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Integrative approach reveals two new species of *Obama* (Platyhelminthes: Tricladida) from the South-Brazilian Atlantic Forest

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Abstract

The genus *Obama* Carbayo *et al*., 2013 includes 38 species, being the most species-rich within Geoplaninae. Species of this genus show a similar anatomy regarding their copulatory apparatus, which may hinder species differentiation. In this study, we describe two new species, presenting a marbled colour pattern, found in two different phytophysionomies of the Atlantic Forest, namely Semi-deciduous Forest and *Araucaria* Forest. Both species can be distinguished from their congeners, as well from each other, by colour pattern and eye arrangement combined with characteristics of the pharynx, penis papilla and prostatic vesicle, confirmed by molecular analyses from cytochrome *c* oxidase subunit I gene (COI). Phylogenetic analyses suggest that both species herein studied are closely related to another species that occurs in areas of *Araucaria* Forest (*O. maculipunctata*). Results also indicate the need to use at least 600 bp of the gene COI in the definitions of interspecific divergences and for species delineation, at least for the genus *Obama*.

Key words: Geoplaninae, land flatworms, Neotropical region, integrative taxonomy, Tricladida

Introduction

Within the subfamily Geoplaninae, the distribution of which is restricted to the Neotropical region, the genus *Obama* Carbayo *et al*., 2013 is the most species-rich, being composed of 38 species (Rossi *et al*. 2015; Carbayo *et al*. 2016a, b). It encompasses all species of Geoplaninae that belonged to the group of "broad and flat land planarians" proposed by Graff (1899).

Species of *Obama* show a similar anatomy regarding their copulatory apparatus, which may hinder species differentiation (Winsor 1998; Negrete *et al.* 2013). In general, for land flatworms, besides external features, such as colour pattern and eye arrangement, the pharyngeal anatomy and the morphology of the reproductive system are used for the identification at the species level (Winsor 1998; Seitenfus & Leal-Zanchet 2004). When this combination of anatomical characters does not provide enough distinguishing features, molecular phylogenetic analyses may be useful to reveal specific differences, having been used in an integrative approach in recent studies of Geoplaninae (Lemos *et al*. 2014; Álvarez-Presas *et al*. 2015; Lago-Barcia *et al*. 2015; Rossi *et al*. 2015; Carbayo *et al*. 2016a, b).

Such studies are especially useful in areas of the Atlantic Forest, which have revealed the highest diversity of land flatworms worldwide, many of them not yet described (Castro & Leal-Zanchet 2005; Antunes *et al*. 2008; Leal-Zanchet & Baptista 2009; Baptista *et al*. 2010; Amaral *et al*. 2014; Negrete *et al.* 2014). Two of these species, belonging to the genus *Obama*, and occurring in areas of the Atlantic Forest with a disjunct distribution, show a marbled colour pattern that is quite distinct in its details. Herein we describe both species through an integrative methodology and investigate their relationships.

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Material and methods

Land planarians were collected during the day by direct sampling in leaf litter and under fallen logs and stones or during the night, when they are more active, by visual search, in two areas located in south Brazil. One of the type-localities is a private reserve named *Araucaria* Natural Heritage Private Reserve (26º20.35'–26º26.13'S; 51º19.49'–51º25.29'W), situated in the Iguassu River Drainage Basin, in General Carneiro, state of Paraná. The other type-locality is an unprotected area represented by a remnant of Semi-deciduous forest, situated in Gravataí (29°48'10.71"S; 50°55'52.33"W), in the metropolitan region of Porto Alegre, state of Rio Grande do Sul.

Morphological analysis. Live specimens were photographed and analysed regarding colour pattern and body shape and dimensions. Before fixation, the posterior tip of 6 specimens was cut and preserved in 100% ethanol for molecular analysis. Afterwards, specimens were euthanised with boiling water and fixed in neutral formalin 10% and subsequently preserved in 70% ethanol. Methods described by Rossi *et al.* (2015) were followed for histological processing of material and analysis of external and internal characters. Histological sections (6 µm thick) were stained with Masson's trichrome method or haematoxylin and eosin (Romeis 1989).

Type-material was deposited in the Museu de Zoologia da Universidade do Vale do Rio dos Sinos, São Leopoldo, state of Rio Grande do Sul, Brazil (MZU), and the Helminthological Collection of Museu de Zoologia da Universidade de São Paulo, São Paulo, state of São Paulo, Brazil (MZUSP).

Nucleic acid isolation and sequence analysis. In Appendix S1 we show the specimens sequenced in this work and downloaded from the GenBank database. We extracted genomic DNA from specimens preserved in 100% ethanol using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Subsequently, we amplified and sequenced a region corresponding to the cytochrome c oxidase subunit I (COI) using primers BarT (Álvarez-Presas *et al*. 2011) and COIR (Lázaro *et al*. 2009). Genomic DNA (20–50 ng) was used as template in a reaction volume of 25 µL, containing 0.2 µM of each primer, 200 µM dNTPs, 1x buffer, 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase (Invitogen, USA) and ultrapure H_2O. Amplification was performed as follows: initial denaturation at 95 °C for 3 min followed by 38 cycles of denaturation at 94 °C for 50 sec, annealing at 50 °C for 60 sec, extension at 72 °C for 50 sec and final extension at 72 °C for 5 min. The PCR products were verified by gel electrophoresis (1% agarose gels) stained with GelRed (Biotium, Hayward, CA, USA), visualized under an UV transilluminator and purified using Shrimp Alkaline Phosphatase (SAP) and exonuclease I (New England Biolabs) following the manufacturer's recommendation. Amplicons were submitted to direct sequencing at Macrogen (Macrogen Inc., Seoul, Korea), and each sample was sequenced in both directions. The nucleotide sequences reported in this study have been deposited in the GenBank databases under the accession numbers shown in Appendix S1.

Sequence and phylogenetic analysis. Quality of sequences was evaluated using ChromasPro 1.5 software (http://www.technelysium.com.au). Sequences were then aligned within ClustalW 2.1 (Thompson *et al*. 1997), inspected manually using Bioedit 7.1.3 (Hall 1999) to refine coding frame and checked using the BLASTn on-line tool for comparison with sequences deposited in the GenBank database (NCBI). The amino acid translation was examined to ensure that no gaps or stop codons were present in the alignment. For the phylogenetic analyses we prepared a dataset with all the COI sequences from this study and from the works by Carbayo *et al*. (2013), Álvarez-Presas *et al*. (2015), Lago-Barcia *et al*. (2015), Rossi *et al*. (2015) and Carbayo *et al*. (2016a, b). A total of 101 COI sequences was aligned and the final dataset consisted of only individuals with at least 653 bp.

Phylogenetic analyses were conducted using both maximum likelihood and Bayesian algorithms. Modeltest version 3.0 (Posada & Crandall 1998) was employed to find the model of DNA substitution that best fits our data. The General-Time-Reversible (GTR) model for nucleotide substitution, considering invariable sites (0.4337) and gamma-distributed (0.3876) substitution rates among sites, was depicted as the best model by both criteria, Akaike Information Criterion (AIC) (Akaike 1974) was implemented in MrBayes 3.2.2 (Ronquist *et al*. 2012). Maximum likelihood analyses were run, using a randomized accelerated maximum likelihood model in RAxML 7.2.8 (Stamatakis *et al*. 2008), on the CIPRES Science Gateway cluster (Miller *et al*. 2010). Relative support of internal nodes was assessed by a rapid bootstrap (–f a -x option) with 1000 replications. We used species of the genus *Cratera* as the out-group. Bayesian inference was performed by MCMC analysis using MrBayes 3.2.2 (Ronquist *et al*. 2012). Two independent analyses were each run for 8,000,000 generations, with four chains, sampling every 1000 generations. Analyses were run until the average standard deviation of the split frequencies approached (<0.01), indicating that two runs converged to a stationary distribution.

