringita Massif, Madagascar, and archived at the Muséum National d'Histoire Naturelle of Paris, were dissected and parasites retrieved and processed as explained above. Host and parasite distribution maps (Fig. 1) were drawn in ArcMap 10.5.1 (Esri, California), and shape files for frog distributions were provided by the IUCN (IUCN, 2016a-e).

2.2. DNA extraction and amplification

All polystome DNA samples used in this study were sourced from Verneau et al. (2009) and Berthier (2011). According to these authors, DNA extractions were conducted in 100–150 μl of 10% Chelex 100 sodium (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) with the proteinase K 1 mg·mL−1 final concentration at 55 °C for the duration of 1 to 1,5 h. Enzymatic reactions were stopped at 100 °C for 15 min and DNA were stored at −20 °C until use. Amplifications of 18S, 28S and COI were conducted following the procedure described in Héritier et al. (2015), regardless of the gene of interest or primers used. The complete 18S rRNA gene and the partial 28S rRNA gene were amplified in two rounds with the respective combinations of primers F18/18Rg and 18F3/IR5 for 18S and LSU5'/IR16 and IF15/LSU3′ for 28S. Primer sequences are reported in Sinnappah et al. (2001), Verneau et al. (2009) and Héritier et al. (2015). COI was amplified using the combination of primers L-CO1p/H-Cox1p2 (Littlewood et al., 1997). All PCR products were sent to the Genoscreen Company (Lille, France) for purification and sequencing with their respective forward and reverse PCR primers. Sequences were read and edited with the software Geneious (Saint Joseph, Missouri, USA) to check chromatograms before use for phylogenetic and distance analyses.

2.3. Phylogenetic and distance analyses

All *Metapolystoma* sequences were edited and aligned independently using Clustal W implemented in MEGA version 7 (Kumar et al., 2016) under default parameters (Thompson et al., 1994). The alignment included five African *Polystoma* species and a single European *Polystoma* species, that is, *Polystoma integerrimum* (Frölich, 1791), which was used as an outgroup according to Bentz et al. (2001). They were subsequently concatenated in a single alignment for Bayesian analysis. The two ribosomal genes were treated as two separate partitions, and the COI genes as three distinct partitions according to their codon position. A two substitution rates model with a proportion of invariable characters was selected for the 18S partition, whereas a GTR + I model was selected for the 28S partition following the Akaike Information Criterion (AIC) implemented in Modeltest 3.06 (Posada and Crandall, 1998). Concerning COI, six types of substitutions and gamma rates each comprising four gamma rate categories were applied to the first and second positions, whereas six types of substitutions and invariable-gamma rates each comprising four gamma rate categories were applied to the third position. Evolutionary parameters were estimated independently for all five partitions. The Bayesian analysis was run using MrBayes 3.04b (Huelsenbeck and Ronquist, 2001), employing four chains running for ten million generations and sampled every 100 cycles. The Bayesian consensus tree was subsequently drawn after removing the first 10 000 trees (10%) as the burn-in phase, and viewed with TreeView version 1.6 (Page, 1996). Corrected pairwise distances were calculated independently for partial 18S, 28S and COI sequences using the Kimura 2-parameter model and 1000 bootstrap replicates in MEGA version 7 (Kimura, 1980).

2.4. Morphology and morphometry

Parasites were examined and photographed using a Zeiss Imager Axio10 compound microscope (Zeiss, Germany) fitted with a Zeiss Axio cam 305 camera (Zeiss, Germany). Morphological structures and organs were measured in micrometres using the Zeiss Zen Blue elements (Zeiss, Germany) software program. Hamuli were respectively measured from the apex behind the hook to the tip of the guard (Length X) and to the handle (Length Y), and the hook from tip to base (Length Z). Full-body images of type specimens were taken using a Nikon AZ100M microscope (Nikon, Netherlands) fitted with a low powered 1× objective. Illustrations were done in Adobe Illustrator CC (Adobe, California). Additional taxonomic measurements for *M. porosissimae* were taken from the type

Table 2
Body measurements in micrometres for all *Metapolystoma* spp. values.

Measurements	*Metapolystoma vencesi* n. sp.	*Metapolystoma falcatum* n. sp.	*Metapolystoma ansuanum* n. sp.	*Metapolystoma theroni* n. sp.	*Metapolystoma multiova* n. sp.
Number of mature specimens	6	1	2	1	2
Total length	9190	7871	2680–3952	9086	4915–5537
Greatest width	3496	3272	1035–1436	3208	2257–2419
Width at vagina	2315	1783	804–1001	2011	1469–2086
Haptor length – Body length ratio	0.16	0.19	0.23–0.25	0.18	0.18
Haptor length	1437	1492	669–912	1634	916
Haptor width	2344	2636	1166–1385	2572	1478
Hamulus length X	269–332 (299 ± 32; 3)	393–407	205	392–408	196–248 (215 ± 29; 3)
Hamulus length Y	225–302 (270 ± 40; 3)	303–314	179	252–257	121–174 (153 ± 23; 4)
Hamulus hook length Z	40–52 (47)	66–70	62	50–55	58.2
Oral disk	391	297	307–329	486	248–271
Pharynx length	302–420	327	256–308	401	302–405
Pharynx width	291–390	290	183–222	385	289–320
Genital bulb diameter	115	86	64–73	65	86
Ovary length	861	772	265–338	479	446
Ovary width	194	196	98–144	385	158
Number of genital spines	8	8	10	7	6
Genital spine length	31.5–33.4 (32.5 ± 0.6; 9)	24.3–31.6 (27.9 ± 1.78; 18)	24.5–28.5 (26.19 ± 1.16; 5)	28.9–34.9 (32.9 ± 4; 3)	40.4–43.7 (42 ± 1.4; 5)
Genital crown diameter	31.8	25.7	29.4–32.8	24.2–27.6 (25.8 ± 1; 10)	24.5
Sucker diameter	366–452(424 ± 37; 12)	435–498(468 ± 29; 6)	241–305(273 ± 25; 12)	404–424 (416 ± 8; 6)	329–356 (339 ± 10; 9)
Maximum number of eggs in utero	367	11	41	176	499+
Egg length	210–230 (219 ± 7; 16)	161–185 (171 ± 8; 11)	196–217 (205 ± 8; 8)	239–265(250 ± 7; 12)	198–228 (212 ± 9; 23)
Egg width	120–147 (130 ± 8; 16)	108–123 (117 ± 5; 11)	119–137 (127 ± 7; 8)	136–152(143 ± 5; 12)	144–165 (154 ± 7; 23)
Marginal hooklet 1 length	29.7–34.2 (32.5 ± 1.3; 13)	32.6–39.7 (36.9 ± 2.3; 13)		34.3–39.4 (936.2 ± 1.4; 21)	28.6–38.7 (33.8 ± 3.3; 11)
Marginal hooklet 2–7 length	20.8–25 (23.6 ± 1; 30)	21.8–27 (23.8 ± 1.52; 30)	22.3–24.79 23.5 ± 1.17; 4)	22.6–27.2 (24.8 ± 1.1; 44)	21.3–26.9 (24.7 ± 2; 17)
Marginal hooklet 8 length	27.4–32.4 (30.5 ± 1.6; 12)	28.8–33.5 (31.3 ± 1.5; 14)		30.5–34.1 (32.2 ± 1; 19)	28.4–36.3 (32.4 ± 2.8; 5)
Widest section from front	57%	66%	16–65%	51%	50%–57%
Times longer than wide	2.7	2.4	2.6–2.8	2.6	2.2–2.3

Measurement	*Metapolystoma porosissimae*[a]	*Metapolystoma brygoonis*[b]	*Metapolystoma cachani*[c]		
Number of mature specimens	15	10	10	3	57
Total length	4150–6710 (5870 ± 0.6; 10)	3400–4800 (4200)	4000	3880–5628 (4594)	3.680–7.250 (5.070)
Greatest width	1570–2360 (2040 ± 0.2; 11)	1220–1500 (1340)	1500	1120–2880 (1793)	1.160–2.880 (1.980)
Width at vagina	1.120–1.700 (1.460 ± 0.2; 11)	650–1030 (800)			880–1.760 (1.220)
Haptor length – Body length ratio	0.26–0.33 (0.29 ± 0.1; 10)	0.22–0.23 (0.22)	0.25	0.17–0.21 (0.19)	0.15–0.32 (0.23)
Haptor length	1.540–1.800 (1.670 ± 01; 12)	760–1070 (900)	1000	720–1072 (864)	910–1.500 (1.15)
Haptor width	2.010–2.660 (2.360 ± 0.2; 12)	1220–1760 (1530)	1500	1248–2800 (1858)	1.300–2.350 (1.730)
Hamulus length X	432–472 (447 ± 14.3; 26)	330–420 (380)	310	280–450 (348)	330–520 (430)
Hamulus length Y	**332.4–385.7 (361.2 ± 18.52; 13)**				
Hamulus hook length Z	**72–86.6 (78.3 ± 3.97; 15)**				
Oral disk	461–566 (521)	220–340 (270)			
Pharynx length	267–348 (310 ± 31.8; 10)				
Pharynx width	234–298 (262 ± 20.4; 10)				
Genital bulb diameter	**93–129.5 (105.7 ± 11.91; 7)**	80	25		
Ovary length	546–755 (651 ± 89.1; 11)	410–460 (440)			
Ovary width	**266**	90–160 (120)			
Number of genital spines	9–10	8	8		
Genital spine length	31/**21.83–25.97 (24.144 ± 1.46; 6)**	30–32 (31)			
Genital crown diameter	**25.18–26.96 (26.35 ± 0.57; 7)**				
Sucker diameter	461–566 (521 ± 30; 65)	260–420 (340)	350–400	272–580 × 288–544 (386 × 379)	300–500 (420)
Maximum number of eggs in utero	250	100–140	70–100	4	8–390 (170)
Egg length	201–229 (214 ± 7.2; 30) **192–207 (197 ± 5.1; 10)**	160–200 (180)	190	176–208 (200)	122–159 (0.138)
Egg width	113–134 (128 ± 6.6; 30) **100–131 (115 ± 9.3; 10)**	90–120 (100)	120	96–112 (101)	
Marginal hooklet 1	35				
Marginal hooklet 2–7	22				
Marginal hooklet 8	33				
Marginal hooklet 1–2		31 – 34 (33)	30		
Marginal hooklet 6–8		31			

Values: (Min – Max (Mean ±Ơ; N)), numbers in bold indicate additional measurements taken from species described prior to the present study.
[a] From Du Preez and Kok (1992a).
[b] From Euzet and Combes (1964).
[c] From Gallien (1956) (left), Kulo (1981) (middle) and Murith (1981) (right).