We also calculated pairwise nucleotide distances between all COI dataset, according to Kimura's 2-parameter model (Kimura 1980), using MEGA version 6 (Tamura *et al*. 2013).

Abbreviations used in the figures: (cg) cyanophil glands; (cmc) common muscle coat; (cov) common glandular ovovitelline duct; (db) dorsal band; (de) dorsal epidermis; (df) dorsal fold; (di) dorsal insertion; (dm) dorsal cutaneous musculature; (dsm) dorsal subcutaneous mesenchymatic musculature; (e) eyes; (eg) erythrophil glands; (ej) ejaculatory duct; (es) oesophagus; (f) flecks; (fa) female atrium; (fc) female canal; (gm) glandular margin; (go) gonopore; (h) halos; (i) intestine; (im) internal musculature; (lu) pharyngeal lumen; (m) mouth; (ma) male atrium; (mg) glands with mixed secretion; (n) nerve cord; (o) ovary; (om) outer musculature; (ov) ovovitelline ducts; (p) penis papilla; (pp) pharyngeal pouch; (ps) paramedian stripe; (pv) prostatic vesicle; (rg) rhabditogen glands; (sg) shell glands; (sbm) sub-intestinal transverse mesenchymatic musculature; (sd) sperm ducts; (sp) sensory pit; (spm) supra-intestinal transverse mesenchymatic musculature; (sv) spermiducal vesicle; (t) testes; (v) vitelline folicles; (ve) ventral epidermis; (vi) ventral insertion; (vm) ventral cutaneous musculature; (xg) xanthophil glands.

Taxonomic part

Family Geoplanidae Stimpson, 1857

Subfamily Geoplaninae Stimpson, 1857

Obama Carbayo *et al*., 2013

Obama allandra sp. nov.

Etymology: The specific name is a composite of the Greek adjective *állos* (different) and Greek noun *andrós* (man), referring to the male atrium which shows a variable aspect in the specimens studied.

Type material. Holotype: MZUSP PL.2139: *leg.* I. Rossi, 07 February 2015, General Carneiro (*Araucaria* Natural Heritage Private Reserve), state of Paraná, Brazil—anterior tip: transverse sections on 18 slides; anterior region at the level of the ovaries: sagittal sections on 26 slides; pre-pharyngeal region: transverse sections on 15 slides; pharynx and copulatory apparatus: sagittal sections on 76 slides.

Paratypes: MZU PL.00283: *leg.* I. Rossi, 06 February 2015, General Carneiro (*Araucaria* Natural Heritage Private Reserve), state of Paraná, Brazil—copulatory apparatus: sagittal sections on 26 slides. **MZU PL.00284**: *leg.* I. Rossi, 07 February 2015, General Carneiro (*Araucaria* Natural Heritage Private Reserve), state of Paraná, Brazil—anterior tip: transverse sections on 19 slides; pre-pharyngeal region: transverse sections on 7 slides; pharynx and copulatory apparatus: sagittal sections on 25 slides.

Diagnosis: species of *Obama* with dorsal ground colour brownish, covered by numerous irregular dark flecks, and median band yellowish on anterior region of body; pharynx cylindrical with dorsal insertion shifted posteriorly; prostatic vesicle with two portions, proximal portion forked, globose or ovoid, and distal portion wide and inverted-U shaped; penis papilla either conical and almost symmetrical or irregular in shape and length; male atrium with a dorso-lateral invagination; male and female atria separated by dorsal fold.

Molecular diagnosis: this species includes all populations that cluster with sequences of specimens included in this study (GenBank accession numbers MH378881- MH378884) with significant support in an adequate molecular delimitation model.

Type-locality. General Carneiro, Paraná (PR), Brazil.

Distribution: known only from the type-locality, General Carneiro, PR, Brazil.

Description. External features. Body elongate with parallel margins and dorsal surface slightly convex; anterior tip rounded and posterior tip pointed (Fig. 1). Live specimens with maximum length of 39 mm when creeping. After fixation, maximum length 29 mm. Mouth at third fourth of body and gonopore at posterior fourth of body (Table 1).

Live specimens with dorsal ground colour brownish and median band yellowish, which is lighter on anterior region of body, becoming greyish on cephalic region (Fig. 1). Irregular blackish or dark-brown flecks occur over dorsal surface, forming paramedian stripes on anterior body half and overlaying median band on posterior half

(Figs. 1, 2). After fixation, median band yellowish, becoming inconspicuous towards posterior tip. Dark irregular flecks longer in anterior body half and smaller and more densely distributed in posterior half, forming paramedian stripes in anterior third of body, excepting cephalic region (Figs. 2, 4–5). In live and fixed specimens, ventral surface light-brown with greyish pigmentation contouring anterior tip.

FIGURE 1. *Obama allandra* **sp. nov.**, holotype: photograph in dorsal view. Anterior tip to the left.

TABLE 1. Measurements, in mm, of specimens of *Obama allandra* **sp. nov.** –: not measured; *: after fixation; ** length; DG: distance of gonopore from anterior end; DM: distance of mouth from anterior end; DMG: distance between mouth and gonopore; DPVP: distance between prostatic vesicle and pharyngeal pouch. The numbers given in parentheses represent the position relative to body length.

	Holotype	Paratype MZU PL.00283	Paratype MZU PL.00284
Maximum length in extension	39	32	25
Maximum width in extension	2	3.5	4
Length at rest	25	24	20
Width at rest	5	5	5
Length*	29	28	25
Width*	5	4.5	4
DM*	20 (69)	18.5 (66)	16 (64)
DG*	24 (83)	23 (82)	19.5 (78)
DMG*	4	4.5	3.5
DPVP*	0.8	–	0.9
Ovaries	8 (27)	–	–
Anteriormost testes	6 (21)	–	–
Posteriormost testes	18 (62)	–	–
Prostatic vesicle**	1.9	1.7	2.3
Penis papilla**	0.1	0.7	1
Male atrium**	1.3	1.1	1.1
Female atrium**	1.1	1.6	0.9

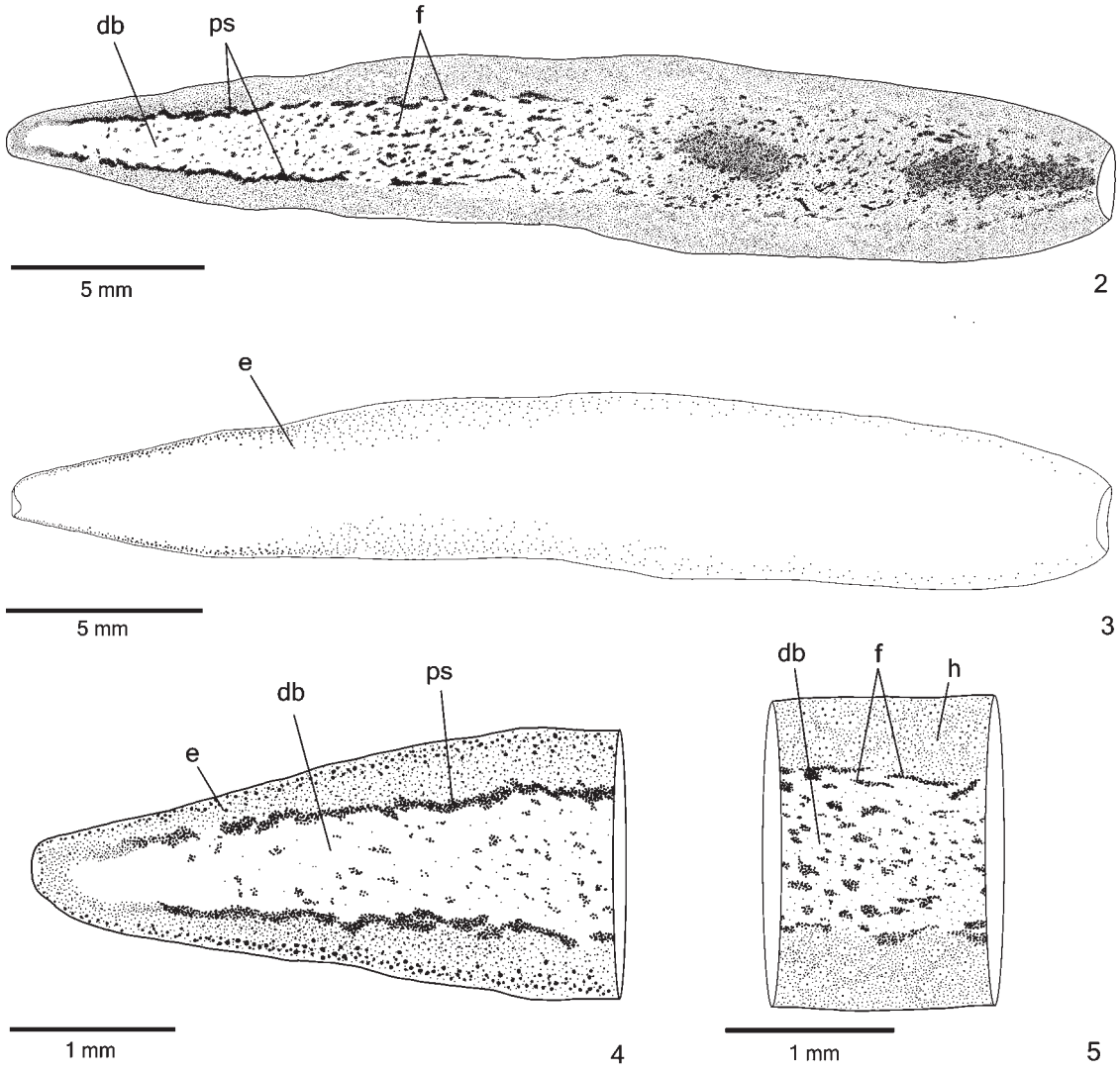
Eyes, initially monolobate and uniserial, surround anterior tip (Figs. 3–4). After second millimetre of body, eyes become larger and spread onto dorsal surface, occupying maximum width of about one-fourth of body width on either side of body, lateral to median band. Eyes become trilobate after anterior fourth of body, remaining

dorsal, but less numerous towards posterior tip (Fig. 3). Clear halos, less noticeable, may occur around dorsal eyes (Fig. 5). Pigment cups about 20–45 µm of diameter.

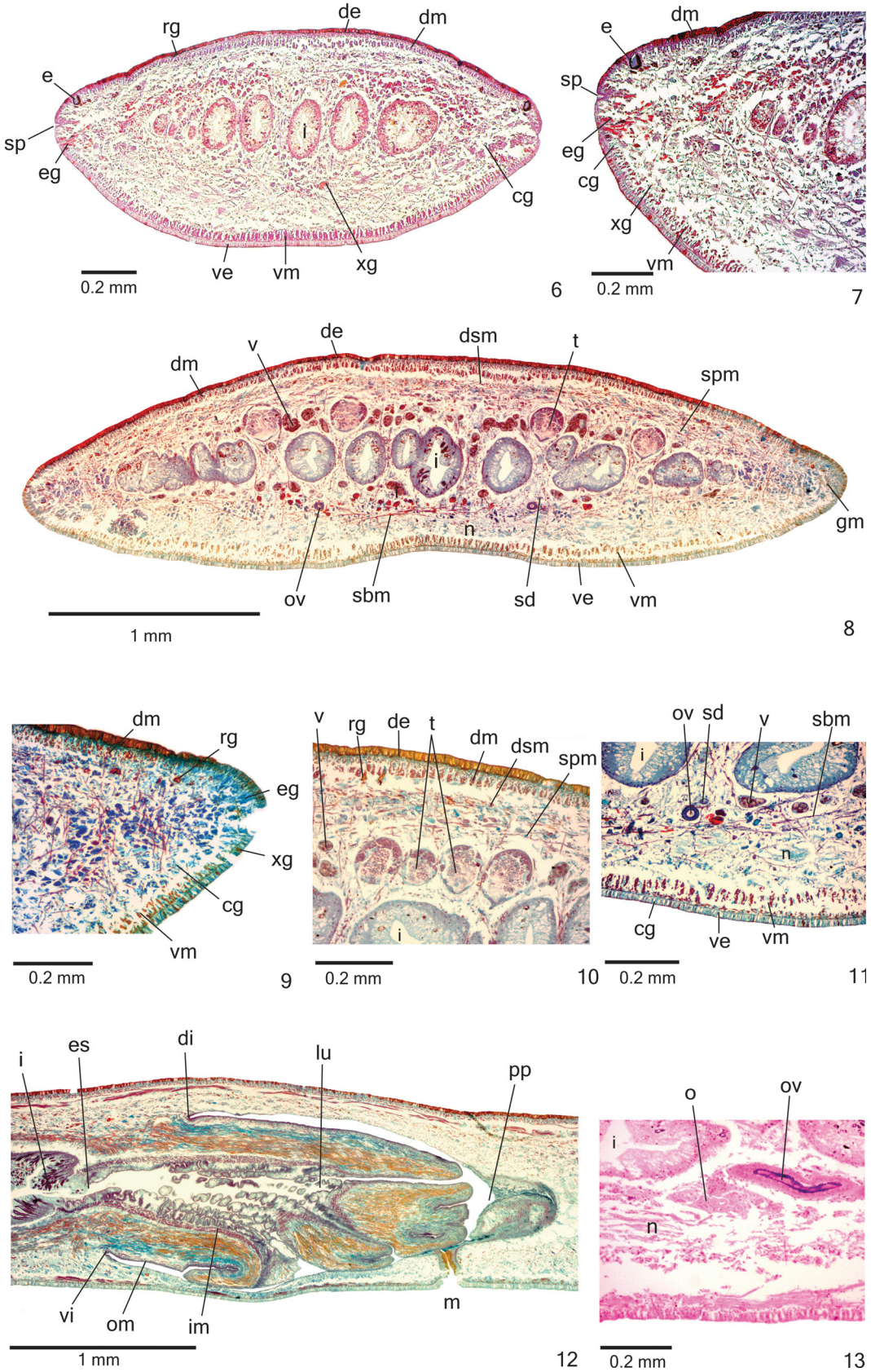
Sensory organs, epidermis and body musculature. Sensory pits (Figs. 6, 7), as simple invaginations (25–45 µm deep), contour anterior tip and occur ventromarginally in irregular, single row in almost the entire anterior half of body (about 45% of body length).

Glands of three types discharge through whole epidermis of pre-pharyngeal region: abundant rhabditogen glands with xanthophil secretion and cyanophil glands with amorphous secretion (Figs. 8–11), besides sparser xanthophil glands with coarse granular secretion. Creeping sole occupies whole body width. Glandular margin (Figs. 8,9) discernible after first millimetre of body. Glands of at least three types constitute glandular margin: glands with cyanophil, finely granular secretion and glands with erythrophil coarse granular secretions, both abundant, besides sparser rhabditogen glands with xanthophil secretion. The same glands discharging through epidermis of pre-pharyngeal region are present in the anterior tip (Figs. 6, 7).

Cutaneous musculature with usual three layers (circular, oblique and longitudinal layers); longitudinal layer with thick bundles (Figs. 8–11, Table 2). Thickness of cutaneous musculature between two and three times that of epidermis (Table 2). Ventral and dorsal musculatures with similar thickness at sagittal plane in pre-pharyngeal region (Table 2). Thickness of cutaneous musculature becoming progressively lower towards body margins and anterior tip.