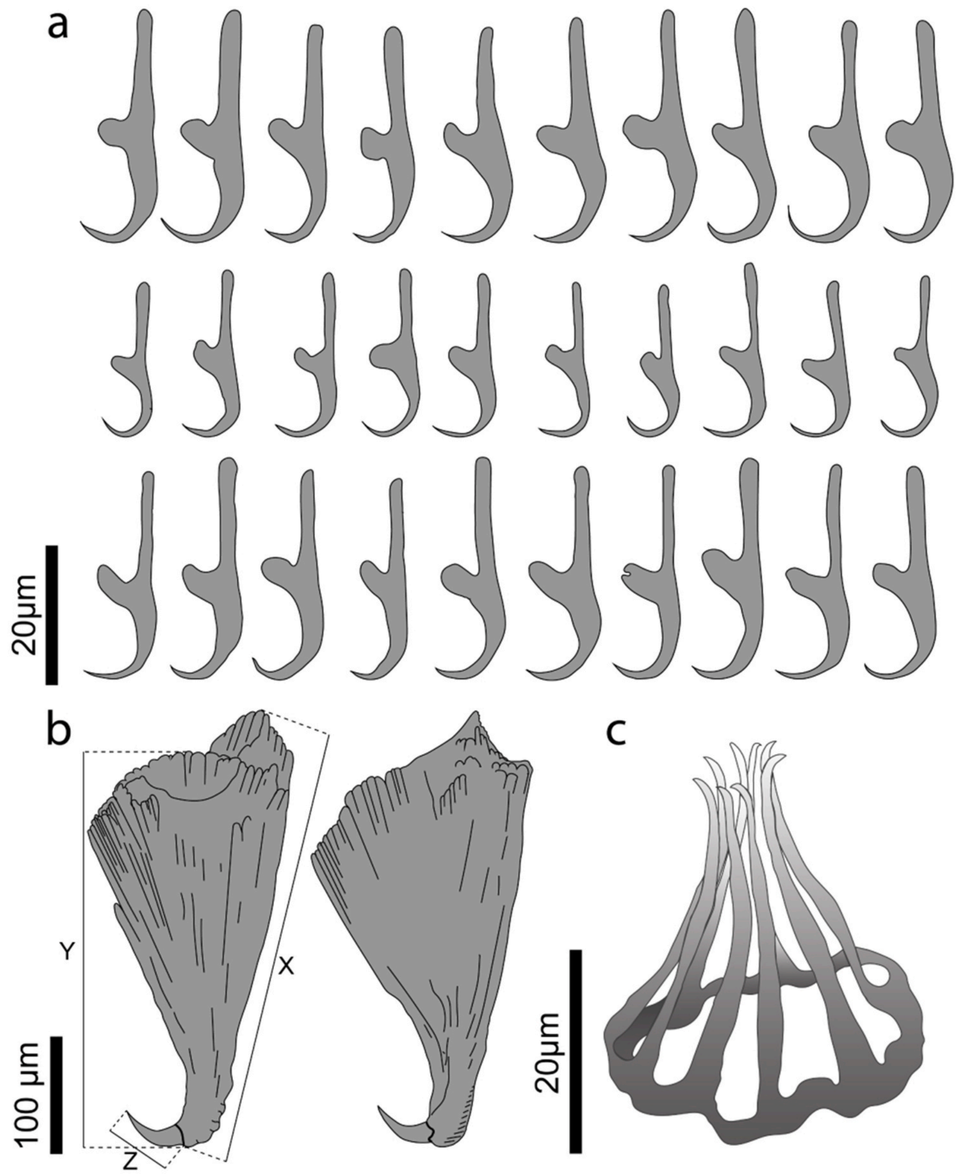

Fig. 3. *Metapolystoma vencesi* n. sp. from *Boophis doulioti.* **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c**, genital crown from holotype. Abbreviations: X, outer length; Y, inner length; Z, hook length.

series (Table 2).

3. Results

3.1. Taxonomic summary

Class: Monogenea van Beneden. 1858 Order: Polystomatidea Lebedev. 1988 Family: Polystomatidae Gamble. 1896 Genus: *Metapolystoma* Combes, 1976.

3.1.1. Metapolystoma vencesi n. sp. (Fig. 2–3; Table 2)

3.1.1.1. Type host. *Boophis albilabris* (Mantellidae)

3.1.1.2. Type locality. Ambatolahy, Madagascar (Fig. 1), (−21.2438667S; 47.4262167E).

3.1.1.3. Site in host. Urinary bladder.

3.1.1.4. Level of infection. One of six frogs examined from Ambatolahy was infected with one mature and four juvenile parasites (prevalence 16.6%). One of four frogs examined from Ambohitantely was infected with seven mature parasites (prevalence 25%). The prevalence for the combined sample was 20% and the mean intensity 6.5.

3.1.1.5. Type material. The morphological description is based on six mature, ten oncomiracidia and four juvenile parasites. Three specimens were sexually mature (Holotype NMBP 578; Paratypes NMBP 579–580) and two immature (Paratypes NMBP 581–582). The holotype and paratypes NMBP 581 and NMBP 582 originated from Ambatolahy, Madagascar, while paratypes NMBP 579 and NMBP 580 originated from Andringita Massif, Madagascar. Juvenile parasites and oncomiracidia were used for marginal hook measurements and drawings. The type material was deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301, South Africa.

3.1.1.6. Voucher material. Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

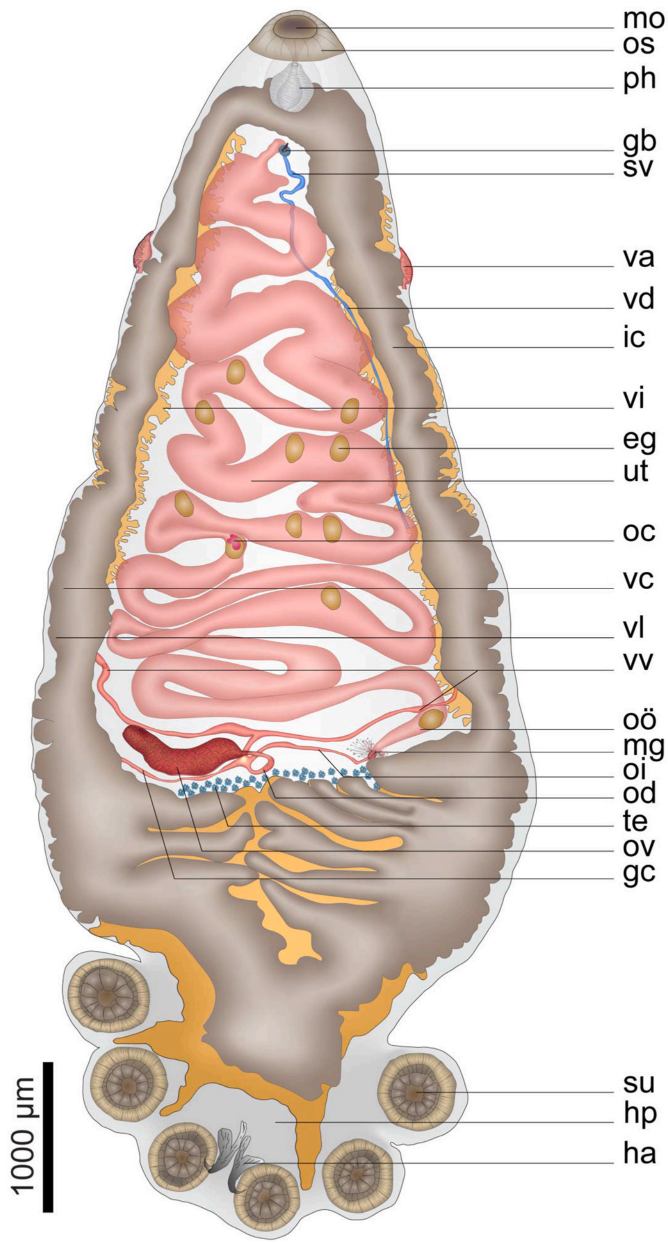

Fig. 4. Ventral view of *Metapolystoma falcatum* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vc, vaginal canal; vd, vas deferens; vi, vitelline; vl, vitelline duct; vv, vitello–vaginal canal.

3.1.1.7. Zoobank registration. The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48 EB-9EA4-881D43956065. The life science identifier (LSID) of the new name *Metapolystoma vencesi* n. sp. Landman et al. is: urn:lsid:zoobank.org:act:680E18A9-64D6-40C9-87D9-1AC5F28A1030.

3.1.1.8. Etymology. In recognition of Professor Miguel Vences, Technical University of Braunschweig, Germany, for his dedication to the study of Madagascan herpetofauna.

3.1.1.9. Description. Measurements reflected in Table 2. Body pyriform (Fig. 2), dorsoventrally flattened, widest section at 57% of total length from anterior end, body length 2.7 times greater than width. Mouth subventral, surrounded by false oral sucker. Posterior haptor 16% of body length, bearing three pairs of haptoral suckers of equal size. Marginal hooklets placed as for other polystomes: pairs one and two between hamuli, pairs three to five embedded in suckers, pairs six to eight found between anterior suckers, pairs one and eight larger than pairs two to seven (Fig. 3a). Well-developed hamuli between posterior-most haptoral suckers without cut between handle and guard (Fig. 3b). Medial pharynx length greater than width, positioned immediately posterior or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx, 8% of total length from most anterior point, converging posteriorly at position of 80% of total length from most anterior point, extending into haptor; no prehaptoral anastomoses. Lateral diverticula length equal to width, found only in last third of intestine. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, positioned in a narrow band posterior to the ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 20–32 (27 ± 5; 1) wide, 179 long, 2% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening on left ventral half, posterior to intestinal ceca bifurcation, 10% of total length from most anterior point. Genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing eight genital spines (Fig. 3c).

Ovary elongate, not lobed, positioned posterior to midbody, length 4.4 times greater than width, measuring 9% of body length. Oviduct 596 long, 29–53 (30 ± 7; 1) wide. Uterus massive and wide, occupying most of body proper, tubiform, convolute, containing 367 ovoid, operculate eggs; some with fully developed oncomiracidia. Hatched intrauterine oncomiracidia present. Mehlis' gland obscure. Two vaginae 180–420 long, 16–31 wide, on lateral margins, bearing multiple marginal openings, vaginal vestibule elongate, positioned at 18% from anterior. Vitellaria extended throughout most of body and haptor, surrounds female reproductive organs. Genito-intestinal canal prominent, 580 long, 40–133 (62 ± 28; 11) wide, situated posterior to ovary.

3.1.2. Metapolystoma falcatum n. sp. (Fig. 4-7; Table 2)

3.1.2.1. Type host. *Boophis doulioti* (Mantellidae).

3.1.2.2. Type locality. Ankarafantsika, Madagascar (Fig. 1), (16.115976S; 47.095631E).

3.1.2.3. Site in host. Gills of tadpoles and urinary bladder of mature frogs.

3.1.2.4. Level of infection. Three of 22 frogs collected were infected. One mature and 13 juvenile parasites were recovered, with a maximum of 12 recovered from a single host (prevalence 13.6%. mean intensity 4.7). Seven of 10 tadpoles collected were infected with a total of 13 neotenic parasites, while as many as three parasites were infecting a single host (prevalence 70%; mean intensity 1.85). Though the name neotenic for some polystomes can be confusing (see Badets and Verneau, 2009), it refers here to egg producing parasites that were recovered from the gills of tadpoles.

3.1.2.5. Type material. Morphological descriptions are based on one mature parasite, eight juveniles and seven neotenics. One sexually mature specimen (Holotype NMBP 560), four immature bladder parasites (Paratypes NMBP 561–564) and four neotenics (Paratypes NMBP 565–568), all from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.1.2.6. Voucher material. Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

3.1.2.7. Zoobank registration.

The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-

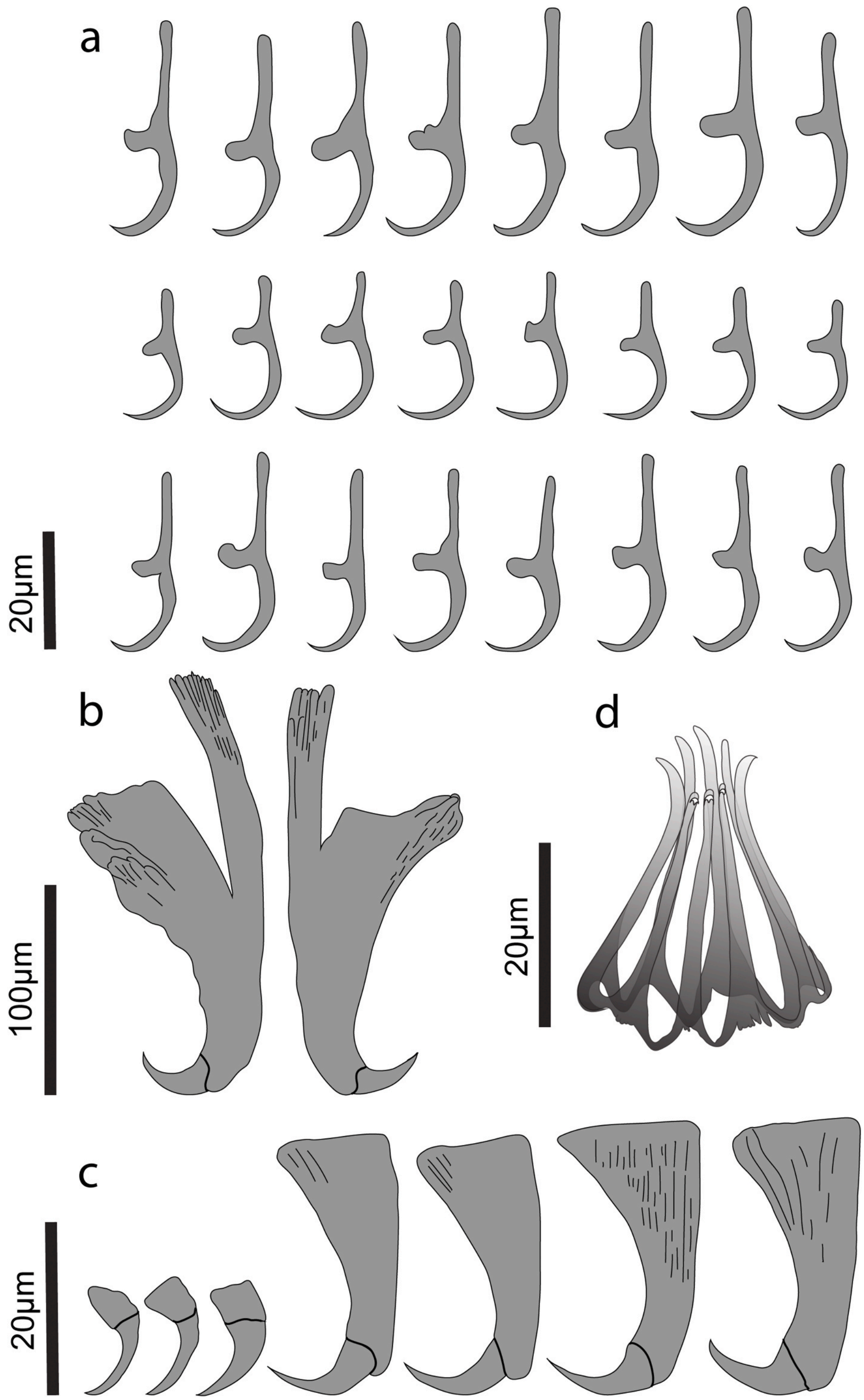

Fig. 5. *Metapolystoma falcatum* n. sp. from *Boophis doulioti*. **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c**, hamulus development; **d**, genital crown from holotype.