FIGURES 2–5. *Obama allandra* **sp. nov.**, holotype: (2) dorsal pattern of pigmentation; (3) eye pattern; detail of eye pattern and pattern of pigmentation at anterior body region (4) and median third of the body (5). Anterior tip to the left.



FIGURES 6–13. *Obama allandra* **sp. nov.**, holotype, microphotographs of transverse (6–11) and sagittal sections (12–13; anterior tip to the left): (6) anterior region of body; (7) detail of anterior region of body; (8) pre-pharyngeal region; (9) detail of body margin of pre-pharyngeal region; (10) detail of dorsal surface of pre-pharyngeal region; (11) detail of ventral surface of pre-pharyngeal region; (12) pharynx; (13) ovary. The ovary is shown in a parasagittal section in order to show the ovovitelline duct opening.

TABLE 2. Body height and cutaneous musculature in the median region of a transverse section of the pre-pharyngeal region, in micrometers, and ratio of the height of cutaneous musculature to the height of the body (mc:h index) of specimens of *Obama allandra* **sp. nov.**

	Holotype	Paratype MZU PL.00284
Dorsal cutaneous musculature	55	65
Ventral cutaneous musculature	50	70
Dorsal epidermis	23	19
Ventral epidermis	23	23
Body height	950	1240
Mc:h (%)	11	11

Mesenchymal musculature (Figs. 8, 10–11) well developed, composed of three layers: (1) dorsal subcutaneous, located mainly close to cutaneous musculature, with decussate fibres (6–12 fibres thick), (2) supra-intestinal transverse (5–9 fibres thick) and (3) sub-intestinal transverse (4–7 fibres thick), besides fibres in various directions. Mesenchymal musculature less developed in anterior region than in pre-pharyngeal region (Fig. 6).

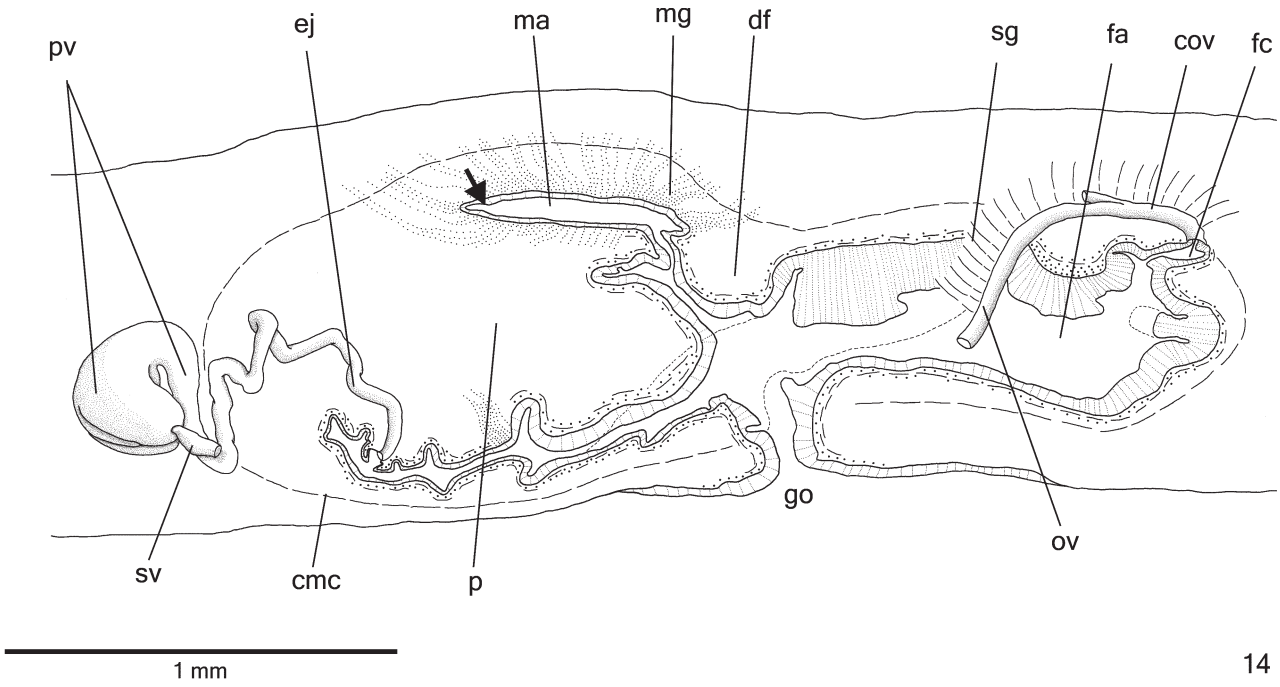
Pharynx. Pharynx cylindrical, nearly 7% of body length, occupies most of the pharyngeal pouch. Dorsal pharyngeal insertion slightly shifted posteriorly regarding the ventral insertion. Mouth located in posterior third of pharyngeal pouch (Fig. 12). Oesophagus short, with folded walls. Oesophagus: pharynx ratio 12–15%.

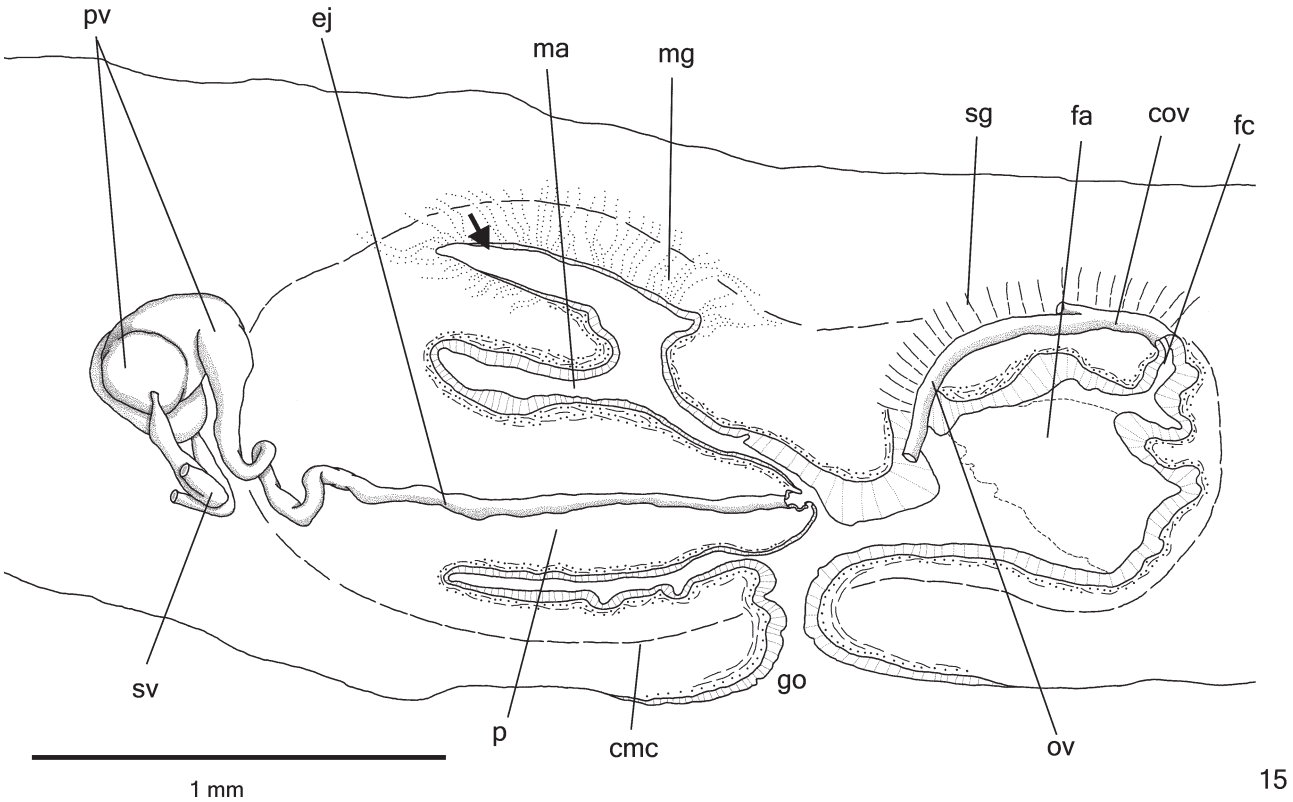
Reproductive organs. Testes in up to four irregular rows on either side of body, located beneath dorsal transverse mesenchymal muscles, between intestinal branches (Figs. 8, 10), begin slightly anteriorly to ovaries, in anterior third of body, and extend laterally to pharynx (Table 1). Sperm ducts dorsal to ovovitelline ducts, above or among fibres of sub-intestinal transverse mesenchymal musculature, in pre-pharyngeal region (Fig. 11). Spermiducal vesicles, beginning laterally to pharynx, enter terminally into proximal portion of prostatic vesicle (Figs. 14–15).