48 EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma falcatum* n. sp. Landman et al. is: urn:lsid:zoobank.org:act:FB1716E2-5C61–4C53-9A2F-BB364D6782A8.

3.1.2.8. Etymology. The species epithet refers to the exceptionally long curved tips of marginal hooklets two to seven (falcatum = sickle shaped, curved, hooked, armed with scythes).

3.1.2.9. Description. Mature parasite (Fig. 4–5).

Measurements reflected in Table 2. Body pyriform (Fig. 4), dorso-ventrally flat, widest section at 66% of total length from anterior end, body length 2.4 times greater than width. Mouth sub-ventral, surrounded by false oral sucker. Posterior haptor 19% of body length bearing three pairs of haptoral suckers equal in size. Marginal hooklets placed as for other polystomes and as described for *M. vencesi* n. sp., pairs one and eight larger than pairs two to seven (Fig. 5a). Well-

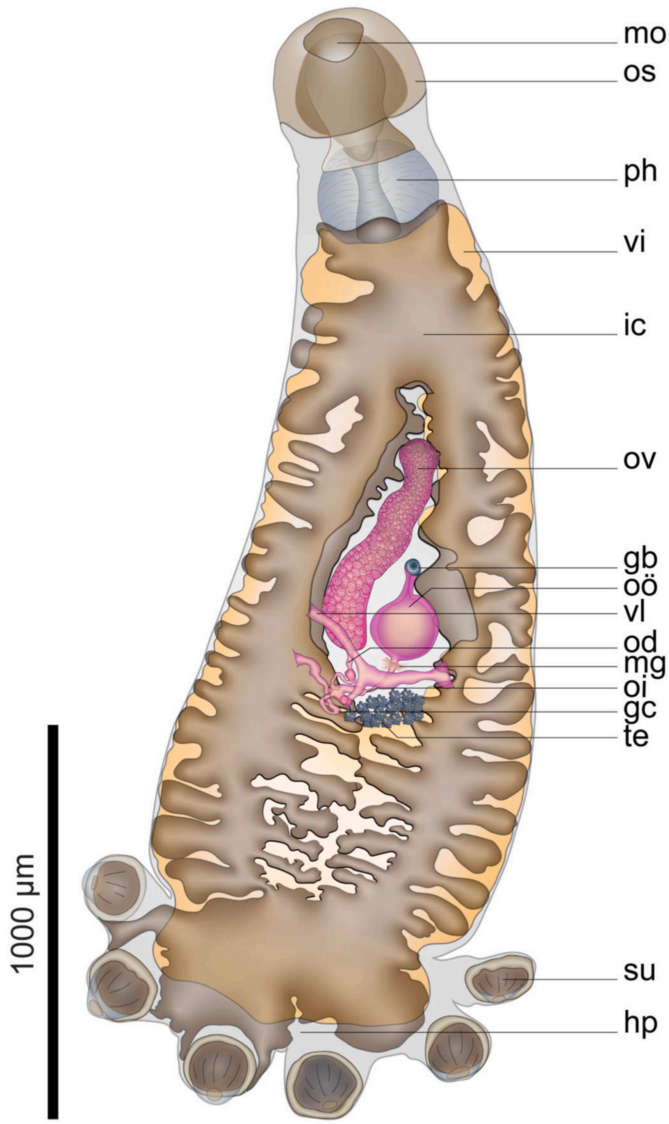

Fig. 6. Ventral view of *Metapolystoma falcatum* n. sp. from *Boophis doulioti*, neotenic form. Abbreviations: gb, genital bulb; gc, genito–intestinal canal; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; te, testis; ut, uterus; vi, vitelline; vl, vitelline duct; vv.

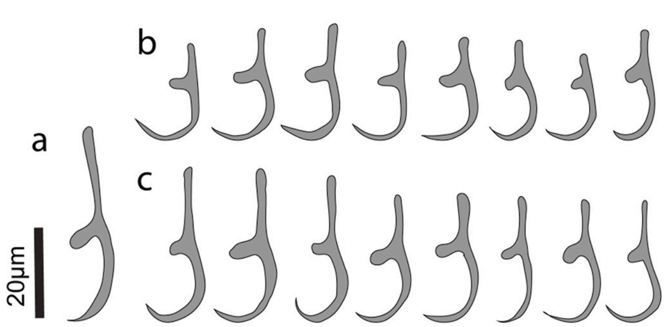

Fig. 7. *Metapolystoma falcatum* n. sp. from *Boophis doulioti*, neotenic form. **a**, marginal hooklets 2–7; **b,** marginal hooklets 8; **c**, marginal hooklet 1.

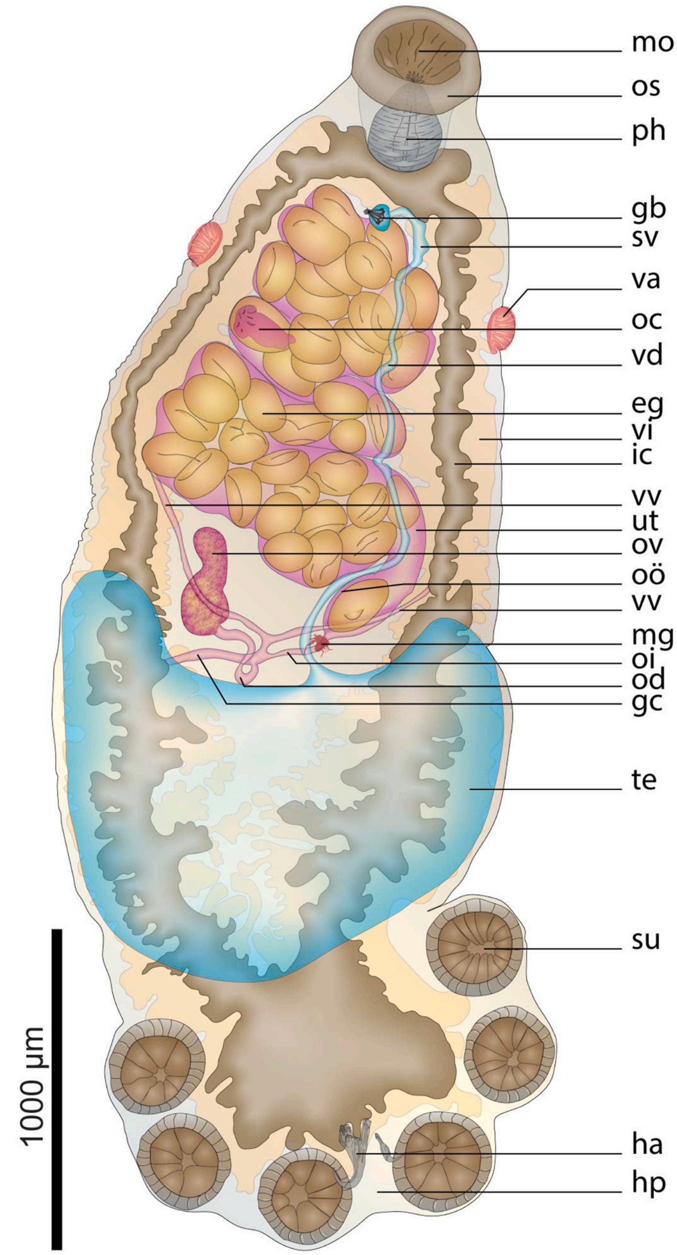

Fig. 8. Ventral view of *Metapolystoma ansuanum* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vd, vas deferens; vi, vitelline; vv, vitello–vaginal canal.

developed hamuli between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 5b). Hamuli development presented in Fig. 5c. Medial pharynx length greater than width, positioned immediately posterior to or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 11% from anterior, converging posteriorly at 82% from anterior, stretching in between haptoral suckers; no prehaptoral anastomoses. Intestine bears lateral diverticula, length equal to width. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, only a small section visible, positioned directly posterior to the ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 23–40 (30 ± 6; 1) wide, 301 long, measuring 4% of total length, narrowing towards genital bulb, opening

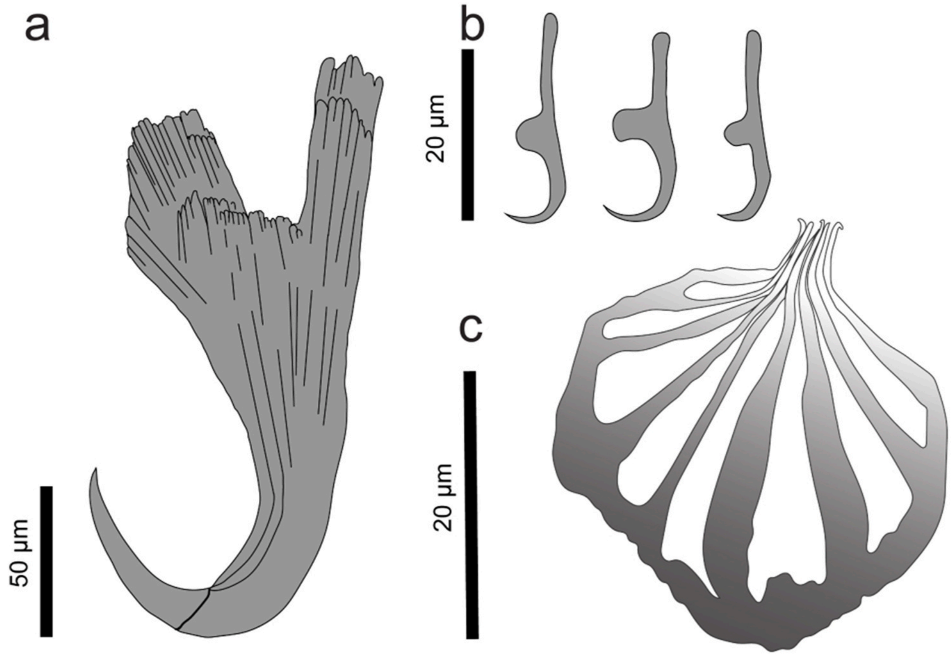

Fig. 9. *Metapolystoma ansuanum* n. sp. from *Boophis luteus*. **a**, hamulus from holotype; **b**, marginal hooklets 2–7; **c**, genital crown from holotype.

in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation at 12% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing eight genital spines (Fig. 5d).

Ovary elongated, not lobed, positioned posterior to midbody, length four times greater than width,10% of body length. Oviduct 411 long, 22–48 (30 7; 1) wide. Uterus massive and narrow, occupying most of the space between intestinal caeca, tubiform, serpentines between oötype and genital bulb. Eggs operculated. Holotype released 171 eggs with only 11 remaining in utero. Some eggs contain fully developed oncomiracidia. Mehlis' gland distinct. Two parallel vaginae 262–235 long, 102 wide, situated on lateral margins, bearing multiple marginal openings. Vaginal vestibule cup-shaped, 21% from anterior. Vitellaria dorsal to intestinal tract, extends throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal canal prominent, 943 long, 10–46 (23 ± 9; 1) wide, situated directly posterior to ovary.