Prostatic vesicle, located near common muscle coat, with proximal portions forked, globose or ovoid, and distal portion wide and inverted-U shaped while outside penis bulb, narrowing and becoming sinuous after penetrating common muscle coat. In paratype MZU PL. 00284, forked portions show asymmetry, with left portion more elongate than right portion (Fig. 15). Forked portions with proximal end closer to ventral epidermis than to dorsal epidermis (Figs. 14–16). Ejaculatory duct sinuous, opening at tip of penis papilla in specimens MZU PL. 00283 and MZU PL. 00284 (Figs. 15, 17, 19), whereas close to ventral insertion of papilla in the holotype (Figs. 14, 20). Male atrium with some folds, sometimes with high and complex folding (Figs. 14–17), and a dorso-lateral invagination. Penis papilla either conical, almost symmetrical, or with irregular shape and asymmetrical (Table 1; Figs. 14–17).

Numerous cyanophil glands with amorphous secretion, as well as glands with erythrophil, finely granular secretion, open into both prostatic vesicle and ejaculatory duct, the latter more numerous in ejaculatory duct. Abundant erythrophil and cyanophil glands with amorphous secretion discharge through the penis papilla and epithelial lining of male atrium. In addition, heavily stained cyanophil glands, with finely granular secretion, open through penis papilla and numerous glands with mixed secretion (granules with a cyanophil peripheral part and an erythrophil core) concentrate their openings through a dorso-lateral invagination of male atrium (Figs 15–18). Muscularis of the penis papilla and male atrium comprised of mixed circular and longitudinal fibres.

Vitelline follicles (Figs. 8, 10–11) well developed in specimens MZU PL. 00283 and MZU PL. 00284, less developed in the holotype. Ovaries oval-elongate, approximately four times longer than wide (0.7 mm in its lateral axis), dorsal to ventral nerve plate, in anterior third of body (Fig. 13, Table 1). Ovovitelline ducts emerge dorso-laterally from median third of ovaries (Fig. 13) and run posteriorly among fibres of sub-intestinal transverse mesenchymal musculature (Fig. 11). Ascending portion of ovovitelline ducts located lateral to female atrium (Figs. 14, 15). Common glandular ovovitelline duct short, located dorsally to posterior third of female atrium (Figs. 14–16). Female genital duct dorso-anteriorly curved (Figs. 14–16). Female atrium oval-elongate with folded walls (Figs. 14–17), with similar length as that of male atrium or somewhat longer (Table 1). Paratype MZU PL. 00283 shows female atrium larger, partially occupied by elongate, conical fold that resembles a penis papilla proceeding from dorsal wall of male atrium (Fig. 17).

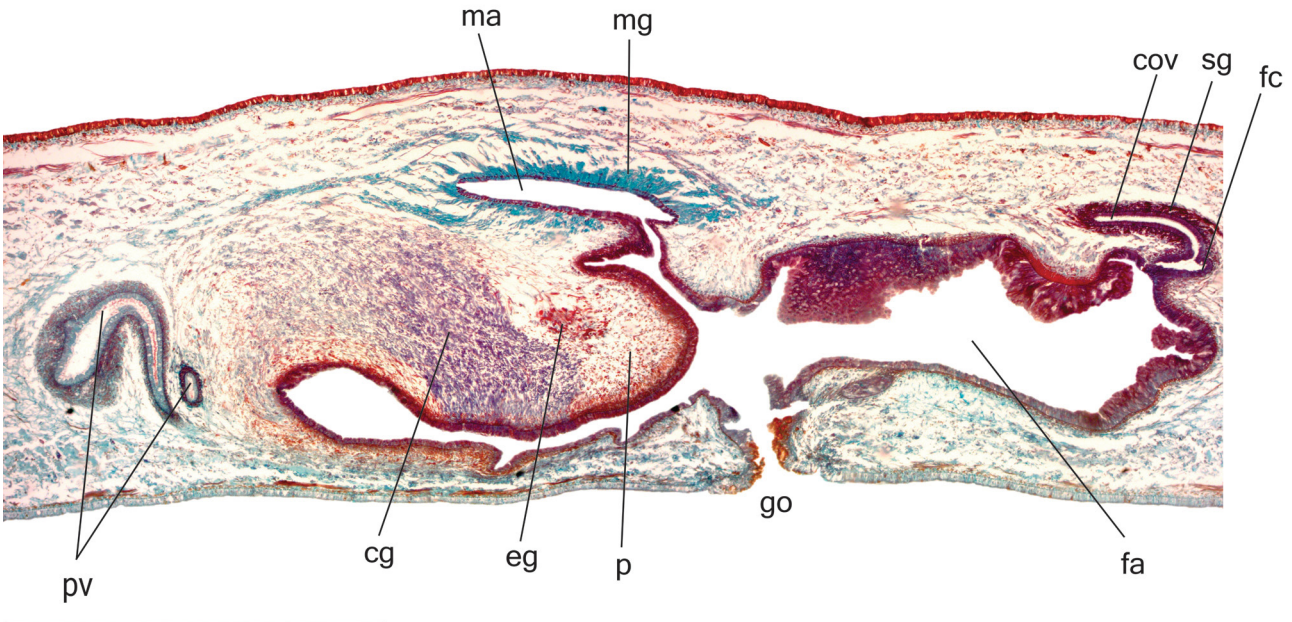




FIGURES 14–15. *Obama allandra* **sp. nov.**, (14–15) sagittal composite reconstructions of copulatory apparatus of the holotype (14) and paratype MZU PL. 00284 (15). Anterior tip to the left. The arrow indicates the invagination of the dorsolateral wall of the male atrium receiving numerous openings from glands with mixed secretion.

Epithelial lining of female genital duct and atrium tall columnar, irregular in height (15–90 μm thick), with stratified appearance in some parts of the female atrium (Fig. 16); epithelial cells with some lacunae containing secretion. Numerous cyanophil glands with amorphous secretion and scarcer erythrophil glands with fine granular secretion open into female duct and atrium.

Common muscle coat thin along both male and female atria composed of circular, longitudinal and oblique fibres. Male and female atria separated by dorsal fold, obliquely crossing the atria (Figs. 14, 17). Gonoduct almost vertical at sagittal plane (Figs. 14–17).



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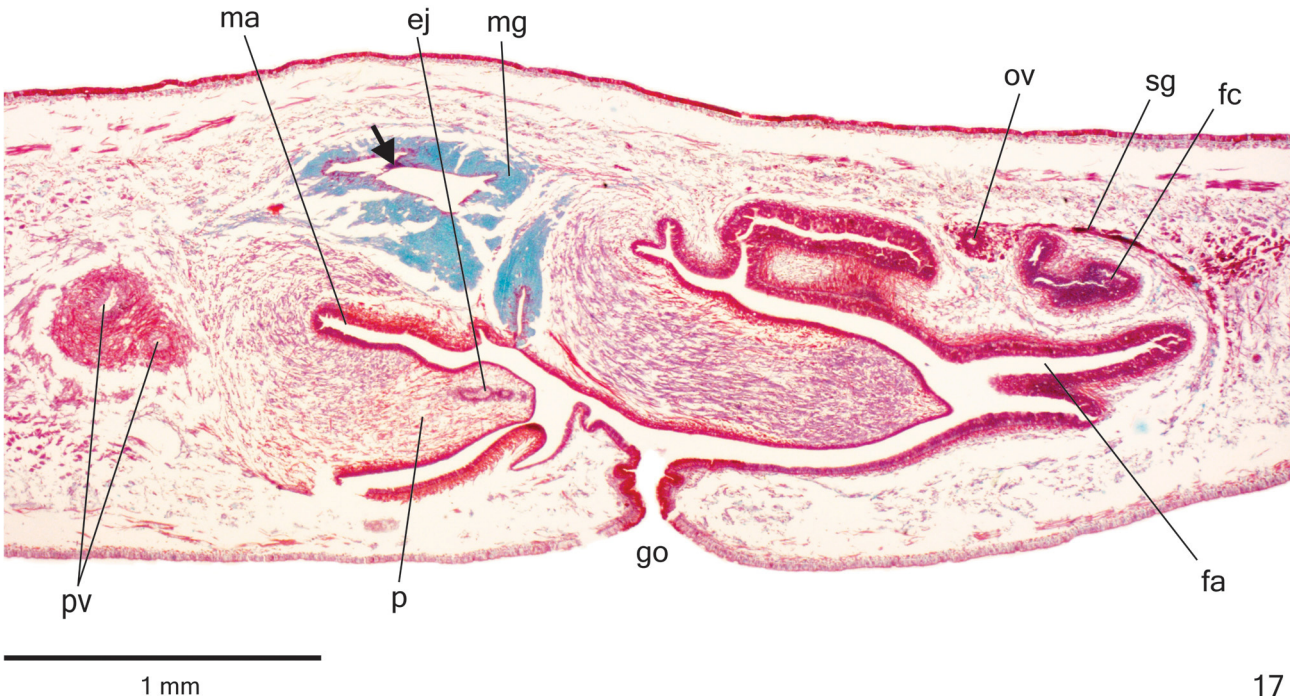

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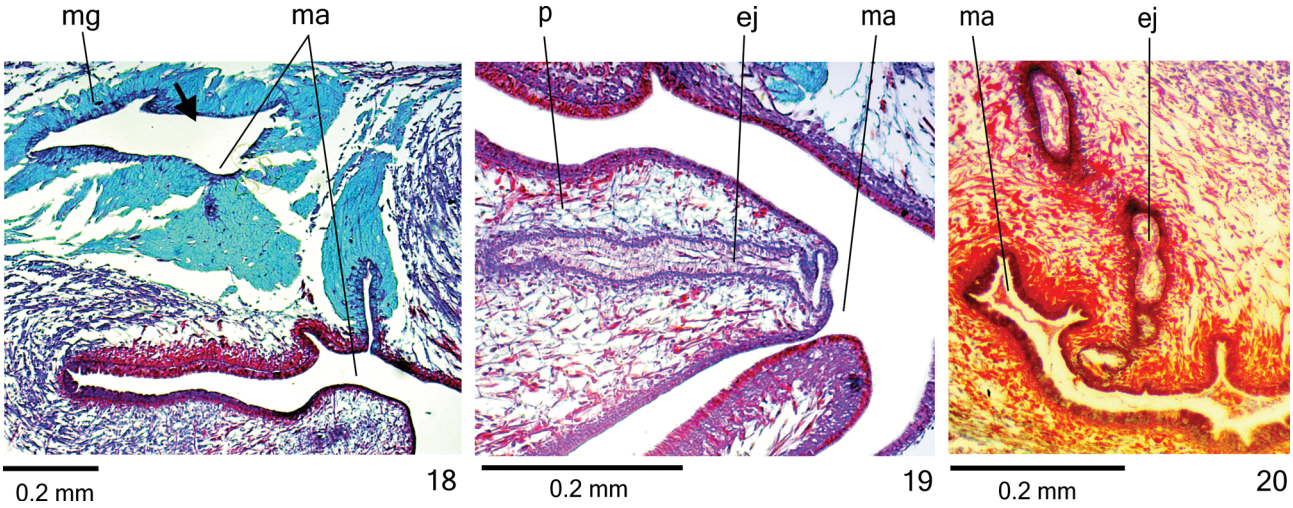
FIGURES 16–17. *Obama allandra* **sp. nov.**, microphotographs of the copulatory apparatus: (16) holotype in sagittal section; (17) paratype MZU PL. 00283 in sagittal section. Anterior tip to the left. The arrow indicates the invagination of the dorso-lateral wall of the male atrium receiving numerous openings from glands with mixed secretion.



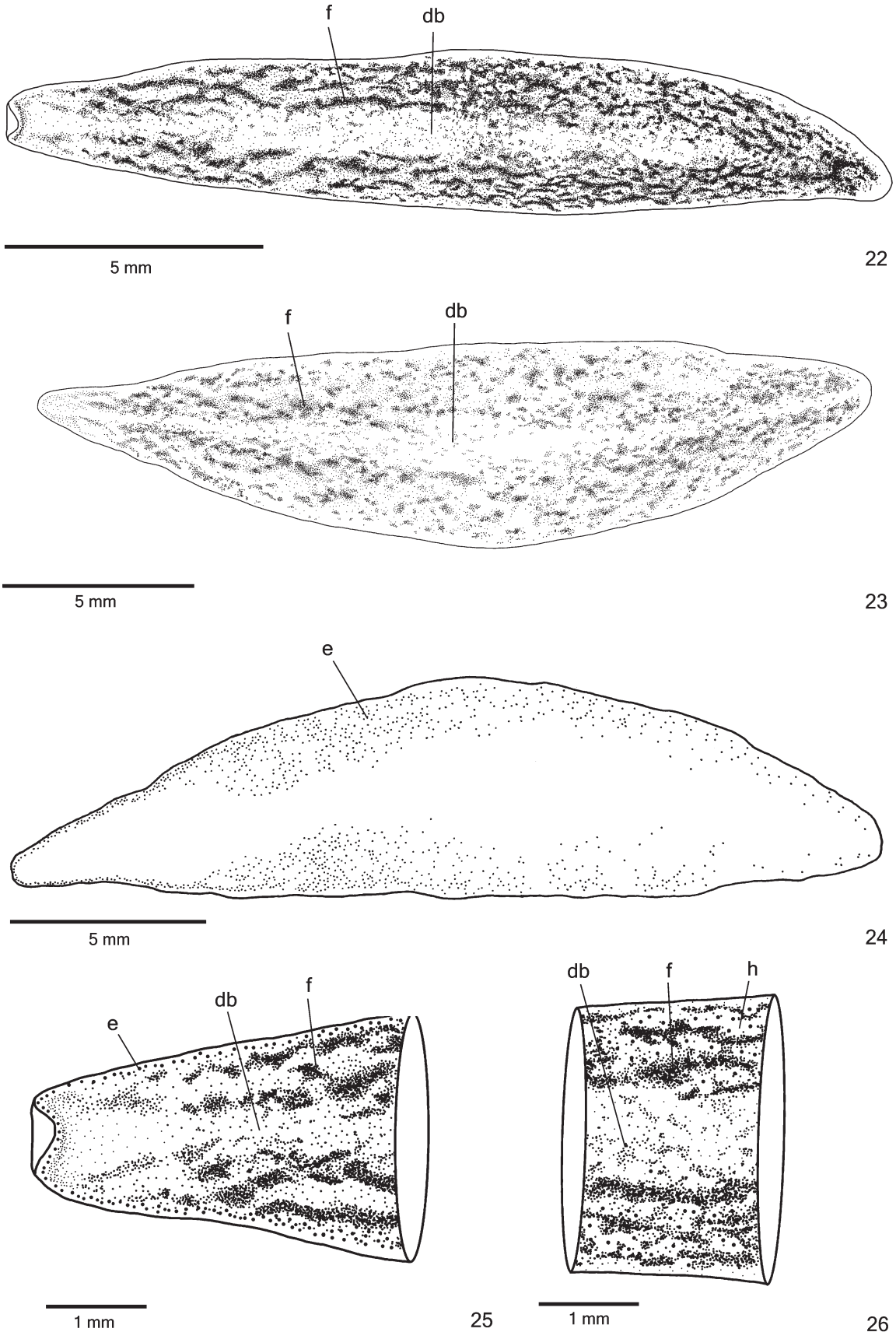
FIGURES 18–20. *Obama allandra* **sp. nov.**, microphotographs of the copulatory apparatus in sagittal sections: (18) detail of male atrium of paratype MZU PL. 00283; (19) detail of penis papilla and ejaculatory duct of paratype MZU PL. 00283; (20) detail of penis papilla and ejaculatory duct of the holotype. Anterior tip to the left. The arrow indicates the invagination of the dorso-lateral wall of the male atrium receiving numerous openings from glands with mixed secretion.