Neotenic parasite (Fig. 6–7).

Measurements obtained from seven egg-producing neotenic parasites. Body pyriform (Fig. 6), dorsoventrally flat, ventrally concave, 1.419–3.307 (2.265 ± 667; 7) long. Maximum width 904–1.376 (1121 ± 368; 3). Body length 2–5 (3 ± 1; 6) times greater than width. Mouth 100–148 (124 ± 17; 5) in diameter, sub-ventral, surrounded by false oral sucker. Posterior haptor 313–526 (411 ± 89; 7) long, 541–1.166 (936 ± 305; 7) wide. Haptor length-body length ratio 0.13–0.24 (0.2 ± 0.04; 6), haptor bearing three pairs of haptoral suckers equal in size 103–226 (178 ± 32; 47). Hamuli absent. Marginal hooklets placed as for *M. vencesi* n. sp., pairs one 28.2 long (Fig. 7a), larger than pairs two to seven 22.6–27.7 (25.5 ± 1.5; 15), (Fig. 7b), pairs eight 27.5–35 (30.5 ± 3.155; 8) long (Fig. 7c). Medial pharynx length 199–312 (244 ± 45; 5) equal to width 207–312 (245 ± 41; 5), positioned immediately posterior or at the margin of false oral sucker. Intestine bifurcates at distance 27–30% (30% ± 2%; 3) from anterior, situated posterior to pharynx, converging posteriorly at 77–81% (80% ± 2.7%; 3) from anterior, stretching into area between haptoral suckers; no prehaptoral anastomosis. Intestine with lateral diverticula, length greater than width. Diverticula in posterior half longer than anterior. Medial diverticula only posterior to ovary, length greater than width, narrower than lateral diverticula.

Testis follicular, though only a small section was visible, positioned directly posterior to vitello-vaginal canal, ventral to intestine. Vas deferens not visible. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation, at distance 42–51% (46% ± 4%; 3) from anterior. Genital bulb diameter 31–51 (41 ± 9; 4), muscular, surrounded by glandular cells, armed with genital crown bearing eight to eleven genital spines 7.9–11.8 (9.8 ± 1; 10) long.

Ovary elongate, 256–580 (401 ± 120; 7) long, 67–117 (91 ± 19; 7) wide, not lobed, situated in middle of body, length three-six times greater than width, measuring 16–21% (17 ± 2.18%; 6) of body length. Oviduct 15 long, 6–28 (13 ± 6; 1) wide. Oötype 171–173 long, ovoid, containing a maximum of one ovoid, operculate egg 171–173 long, 132–142 wide. Uterus absent, eggs laid immediately after production. Mehlis' gland distinct. Vaginae absent. Vitellaria dorsal to intestinal tract, extended throughout most of body, except area occupied by female reproductive organs. Genito-intestinal canal prominent, 257 long, 7–47 (18 ± 8; 1) wide, situated posterior to ovary.

3.1.3. *Metapolystoma ansuanum n. sp.* (Fig. 8–9; Table 2)

3.1.3.1. *Type host.* *Boophis luteus* (Mantellidae).

3.1.3.2. *Type locality.* Cascade des Nymphes, Isalo National Park, Madagascar (Fig. 1), (22.46977S; 45.260701E).

3.1.3.3. *Site in host.* Urinary bladder.

3.1.3.4. *Level of infection.* One of nine frogs collected were infected with two mature parasites (prevalence 11.1%).

3.1.3.5. *Type material.* The morphological descriptions are based on two mature parasites (Holotype NMBP 569; Paratype NMBP 570) collected from the same locality in Cascade des Nymphes. The type material was deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.1.3.6. *Zoobank registration.* The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48 EB-9EA4-881D43956065. The LSID of the

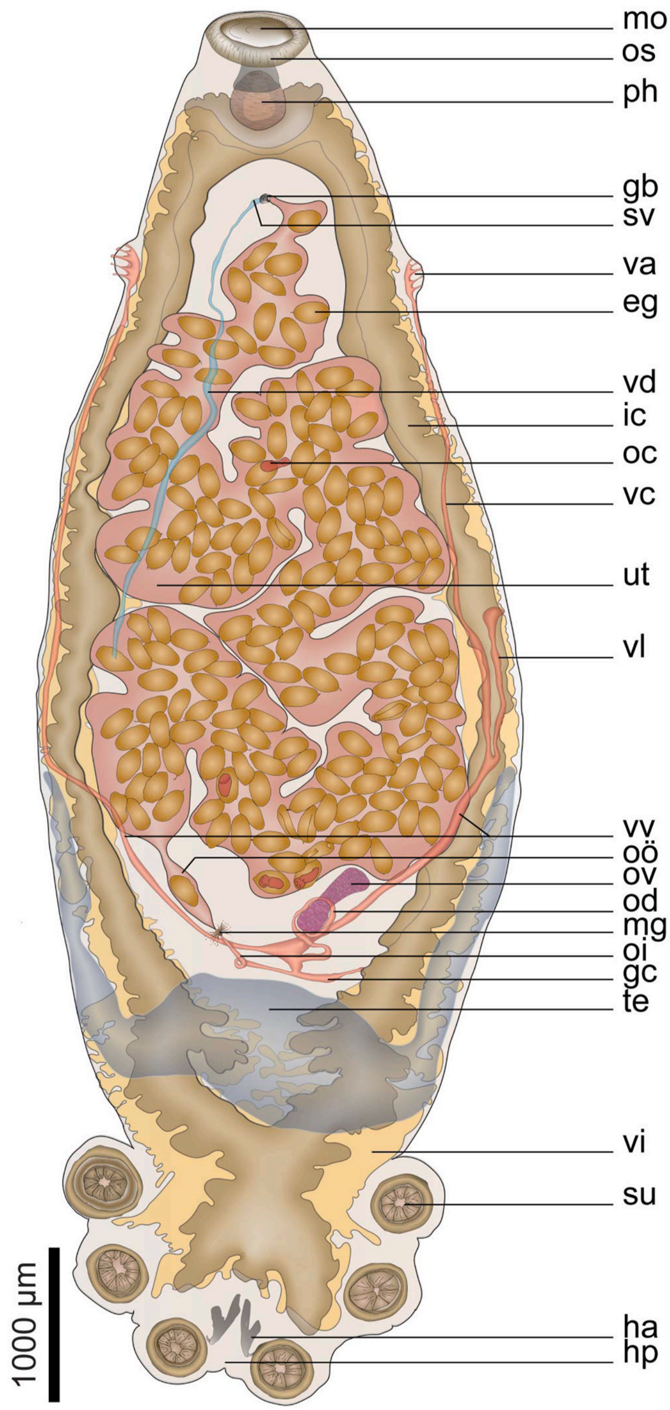

Fig. 10. Ventral view of *Metapolystoma theroni* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mg, Mehlis gland; mo, mouth; oc, oncomiracidium; od, oviduct; oi, oö–vitelline canal; oö, oötype; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; sv, semen vesicle; te, testis; ut, uterus; va, vagina; vc, vaginal canal; vd, vas deferens; vi, vitelline; vl, vitelline duct; vv, vitello–vaginal canal.

new name *Metapolystoma ansuanum* n. sp. Landman et al. is: urn:lsid: zoobank.org:act:4761BF6E-F309-4625-A7D7-CA64F3A3F6F8.

3.1.3.7. Etymology. This species is named for Mrs Anna-Susan van der Linde, known as Ansu, in acknowledgement of her teaching and inspiration of many secondary school pupils in the field of biology.

3.1.3.8. Description. Measurements reflected in Table 2. Body pyriform (Fig. 8), dorsoventrally flat, ventrally concave, widest section at position 16–65% from anterior end of body. Body length 2.6–2.8 times greater than width. Mouth sub-ventral, surrounded by false oral sucker. Posterior haptor measures 24% of total length, bearing three pairs of haptoral suckers of equal size. Well-developed hamuli positioned between posterior-most haptoral suckers with deep cut between handle and guard, hook length Z long relative to Length X (Fig. 9a). Marginal hooklets placed as for other polystomes: pairs one and two between hamuli, pairs three to five embedded in haptoral suckers, pairs six to eight positioned between anterior-most haptoral suckers (Fig. 9b). Medial pharynx length greater than width, positioned immediately posterior to or at margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 15–18% from anterior, converging posteriorly at 79–81% from anterior; no prehaptoral anastomoses. Lateral intestinal diverticula length equal to width in anterior half, length greater than width in posterior half. Medial diverticula only posterior to ovary.

Testis follicular, large, kidney-shaped, positioned posterior to ovary, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 17–31 (26 ± 4; 1) wide, 422 long, measuring 11% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation at position 16–18% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing 10 genital spines (Fig. 9c).

Ovary, elongate, not lobed, positioned posterior to midbody, length 2.3–2.7 times greater than width, measuring 9–10% of body length. Oviduct 345 long, 19–39 (28 ± 6; 1) wide. Uterus large, occupying one-third of body proper, tubiform, convolute. Uterus contains 41 ovoid, operculate eggs, some contain fully developed oncomiracidia. Intra-uterine oncomiracidia present. Mehlis' gland distinct. Two parallel vaginae, each 94–132 long (119 ± 18; 4), 66–81 (75 ± 8; 4) wide, found on lateral margins, bearing multiple marginal openings; vaginal vestibule cup-shaped at 21–26% from anterior. Vitellaria dorsal to intestinal tract, extending throughout most of body, except area occupied by female reproductive organs. Genito-intestinal canal prominent, 264 long, 30–40 (37 ± 4; 1) wide, situated directly posterior to ovary.

3.1.4. Metapolystoma theroni n. sp. (Fig. 10–11; Table 2)

3.1.4.1. Type host. *Boophis madagascariensis* (Mantellidae).

3.1.4.2. Type locality. Indri Reserve in Andasibe, Madagascar (Fig. 1), (18.930856S; 48.413611E).

3.1.4.3. Site in host. Urinary bladder.

3.1.4.4. Level of infection. Three of 30 frogs collected were infected with a total of one mature and 71 juvenile parasites, while as many as 40 parasites were infecting a single host (prevalence of 10%, mean intensity 24).

3.1.4.5. Type material. The morphological descriptions are based on one mature and 27 juvenile parasites. One sexually mature specimen (Holotype 573) and four immature ones (Paratypes 574–577), all from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.1.4.6. Voucher material. Remaining specimens in polystome collection, North-West University, Potchefstroom, South Africa.

3.1.4.7. Zoobank registration. The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48 EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma theroni* n. sp. Landman et al. is: urn:lsid:

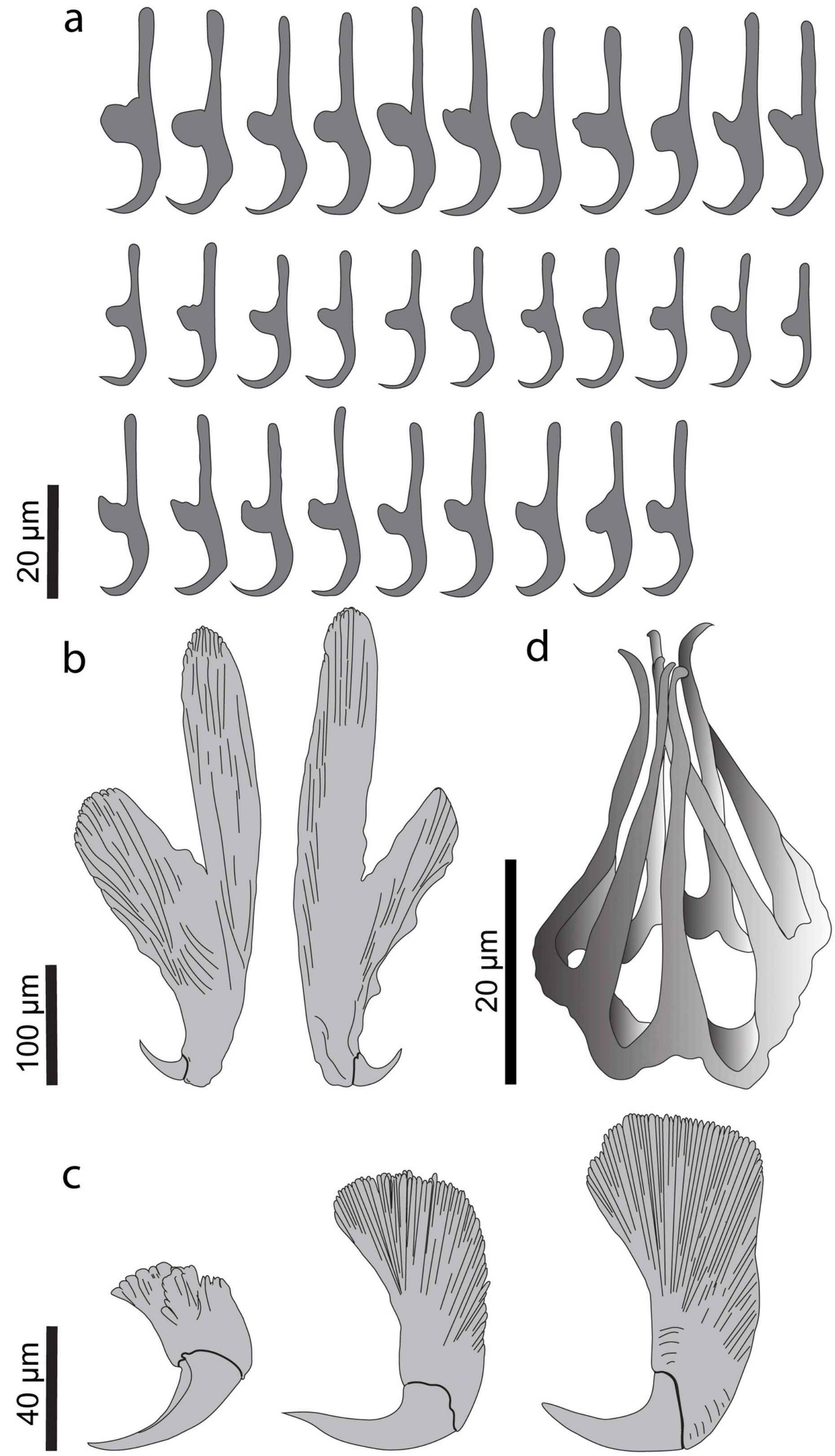

Fig. 11. *Metapolystoma theroni* n. sp. from *Boophis madagascariensis*. **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype; **c,** hamulus development; **d**, genital crown from holotype.

zoobank.org:act:55203AE0-217D-45D0-B3C8-07DDE53FA9A3.

3.1.4.8. Etymology. This species is named in honour of emeritus Professor Pieter Daniel Theron at the North-West University, South Africa, in recognition of 54 years of inspiring teaching and dedication to the field of zoology.

3.1.4.9. Description. Measurements reflected in Table 2. Body pyriform

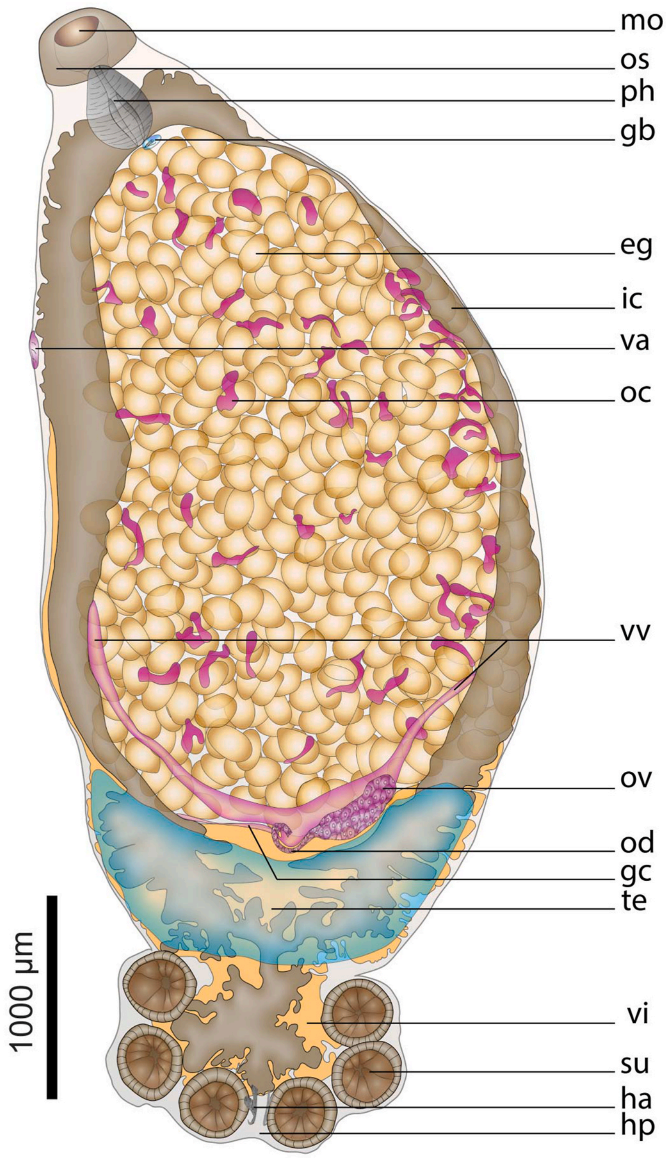

Fig. 12. Ventral view of *Metapolystoma multiova* n. sp. holotype. Abbreviations: eg, egg; gb, genital bulb; gc, genito–intestinal canal; ha, hamuli; hp, haptor; ic, intestinal caecum; mo, mouth; oc, oncomiracidium; od, oviduct; os, false oral sucker; ov, ovarium; ph, pharynx; su, sucker; te, testis; va, vagina; vi, vitelline; vv, vitello–vaginal canal.

(Fig. 10) dorsoventrally flat, widest section at 51% from anterior end, body length 2.6 times greater than width, mouth sub-ventral, surrounded by false oral sucker. Posterior haptor occupying 18% of total body length, bearing three pairs of haptoral suckers, equal in size. Marginal hooklets placed as for other polystomes, pairs one and two between hamuli, pairs three to five embedded in suckers, pairs six to eight in area between anterior-most suckers, pairs one and eight larger than pairs two to seven (Fig. 11a). Well-developed hamuli positioned between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 11b). Hamuli development presented in Fig. 11c. Medial pharynx length greater than width, positioned immediately posterior to or at margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 10% from anterior, converging posteriorly at 80% from anterior; no prehaptoral anastomoses. Lateral intestinal diverticula in first three quarters length equal to width, in last quarter length greater than width. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, u-shaped, mainly positioned posterior to the ovary with two lateral processes extending forward along the lateral line past the ovary up to one-third of the body proper, ventral to intestine. Vas deferens widens anteriorly to form sinuous semen vesicle 23–65 (46 ± 18; 1) wide, 122 long, measuring 1% of body length, narrowing towards genital bulb, opening in common genital opening. Genital pore opening mid-ventral, posterior to intestinal ceca bifurcation, positioned 13% from anterior, genital bulb muscular, surrounded by glandular cells, armed with genital crown bearing seven genital spines (Fig. 11d).

Ovary elongate, not lobed, positioned posterior to midbody, length 1.2 times greater than width, measuring 5% of body length. Oviduct 1167 long, 20–51 (33 ± 9; 1) wide. Uterus massive, occupying 50% of body proper, tubiform, serpentines between posterior connection at oötype and anterior connection at genital bulb, containing 176 ovoid, operculate eggs, some contain fully developed oncomiracidia. Hatched intrauterine oncomiracidia present. Mehlis' gland distinct. Two parallel vaginae 270–304 long, 132–177 wide, on lateral margins, with multiple marginal openings, vaginal vestibule cup-shaped at 18% from anterior. Vitellaria dorsal to intestinal tract, extended throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal canal prominent 457 long, 30–77 (52 ± 16; 1) wide, situated posterior to ovary.

3.1.5. Metapolystoma multiova n. sp. (Fig. 12–13; Table 2)

3.1.5.1. Type host. *Boophis occidentalis* (Mantellidae).

3.1.5.2. Type locality. Cascade des Nymphes, Isalo National Park, Madagascar (Fig. 1), (22.46977S; 45.260701E).

3.1.5.3. Site in host. Urinary bladder.

3.1.5.4. Level of infection. One of five frogs collected were infected with two adult parasites (prevalence 20%).

3.1.5.5. Type material. The morphological descriptions are based on two mature parasites. Two sexually mature specimens (Holotype 571, Paratype 572), both from the type locality, were deposited in the parasitic worm collection, National Museum, Aliwal Street, Bloemfontein 9301.

3.1.5.6. Zoobank registration. The Life Science Identifier (LSID) of the article is: 59F6A99A-C667-48 EB-9EA4-881D43956065. The LSID of the new name *Metapolystoma multiova* n. sp. Landman et al. is: urn:lsid:zoobank.org:act:6854087D-44B5–492B-9750-3647188F0F26.

3.1.5.7. Etymology. The species epithet is derived from Latin and related to the vast number of eggs (±500) carried by this species in contrast with other currently known species in the genus (multi = many + ova = eggs).

3.1.5.8. Description. Measurements reflected in Table 2. Body pyriform (Fig. 12), dorsoventrally flat, ventrally concave, widest section at 50–57% from anterior end of body. Body length 2.2–2.3 times greater than width. Mouth directed sub-ventrally, surrounded by false oral sucker. Posterior haptor measures 18% of total length bearing three pairs of haptoral suckers of equal size. Marginal hooklet pairs six to eight situated between anterior-most haptoral suckers, pairs one and eight larger than pairs two to seven, pairs one and two between hamuli, pairs three to five embedded in suckers (Fig. 13a). Well-developed hamuli situated between posterior-most haptoral suckers with deep cut between handle and guard (Fig. 13b). Medial pharynx length greater than width, positioned immediately posterior to or at the margin of false oral sucker. Intestine bifurcates immediately posterior to pharynx at 13% from anterior, converging posteriorly at 79–85% from anterior; no

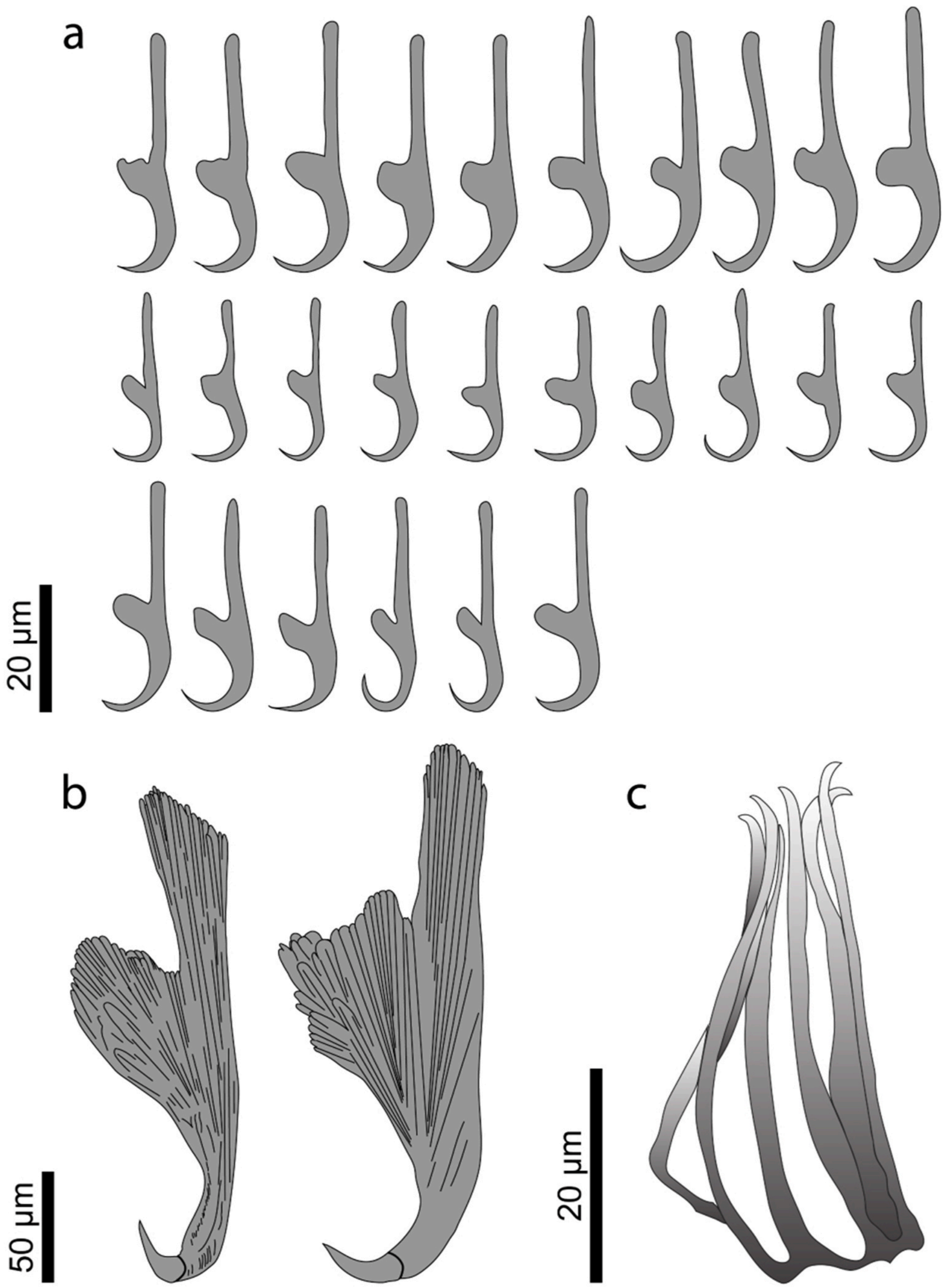

Fig. 13. *Metapolystoma multiova* n. sp. from *Boophis occidentalis*. **a**, marginal hooklets 1 (top), 2–7 (middle) and 8 (bottom) from holotype and paratypes; **b**, hamuli from holotype (left) and from paratype (right); **c**, genital crown from holotype.

prehaptoral anastomoses. Lateral intestinal diverticula situated in first third of intestine, where length is equal to width, absent in the second third while length is greater than width in the posterior third. Medial diverticula only posterior to ovary, length greater than width.