Obama tribalis sp. nov.

Etymology: The specific name, from latin *tribālis* (tribal), refers to the dorsal pigmentation which resembles the crossed stripes pattern of the tribal style drawings.

Type-material. Holotype: **MZUSP PL.2140**: *leg.* E. Federolf, 30 June 2013, Gravataí, state of Rio Grande do Sul, Brazil—anterior tip: transverse sections on 7 slides; anterior region at the level of the ovaries: sagittal sections on 42 slides; pre-pharyngeal region: transverse sections on 26 slides; copulatory apparatus: sagittal sections on 43 slides.

FIGURE 21. *Obama tribalis* **sp. nov.**, paratype MZU PL. 00288: photograph in dorsal view. Anterior tip to the left.



FIGURES 22–26. *Obama tribalis* **sp. nov.**: (22) dorsal pattern of pigmentation of paratype MZU PL. 00287; (23) dorsal pattern of pigmentation of the holotype; (24) eye pattern of the holotype; detail of eye pattern and pattern of pigmentation at anterior region (25) and median third (26) of body of paratype MZU PL. 00287. Anterior tip to the left.

Paratypes: MZU PL.00287: *leg*. E. Federolf, 30 June 2013, Gravataí, state of Rio Grande do Sul, Brazil—anterior tip: transverse sections on 13 slides; anterior region at the level of the ovaries: sagittal sections on 10 slides; pre-pharyngeal region: transverse sections on 4 slides; pharynx and copulatory apparatus: sagittal sections on 23 slides. **MZU PL.00288:** *leg.* E. Federolf, 30 June 2013, Gravataí, state of Rio Grande do Sul, Brazil—copulatory apparatus: horizontal sections on 12 slides.

Diagnosis: species of *Obama* with dorsal ground colour yellowish covered by irregular flecks, more concentrated laterally; pharynx cylindrical; prostatic vesicle with two portions, proximal portion forked and globose and distal portion funnel-shaped; penis papilla long, conical and symmetrical; male and female atria with slightly folded walls, and with ample communication.

Molecular diagnosis: this species includes all populations that cluster with sequences of specimens included in this study (GenBank accession numbers MH378885–MH378886) with significant support in an adequate molecular delimitation model.

Type-locality. Gravataí, Rio Grande do Sul (RS), Brazil.

Distribution: known only from the type-locality, Gravataí, RS, Brazil.

Description. External features. Body foliaceous and dorsal surface slightly convex; anterior and posterior tips rounded (Fig. 21). When creeping, maximum length 33 mm. After fixation, maximum length 23 mm. Mouth at third-fourth of body and gonopore at posterior fourth of body, in most specimens (Table 3).

Live specimens with dorsal ground colour yellowish, covered by greyish pigmentation in cephalic region (Fig. 21). Blackish pigmentation contours cephalic region and constitutes irregular flecks over dorsal surface, more concentrated laterally, sometimes forming a pattern that resembles crossed stripes at some points (Figs. 21–23). Median region of dorsal surface almost without flecks. Paratype MZU PL. 00287 with more abundant overlaying black pigmentation than holotype (Fig. 22); paratype MZU PL. 00288 with very few irregular flecks over dorsal surface (Fig. 21). Ventral surface greyish with yellowish margins. After fixation, dorsal ground colour fades.

Eyes, initially monolobate and uniserial, surround anterior tip (Figs. 24–25). After first millimetre of body, eyes become larger and spread onto dorsal surface, occupying maximum width of about one-third of body width on either side of body. Eyes become trilobate after one-fifth of body length and remain dorsal, but less numerous towards posterior tip (Fig. 24). Inconspicuous clear halos may occur around dorsal eyes (Fig. 26). Diameter of pigment cups about 30 μm.

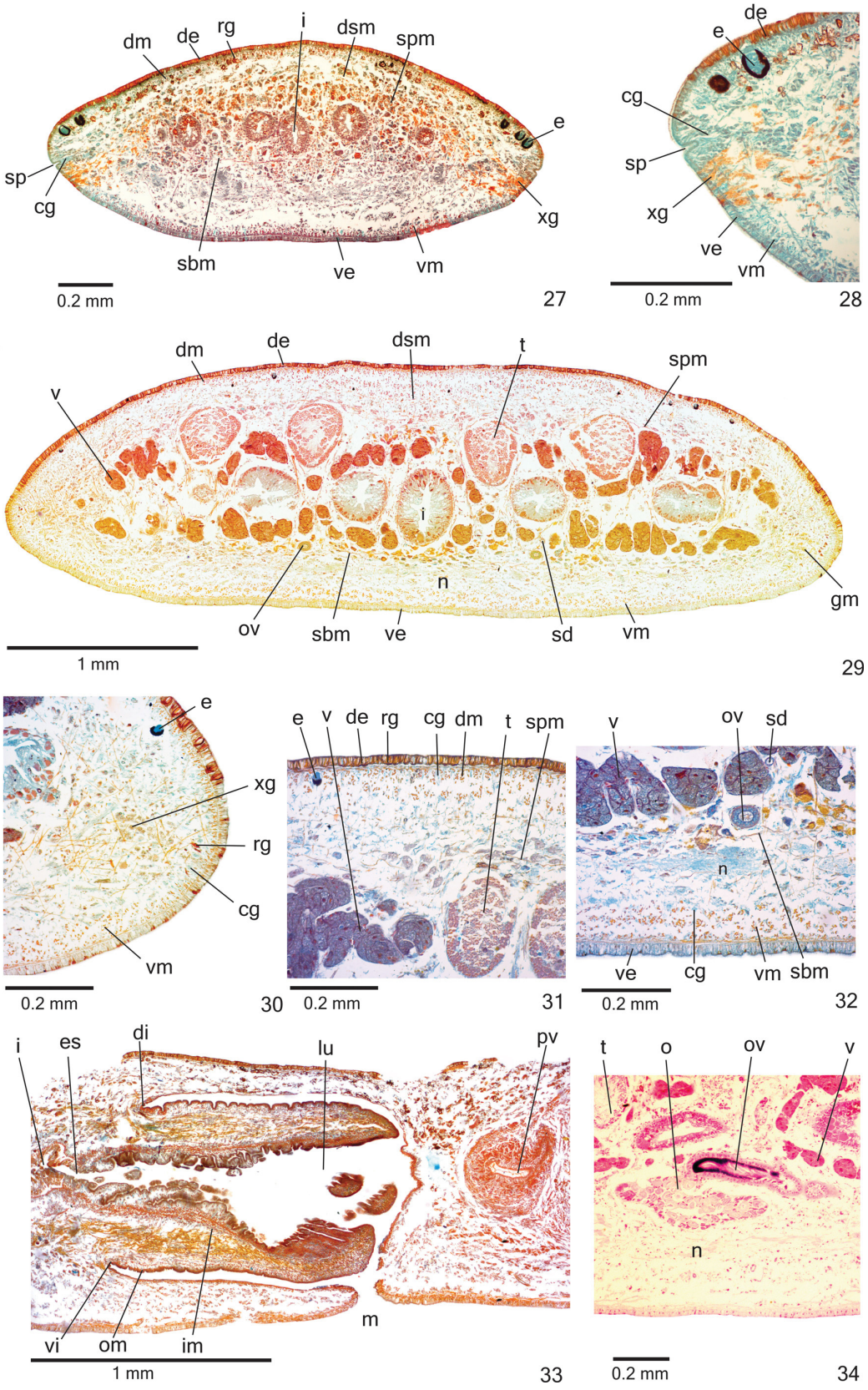
Sensory organs, epidermis and body musculature. Sensory pits (Figs. 27–28), as simple invaginations (20–40 μm deep), contour anterior tip and occur ventromarginally in irregular, single row in anterior third of body.