Testis follicular, sickle-shaped, posterior to ovary, ventral to intestine. Vas deferens obscured. Genital pore opening mid-ventrally situated on anterior margin of intestinal ceca bifurcation at 4% from anterior, genital bulb muscular, armed with genital crown bearing six genital spines (Fig. 13c).

Ovary elongate, not lobed, positioned posterior to midbody, length 2.8 times greater than width, measuring 8% of body length. Oviduct 988 long, 20–48 (31 ± 9; 1) wide. Uterus contains 336–499 ovoid, operculate eggs, some contain fully developed oncomiracidia, some hatched intrauterine oncomiracidia present. Mehlis' gland obscured. Two parallel vaginae, each 266–311 long, 58–62 wide, situated on lateral margins bearing multiple marginal openings. Vaginal vestibule cup-shaped, situated at 18% from anterior. Vitellaria dorsal to intestinal tract, extended throughout most of body and haptor, except areas occupied by female reproductive organs. Genito-intestinal cannel prominent 633 long, 7–103 (24 ± 24; 1) wide, situated at level of ovary.

3.2. Genetic divergences between Metapolystoma species and parasite phylogeny

Some of the 18S, 28S and COI sequences used in this study were retrieved from GenBank, while the others were obtained and submitted under accession numbers MW053457, MW053458 and MW054236 to MW054249 (Table 3). The final alignment, which had resulted in 3.977

Table 3
Polystome species investigated, host species, locality and GenBank accession numbers for 18S, 28S and COI.

Polystome species	Host species	Locality	18S Accession number	28S Accession number	COI Accession number
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ambatolampy	**FM897287**[a]	**FM897270**[a]	**FM897300**[a]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ambatolampy	**MW054243**[g]	**JN800281**[b]	**MW053457**[g]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ankarafantsika	**MW054242**[g]	**MW054236**[g]	**JN800285**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Makira	**MW054246**[g]	**MW054239**[g]	**JN800288**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Makira	**MW054247**[g]	**MW054240**[g]	**JN800289**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Ranomafana	**MW054245**[g]	**MW054238**[g]	**JN800286**[b]
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar: Vohiparara	**MW054244**[g]	**MW054237**[g]	**JN800287**[b]
Metapolystoma cachani	*Ptychadena longirostris*	Africa: Nigeria	**FM897280**[a]	**FM897262**[a]	**JN800294**[b]
Metapolystoma falcatum n. sp.	*Boophis doulioti*	Madagascar: Ankarafantsika	**MW054248**[g]	**JN800283**[b]	**JN800291**[b]
Metapolystoma falcatum n. sp.	*Boophis doulioti*	Madagascar: Ankarafantsika	**FM897286**[a]	**FM897269**[a]	**MW053458**[g]
Metapolystoma multiova n. sp.	*Boophis occidentalis*	Madagascar: Isalo	**FM897285**[a]	**FM897268**[a]	**FM897301**[a]
Metapolystoma theroni n. sp.	*Boophis madagascareniensis*	Madagascar: Andasibe	**FM897284**[a]	**FM897267**[a]	**FM897298**[a]
Metapolystoma theroni n. sp.	*Boophis madagascareniensis*	Madagascar: Andasibe	**MW054249**[g]	**MW054241**[g]	**JN800293**[b]
Polystoma claudecombesi	*Amietia delalandii*	South Africa	**FM897281**[a]	**FM897263**[a]	(–)
Polystoma dawiekoki	*Ptychadena anchietae*	South Africa	**AM051069**[c]	**AM157204**[d]	**AM913856**[e]
Polystoma integerrimum	*Rana temporaria*	France	**AM051071**[c]	**AM157206**[d]	**JF699306**[f]
Polystoma marmorati	*Hyperolius marmoratus*	South Africa	**AM051073**[c]	**AM157208**[d]	**AM913859**[e]
Polystoma occipitalis	*Hemisus marmoratus*	Ivory Coast	**AM051075**[c]	**FM897264**[a]	(–)
Polystoma testimagna	*Strongylopus fasciatus*	South Africa	**AM157194**[c]	**AM157217**[d]	**AM913860**[e]

[a] From Verneau et al. (2009).
[b] From Berthier and Verneau, unpublished.
[c] From Bentz et al. (2006).
[d] From Badets et al. (2011).
[e] From du Preez et al. (2007).
[f] From Raharivololoniaina et al. (2011).
[g] Sequences reported in the present study; (–) Not available.

Table 4
Mean genetic distances (below diagonal) and total character differences (above diagonal) between species groups as inferred from comparisons of 18S rDNA sequences (1931 characters).

		1	2	3	4	5	6	7	8	9	10	11
1	*M. brygoonis*		1	2	2	2	11	10	13	10	9	25
2	*M. falcatum* n. sp. (B. d.)	0,001		1	1	1	10	9	12	9	8	24
3	*M. multiova* n. sp. (B. o.)	0,001	0001		0	2	11	10	13	10	9	25
4	*M. theroni* n. sp. (B. m)	0,001	0001	0,000		2	11	10	13	10	9	25
5	*M. cachani*	0,001	0001	0,001	0001		9	8	11	10	9	23
6	*P. occipitalis*	0,006	0005	0,006	0006	0,005		6	16	15	14	28
7	*P. dawiekoki*	0,005	0005	0,005	0005	0,004	0003		16	15	14	28
8	*P. claudecombesi*	0,007	0006	0,007	0007	0,006	0008	0,008		11	10	24
9	*P. marmorati*	0,005	0005	0,005	0005	0,005	0008	0,008	0006		1	25
10	*P. testimagna*	0,005	0004	0,005	0005	0,005	0007	0,007	0005	0,001		24
11	*P. integerrimum*	0,013	0013	0,013	0013	0,012	0015	0,015	0013	0,013	0,0126	

Abbreviations: B. d. = *Boophis doulioti*; B. o. = *Boophis occidentalis*; B. m. = *Boophis madagascariensis*

Table 5
Mean genetic distances (below diagonal) and total character differences (above diagonal) between species groups as inferred from comparisons of 28S rDNA sequences (1418 characters).

		1	2	3	4	5	6	7	8	9	10	11	12
1	*M. brygoonis* (SlA)	–	2	8	13	15	20	29	29	37	38	37	58
2	*M. brygoonis* (SlB)	0,001	–	6	11	13	18	27	27	35	36	35	59
3	*M. falcatum* n. sp. (B. d.)	0,006	0004	–	11	13	20	26	26	34	35	34	56
4	*M. multiova* n. sp. (B. o.)	0,009	0008	0,008	–	4	23	32	32	40	39	38	62
5	*M. theroni* n. sp. (B. m.)	0,011	0009	0,009	0003	–	27	34	34	40	39	38	62
6	*M. cachani*	0,014	0013	0,014	0016	0,019	–	32	33	41	42	41	68
7	*P. occipitalis*	0,020	0019	0,019	0023	0,024	0023	–	7	36	38	35	59
8	*P. dawiekoki*	0,021	0019	0,019	0023	0,024	0024	0,005	–	35	35	32	59
9	*P. claudecombesi*	0,027	0025	0,024	0,09	0,029	0030	0,026	0025	–	22	23	53
10	*P. marmorati*	0,027	0026	0,025	0028	0,028	0030	0,027	0025	0,016	–	5	53
11	*P. testimagna*	0,027	0025	0,024	0027	0,027	0030	0,025	0022	0,016	0004	–	52
12	*P. integerrimum*	0,042	0043	0,041	0045	0,045	0050	0,043	0043	0,039	0039	0,038	–

Abbreviations: SlA = sublineage A; SlB = sublineage B; B. d. = *Boophis doulioti*; B. o. = *Boophis occidentalis*; B. m. = *Boophis madagascariensis*.

characters, contained 19 taxa, among which 13 specimens of *Metapolystoma* and six specimens characterizing distinct species of *Polystoma*.

Minimum and maximum distances with standard deviation as estimated among *Metapolystoma* species were as follows. *M. falcatum* n. sp. (18S: 0.051% ± 0.0003–0.051 ± 0.0005%; 28S: 0.42% ± 0.001–1.42% ± 0.002; COI: 8.6% ± 0.016–15.68% ± 0.023); *M. multiova* n. sp. (18S: 0–0.1% ± 0.0007); 28S: 0.28% ± 0.001–1.64% ± 0.003; COI: 12.54% ± 0.020–13.78% ± 0.022); *M. theroni* n. sp. (18S: 0–0.1% ± 0.0007; 28S:

Table 6
Mean genetic distances (below diagonal) and total character differences (above diagonal) between specimens of *Metapolystoma* species as inferred from comparisons of COI sequences (323 characters).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*M. brygoonis* (Ankarafantsika)	–	1	2	21	21	13	17	27	27	40	39	41	26	36	35	38	55
2	*M. brygoonis* (Ambatolampy)	0,003	–	3	20	20	12	16	26	26	39	38	40	25	35	34	37	55
3	*M. brygoonis* (Ambatolampy)	0,006	0009	–	21	21	13	17	26	26	39	39	41	28	36	35	38	56
4	*M. brygoonis* (Vohiparara)}	0,068	0065	0,068	–	1	13	11	30	30	38	37	39	30	43	40	41	58
5	*M. brygoonis* (Ranomafana)	0,068	0065	0,068	0003	–	13	12	30	30	38	36	39	29	43	40	41	57
6	*M. brygoonis* (Makira)	0,041	0038	0,041	0042	0,042	–	8	25	25	37	34	36	22	35	32	36	54
7	*M. brygoonis* (Makira)	0,055	0051	0,055	0035	0,038	0025	–	30	30	38	36	38	29	40	37	40	55
8	*M. falcatum* n. sp. (B. d.)	0,089	0086	0,086	0100	0,099	0082	0,100	–	0	38	43	45	32	38	33	37	60
9	*M. falcatum* n. sp. (B. d.)	0,089	0086	0,086	0100	0,099	0082	0,100	0000	–	38	43	45	32	38	33	37	60
10	*M. multiova* n. sp. (B. o.)	0,137	0133	0,133	0129	0,129	0125	0,129	0129	0,129	–	40	38	40	49	37	42	61
11	*M. theroni* n. sp. (B. m.)	0,133	0129	0,133	0125	0,121	0114	0,121	0149	0,149	0138	–	4	36	48	41	45	64
12	*M. theroni* n. sp. (B. m.)	0,141	0137	0,141	0132	0,133	0121	0,129	0157	0,157	0130	0,013	–	40	50	41	46	65
13	*M. cachani*	0,085	0082	0,092	0099	0,096	0071	0,096	0106	0,106	0136	0,121	0136	–	35	31	34	51
14	*P. dawiekoki*	0,121	0117	0,121	0147	0,147	0117	0,135	0128	0,128	0170	0,166	0174	0,117	–	38	42	57
15	*P. marmorati*	0,117	0114	0,117	0136	0,135	0106	0,124	0110	0,110	0125	0,139	0139	0,103	0129	–	25	54
16	*P. testimagna*	0,128	0124	0,128	0139	0,139	0121	0,135	0124	0,124	0143	0,155	0158	0,113	0143	0,082	–	53
17	*P. integerrimum*	0,193	0193	0,198	0205	0,201	0189	0,193	0214	0,214	0218	0,231	0235	0,177	0202	0,189	0185	–

Abbreviations: B. d. = *Boophis doulioti*; B. o. = *Boophis occidentalis*; B. m. = *Boophis madagascariensis*

0.28% ± 0.001–1.93% ± 0.003; COI: 11.4% ± 0.019–15.68% ± 0.023). With regard to 18S, total differences between species showed no character difference between *M. multiova* n. sp. and *M. theroni* n. sp. while the total 18S differences among all other *Metapolystoma* spp. ranged from 1 to 2 (Table 4). Regarding total 28S differences (Table 5), values ranged from 4 to 27 among all *Metapolystoma* spp., while the lowest value occurred between *M. multiova* n. sp. and *M. theroni* n. sp. Finally, regarding total COI differences (Table 6), values ranged from 25 to 45 between all *Metapolystoma* spp. Though no 18S character difference was detected between *M. multiova* n. sp. and *M. theroni* n. sp., the high levels of genetic divergence between *M. falcatum* n. sp., *M. multiova* n. sp., *M. theroni* n. sp., *M. brygoonis* and *M. cachani* (as estimated on the basis of 28S and COI sequences) led us to consider three new *Metapolystoma* species.

When 18S, 28S and COI sequences were combined for Bayesian analysis (Fig. 14), *M. cachani* appears as the most basal species within *Metapolystoma* and Malagasy *Metapolystoma* form a clade. Within this clade, *M. multiova* n. sp. and *M. theroni* n. sp. are sister species; two sublineages are well differentiated within *M. brygoonis*. Sublineage A comprises polystomes found in localities at Makira, Ranomafana and Vohiparara (Fig. 1), while sublineage B comprises polystomes of the Ambatolampy and Ankarafantsika areas (Fig. 1). Because high levels of 28S and COI genetic divergence (Tables 5 and 6) were also observed these two sublineages (28S: 0.14% ± 0.0009; COI: 5.71% ± 0.011), it suggests that there could be two genetic entities within *M. brygoonis* infecting the same host species in Madagascar, that is, *Ptychadena mascareniensis*.

3.3. Remarks

The phylogenetic position of *M. falcatum* n. sp., *M. multiova* n. sp. and *M. theroni* n. sp. within the Polystomatidae (Fig. 14) confirms that they are members of *Metapolystoma* (Polystomatidae). Genetic divergence estimates among *Metapolystoma* taxa (Tables 4–6) sustain morphological descriptions, for these three species.

The lengths of *M. vencesi* n. sp. (9190) and *M. theroni* n. sp. (9086) differ from all other Malagasy metapolystomes, which vary in length from 2680 to 6710. *M. theroni* n. sp. has a haptor length of 1,634, separating it from all other known Malagasy metapolystomes. Haptor length of *M.falcatum* n. sp. (1492) and *M. vencesi* n. sp. (1437) overlap but differ substantially from *M. ansuanum* n. sp. (669–912), *M. brygoonis* (760–1070) and *M. multiova* n. sp. (916). Haptor length-body length ratio of *M. vencesi* n. sp. (0.16) separates it from all other species while, in the overlapping cases of *M. falcatum* n. sp. (0.19), *M. multiova* n. sp. (0.18) and *M. theroni* n. sp. (0.18), there is a marked difference from *M. ansuanum* n. sp. (0.23–0.25) and *M. brygoonis* (0.22–0.23), the latter two of which overlap in turn.

With a hamulus length X (Fig. 3b) of 269–332, *M. vencesi* n. sp. differs from all other known Malagasy metapolystomes, which range in length between 196 and 420. It is also the single species that has no separation between the hamulus handle and guard. *Metapolystoma brygoonis* (330–420), *M. falcatum* n. sp. (393–407) and *M. theroni* n. sp. (392–408) overlap but differ from *M. ansuanum* n. sp. (205) and *M. multiova* n. sp. (196–248), while the latter two also overlap. Hamulus hook length Z (Fig. 3b) separates all newly described metapolystomes from one another, ranging from *M. vencesi* n. sp. (40–52) with the smallest hooks to *M. falcatum* n. sp. (66–70) with the largest. Besides *M. ansuanum* n. sp. differs from all other metapolystomes in that it has an exceptional long hamulus hook relative to the rest of the hamulus and *M. falcatum* n. sp. differs from all others in that it has an exceptional long curved marginal hooklet tip on hooks two to seven.

With a genital bulb diameter of 115, *M. vencesi* n. sp. has the largest diameter ranging between 64 and 86. *Metapolystoma. brygoonis* (80), *M. falcatum* n. sp. (86), and *M. multiova* n. sp. (86) overlap but are separated from *M. ansuanum* n. sp. (64–73) and *M. theroni* n. sp. (65), while the latter two overlap. *Metapolystoma falcatum* n. sp. (26),

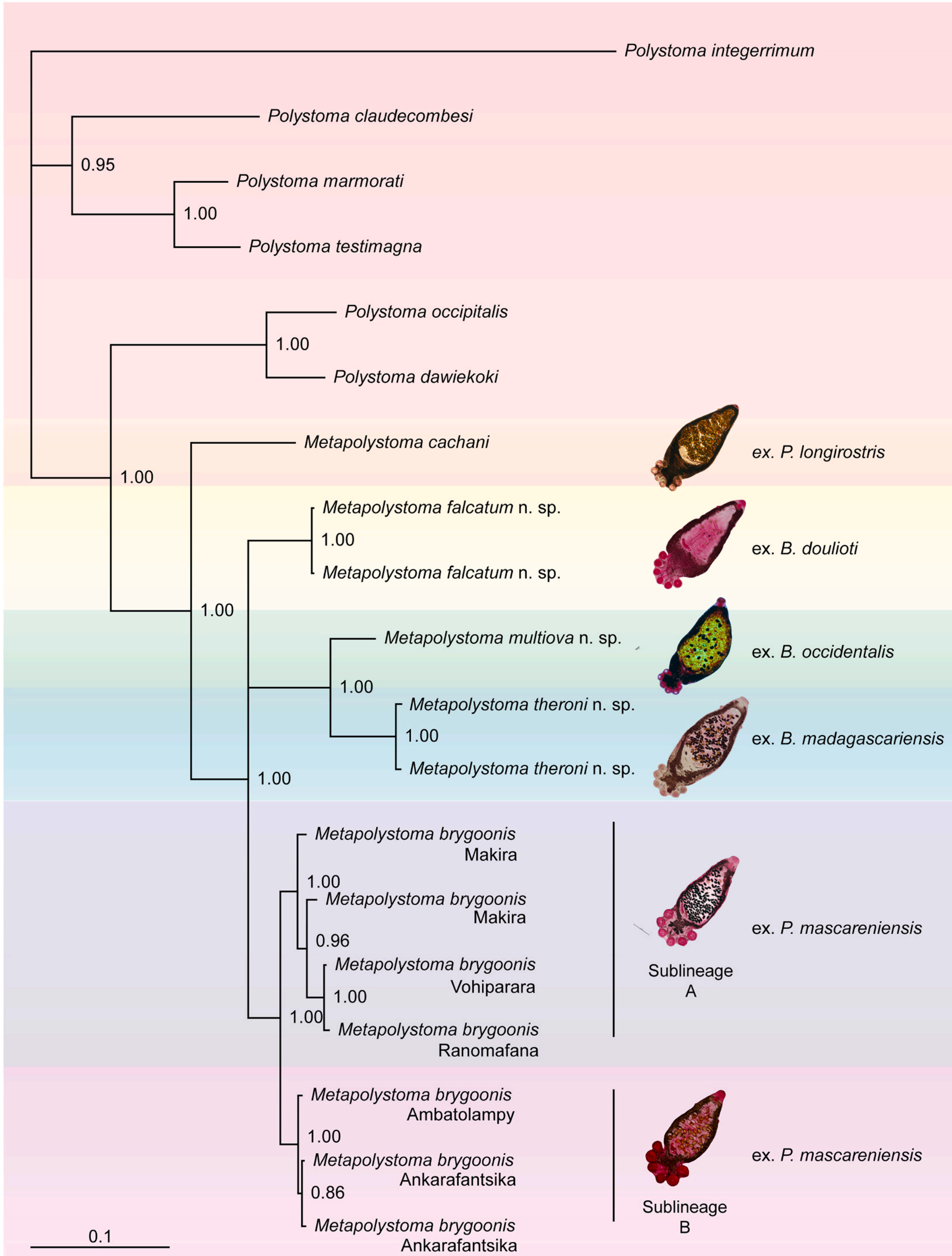

Fig. 14. Bayesian tree inferred from the analysis of concatenated 18S, 28S and COI gene sequences. Node values indicate Bayesian posterior probabilities.

M. multiova n. sp. (25), and *M. theroni* n. sp. (24–28) overlap in genital crown diameter but are separated from *M. ansuanum* n. sp. (29–33) and *M. vencesi* n. sp. (32), while the latter two overlap. *Metapolystoma brygoonis*, *M. falcatum* n. sp. and *M. vencesi* n. sp. all bear eight genital spines and differ from *M. ansuanum* n. sp., *M. multiova* n. sp. and *M. theroni* n. sp. that respectively bear ten, six and seven genital spines. With the greatest genital spine length of 40.4–43.7 *M. multiova* n. sp. is separated from all other known Malagasy metapolystomes, except *M. brygoonis*, which ranges between 24.3 and 34.9.

Metapolystoma brygoonis (410–460), *M. multiova* n. sp. (446) and *M. theroni* n. sp. (479) overlap in ovary length but differ from *M. ansuanum* n. sp. (265–338), *M. falcatum* n. sp. (772) and *M. vencesi* n. sp. (861), the latter which also differ from one another. With the greatest egg length of 239–265, *M. theroni* n. sp. differs from all other Malagasy metapolystomes, which range from 160 to 230. *Metapolystoma ansuanum* (196–217), *M. multiova* n. sp. (198–228), and *M. vencesi* n. sp. (210–230) overlap but differ from *M. brygoonis* (160–200) and *M. falcatum* n. sp. (161–185), the latter two which overlap.

To conclude, due to the small number of parasite specimens that were investigated, we can not exclude that the morphometric variability may represent intra specific variation. However, worms under investigation show a combination of morphological characters, namely shape of the hamulus handle and guard, shape of the hamulus hook, shape of the marginal hooklet tip on hooks and genital spine number that allow the differentiation of five distinct metapolystome species.

4. Discussion

Metapolystoma was until now reported only from the Afrotropical realm anuran hosts belonging to *Ptychadena* (Ptychadenidae). *Ptychadena* is a successful and widespread genus in Africa being a well-suited host for polystomes (Du Preez and Kok, 1992a). Of the 56 known *Ptychadena* species (Frost, 2020), 11 are known to host polystomes, including 14 *Polystoma* and three *Metapolystoma* species (see Du Preez and Kok, 1992a). According to Verneau et al. (2009), *Metapolystoma* may have originated in Africa within the time window 19.8–4.3 million years ago (Mya) from ancestors close to *Polystoma* and further dispersed to Madagascar following natural transoceanic dispersal of the ancestor of *Pt. mascareniensis* at about 14.2–2.3 Mya (Verneau et al., 2009). Because *M. brygoonis* forms a sister group to all other members of Malagasy *Metapolystoma*, *M. cachani* being basal within *Metapolystoma*, a host switch was suggested from the ancestor of *Pt. mascareniensis* to ancestral *Boophis* (Mantellidae) (Verneau et al., 2009), which is confirmed in the present phylogenetic study. Even though *Ptychadena* and *Boophis* are not phylogenetically closely related, they both display pleisiomorphic reproductive modes, favouring the possibility of an ancestral host switch in Madagascar (see Verneau et al., 2009), and ultimately the diversification of *Metapolystoma* within *Boophis*. While *Boophis* is not the single representative of the family Mantellidae infected by polystomes in Madagascar, mantellid frogs of the two genera *Mantella* and *Blommersia* which both exhibit a derived mode of reproduction, are infected by polystomes of another genus, i.e. *Madapolystoma* (see Verneau et al., 2009; Du Preez et al., 2010; Landman et al., 2018). In addition, Verneau et al. (2009) reported another undescribed species of *Metapolystoma* from *Aglyptodactylus madagascariensis* (Duméril, 1853). Therefore, the five new *Metapolystoma* species described along this study clearly indicate that a larger diversity of metapolystomes can be expected. This is especially true since Madagascar is inhabited by 79 *Boophis* species (Frost, 2020) that may serve as hosts for *Metapolystoma* species. Furthermore, *M. brygoonis* can be divided in two separate lineages according to genetic differentiation (Fig. 14). These results strengthen the fact that *Metapolystoma* in Madagascar still continue to diversify and call for further sampling and investigation.

The validity of *Metapolystoma* has long been disputed. It was suggested that the uterine structure was achieved convergently within *Metapolystoma* (Tinsley, 1974), through the ability of the parasite to adapt to the ecology of its host (Kok and Seaman, 1987; Murith, 1981; Tinsley, 1983). Bentz et al., (2001) claimed it to be invalid and ascribed its morphological differences from *Polystoma* in terms of homoplastic characters. Nevertheless our phylogeny supports the monophyly of *Metapolystoma*, which is however nested within the paraphyletic *Polystoma*. If the morphological differences between *Metapolystoma* and *Polystoma* were the product of reproductive plasticity, *Metapolystoma* spp. would not have clustered together on a molecular level (see Fig. 14). The fact that they do, therefore, suggests that the long uterus may have been inherited by descent. It is however intriguing to note that two polystome species *M. porosissimae* and *P. sodwanensis*, which display completely different life-history strategies, can occur simultaneously in the same host species, i.e. *P. porosissima* in Africa (Du Preez and Kok, 1992b). *Metapolystoma porosissimae* displays a strategy where many eggs are stored in a large uterus, which is typical of polystomes that infest hosts within arid environments (Du Preez, 2015; Du Preez and Kok, 1992b). Conversely, *P. sodwanensis* has a small uterus containing only a few eggs, which is in line with a water-dependent host (Du Preez and Kok, 1992b). Even though morphological differences between the two species are distinct, the simultaneous occurrence of these two species in the same individuals of *Pt. porosissima* have reinforced the dispute (Du Preez and Kok, 1992b). Though the validity of *Metapolystoma* at this stage cannot be ruled out, a more in depth genetic investigation of the two African polystome species *M. porosissimae* and *P. sodwanensis* should help to conclude.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgements

We are grateful to all the colleagues, assistants and students who assisted in field work, in particular to Miguel Vences, Che Weldon and various Malagasy students. Research was conducted in a collaborative effort between the North-West University, the Association Nationale pour la Gestion des Aires Protégées and the Département de Biologie Animale de l'Université d'Antananarivo. We are grateful to the Malagasy authorities who provided the necessary research and export permits. We are also grateful to Annemarie Ohler at the Muséum National d'Histoire Naturelle de Paris who allowed us to dissect several museum specimens for additional parasite material. Funding was provided by the Volkswagen Foundation , Grant number: VE247/2-1, the Deutsche Forschungsgemeinschaft and the CNRS.

References

Andreone, F., Carpenter, A.I., Cox, N., Du Preez, L., Freeman, K., Furrer, S., Garcia, G., Glaw, F., Glos, J., Knox, D., Köhler, J., Mendelson III, J.R., Mercurio, V., Mittermeier, R.A., Moore, R.D., Rabibisoa, N.H.C., Randriamahazo, H., Randrianasolo, H., Raminosoa, N.R., Ramilijaona, O.R., 2008. The challenge of conserving amphibian megadiversity in Madagascar. PLoS Biol. e118.

Badets, M., Verneau, O., 2009. Origin and evolution of alternative developmental strategies in amphibious sarcopterygian parasites (Platyhelminthes, Monogenea, Polystomatidae). Org. Divers. Evol. 9, 155–164.

Badets, M., Whittington, I., Lalubin, F., Allienne, J.F., Maspimby, J.L., Bentz, S., Du Preez, L.H., Barton, D., Hasegawa, H., Tandon, V., Imkongwapang, R., Ohler, A., Combes, C., Verneau, O., 2011. Correlating early evolution of parasitic platyhelminths to Gondwana breakup. Syst. Biol. 60 (6), 762–781. https://doi.org/10.1093/sysbio/syr078.

Bentz, S., Leroy, S., Du Preez, L., Mariaux, J., Vaucher, C., Verneau, O., 2001. Origin and evolution of African *Polystoma* (Monogenea: polystomatidae) assessed by molecular methods. Int. J. Parasitol. 31, 697–705.

Bentz, S., Sinnappah-Kang, N.D., Lim, L., Lebedev, B., Combes, C., Verneau, O., 2006. Historical biogeography of amphibian parasites, genus *Polystoma* (Monogenea: Polystomatidae). J. Biogeogr. 33 (4), 742–749. https://doi.org/10.1111/j.1365-2699.2005.01402.x.

Berthier, P., 2011. Conservation animale: Utilisation des parasites comme marqueurs de l'écologie évolutive des amphibiens. University of Perpignan, France, pp. 1–185.

Combes, C., 1976. World biogeography of polystomatid monogeneans. Acad. Sci. Proc. 34, 55–63.

Du Preez, L.H., 2015. *Eupolystoma namibiensis* n. sp. (Monogenea: polystomatidae) parasitic in *Poyntonophrynus hoeschi* (Ahl, 1934) of Namibia. Afr. Zool. 50, 141–145.

Du Preez, L.H., Kok, D.J., 1992a. Syntopic occurrence of new species of Polystoma and metapolystoma (Monogenea: polystomatidae) in *Ptychadena porosissima* in South Africa. Syst. Parasitol. 22, 141–150.

Du Preez, L.H., Kok, D.J., 1992b. The frog genus *Ptychadena* as host for polystomatid (Monogenea) parasites in Africa. Afr. J. Herpetol. 40, 47–49.

Du Preez, L.H., Raharivololoniaina, L., Verneau, O., Vences, M., 2010. A new genus of polystomatid parasitic flatworm (Monogenea: polystomatidae) without free-swimming life stage from the Malagasy poison frogs. Zootaxa 2722, 54–68.

Du Preez, L.H., Verneau, O., Gross, T.S., 2007. *Polystoma floridana* n. sp.(Monogenea: Polystomatidae) a parasite in the green tree frog, *Hyla cinerea* (Schneider), of North America. Zootaxa 1663 (1), 33–45. https://doi.org/10.5281/zenodo.179988.

Euzet, L., Combes, C., 1964. Sur un polystomatidae (Monogenea) recolté à Madagascar chez *Rana mascareniensis* Duméril et Bribon. Bul. Soc. Zool. 392–401.

Frost, D.R., 2020. In: Amphibian Species of the World: an Online Reference. American Museum of Natural History, New York, USA. Version 6 (10 April 2020). Electronic database accessible at. http://research.amnh.org/herpetology/amphibia/index.html.

Gallien, L., 1956. Deux espèces nouvelles de polystomes africains. Bull. Soc. Zool. Fr. 81, 369–374.

Glaw, F., Vences, M., 2007. In: A Field Guide to the Amphibians and Reptiles of Madagascar, third ed. Köln, Vences and Glaw, ISBN 978-3-929449-03-7, pp. 146–178.

Heritier, L., Badets, M., Du Preez, L.H., Aisien, M.S., Lixian, F., Combes, C., Verneau, O., 2015. Evolutionary processes involved in the diversification of chelonian and mammal polystomatid parasites (Platyhelminthes, Monogenea, Polystomatidae) revealed by palaeoecology of their hosts. Mol. Phylogenet. Evol. 92, 1–10.

Huelsenbeck, J.P., Ronquist, F., 2001. Mrbayes: Bayesian inference of phylogenetic trees. Bioinformation 17, 754–755.

IUCN, 2016a. International union for conservation of nature and natural resources. https://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T57386A49392305.en. Date of access: Downloaded on 09 April 2020.
IUCN, 2016b. International union for conservation of nature and natural resources. https://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T57397A84162240.en. Date of access: Downloaded on 09 April 2020.
IUCN, 2016c. International union for conservation of nature and natural resources. https://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T57410A84163237.en. Date of access: Downloaded on 09 April 2020.
IUCN, 2016d. International union for conservation of nature and natural resources. https://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T49545913A67038125.en. Date of access: Downloaded on 09 April 2020.
IUCN, 2016e. International union for conservation of nature and natural resources. https://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T68206465A68200098.en. Date of access: Downloaded on 09 April 2020.
Junker, K., Lhermitte-Vallarino, N., Barbuto, M., Ineich, I., Wanji, S., Bain, O., 2010. New species of *Rhabdias* (Nematoda: rhabdiasidae) from afrotropical anurans, including molecular evidence and notes on biology. Folia Parasitol. 57, 47–61.
Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
Kok, D.J., Seaman, M.T., 1987. Polystomatidae (Monogenea) parasitic in the anuran genus *Natalobatrachus* in South Africa. S. Afr. J. Zool. 22, 258–263.
Kulo, S.D., 1981. Présence au Togo de *Metapolystoma cachani* (Gallien, 1957). Combes, 1976 (Monogenea) chez l'amphibien *Ptychadena longirostris* (Peters, 1870). Bull. Soc. Zool. Fr. 105, 177–181.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Kuzmin, Y., Junker, K., Du Preez, L., Bain, O., 2013. A new species of *Rhabdias* Stiles et Hassall, 1905 (Nematoda: rhabdiasidae) from *Blommersia domerguei* (Guibe) (Amphibia: Mantellidae) in Madagascar. Folia Parasitol. 60, 469–474.
Landman, W.J., Verneau, O., Du Preez, L.H., 2018. 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*. Int. J. Parasitol Parasites Wildl. 7, 343–354.
Littlewood, D.T.J., Rohde, K., Clough, K.A., 1997. Parasite speciation within or between host species? Phylogenetic evidence from site-specific polystome monogeneans. Int. J. Parasitol. 27, 1289–1297.
Murith, D., 1981. Contribution à l'étude de la systématique des polystomes (Monogènes, Polystomatidae) parasites d'amphibiens anoures de basse Côte-d'Ivoire. Rev. Suisse Zool. 88, 475–533.
Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858.
Olson, P.D., Tkach, V.V., 2005. Advances and trends in the molecular systematics of the parasitic Platyhelminthes. Adv. Parasitol. 60, 165–243.
Page, R.D., 1996. Treeview: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.
Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformation 14, 817–818.
Raharivololoniaina, L., Verneau, O., Berthier, P., Vences, M., Du Preez, L.H., 2011. First monogenean flatworm from a microhylid frog host: *Kankana*, a new polystome genus from Madagascar. Parasitol. Int. 60 (4), 465–473. https://doi.org/10.1016/j.parint.2011.08.001.
Rocha, R., Borda, E., Andreone, F., Rosa, G.M., 2012. First reports of leech parasitism in Malagasy Anurans. Comp. Parasitol. 79, 352–356.
Sinnappah, N.D., Lim, L.H., Rohde, K., Tinsley, R., Combes, C., Verneau, O., 2001. A paedomorphic parasite associated with a neotenic amphibian host: phylogenetic evidence suggests a revised systematic position for Sphyranuridae within anuran and turtle polystomatoineans. Mol. Phylogenet. Evol. 18, 189–201.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Tinsley, R.C., 1974. Observations on *Polystoma africanum* Szidat with a review of the inter–relationships of *Polystoma* species in Africa. J. Nat. Hist. 8, 355–367.
Tinsley, R.C., 1983. Ovoviviparity in platyhelminth life-cycles. Parasitology 86, 161–196.
Verneau, O., Bentz, S., Sinnappah, N.D., Du Preez, L.H., Whittington, I., Combes, C., 2002. A view of early vertebrate evolution inferred from the phylogeny of polystome parasites (Monogenea: polystomatidae). Proc. Biol. Sci. 269, 535–543.
Verneau, O., Du Preez, L.H., Laurent, V., Raharivololoniaina, L., Glaw, F., Vences, M., 2009. The double odyssey of Madagascan polystome flatworms leads to new insights on the origins of their amphibian hosts. Proc. R. Soc. Lond. B Biol. Sci. 276, 1575–1583.
Wohltmann, A., Du Preez, L., Rodel, M.-O., Kohler, J., Vences, M., 2007. Endoparasitic mites of the genus *Endotrombicula* ewing, 1931 (Acari: Prostigmata: Parasitengona: trombiculidae) from African and madagascan anurans, with description of a new species. Folia Parasitol. 54, 225–235.