Glands of three types discharge through whole epidermis of pre-pharyngeal region: rhabditogen cells with xanthophil secretion (scarcer ventrally and with smaller rhabdites), cyanophil glands with finely granular secretion (Figs. 31–32) and sparser xanthophil glands with finely granular secretion. Creeping sole occupies whole body width. Glands of at least four types constitute the inconspicuous glandular margin (Figs. 29, 30), which is noticeable after first millimetre of body: numerous xanthophil glands and sparser cyanophil glands, both with coarse granular secretions, besides xanthophil and cyanophil glands with fine granules. Glands discharging through anterior tip of body similar to those of pre-pharyngeal region (Figs. 27–28).

Cutaneous musculature with usual three layers (circular, oblique and longitudinal layers); longitudinal layer with thick bundles (Figs. 29–32). Thickness of cutaneous musculature two times that of epidermis (Table 4). Ventral musculature slightly thicker than dorsal at sagittal plane in pre-pharyngeal region (Table 4). Thickness of cutaneous musculature gradually diminishes towards body margins and anterior tip.

Mesenchymal musculature (Figs. 29, 31–32) well developed, mainly composed of three layers: (1) dorsal subcutaneous, with decussate fibres (about 5–7 fibres thick), (2) supra-intestinal transverse (about 7–9 fibres thick) and (3) sub-intestinal transverse (about 7–13 fibres thick), besides fibres in various directions. Mesenchymal musculature less developed in anterior region than in pre-pharyngeal region (Fig. 27).

Pharynx. Pharynx cylindrical, nearly 7% of body length, occupies most of the pharyngeal pouch (Fig. 33). Pharyngeal insertions almost at same transversal level. Mouth located close to end of pharyngeal pouch (Fig. 33). Oesophagus short, with folded walls. Oesophagus: pharynx ratio 11–13%.



FIGURES 27–34. *Obama tribalis* **sp. nov.**, microphotographs of transverse (27–32) and sagittal sections (33–34; anterior tip to the left): (27) anterior region of body and (28) detail of the anterior region of body of paratype MZU PL. 00287; (29) pre-pharyngeal region; (30) detail of body margin of pre-pharyngeal region; (31) detail of dorsal surface of pre-pharyngeal region and (32) detail of ventral surface of pre-pharyngeal region of the holotype; (33) pharynx and (34) ovary of paratype MZU PL. 00287.

TABLE 3. Measurements, in mm, of specimens of *Obama tribalis* **sp. nov**. –: not measured; *: after fixation; DG: distance of gonopore from anterior end; DM: distance of mouth from anterior end; DMG: distance between mouth and gonopore; DPVP: distance between prostatic vesicle and pharyngeal pouch. The numbers given in parentheses represent the position relative to body length.

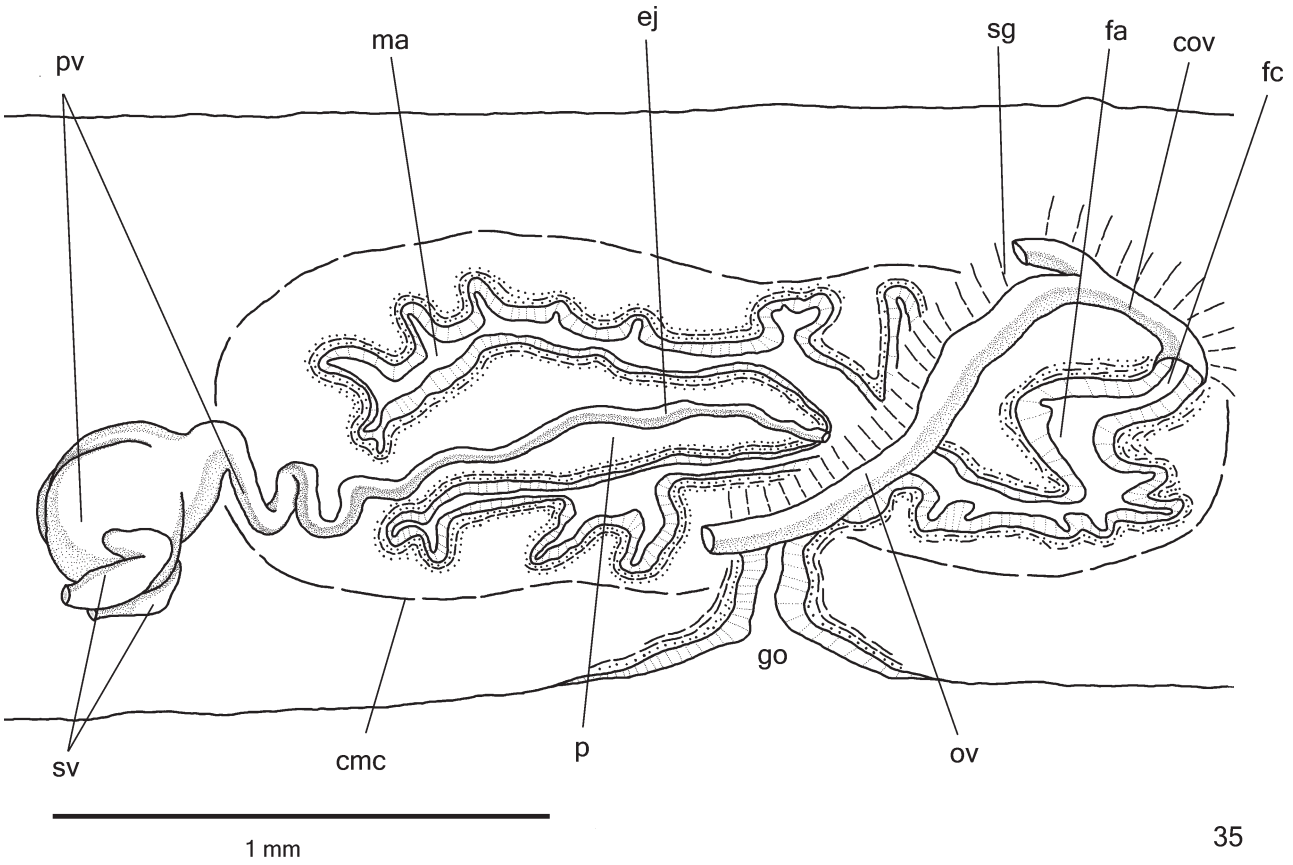
	Holotype	Paratype MZU PL.00287	Paratype MZU PL.00288
Maximum length in extension	32	21	29
Maximum width in extension	3.5	2	3
Length at rest	17	15	16
Width at rest	5	3.5	6
Length*	23	18	19
Width*	6	4	5
DM*	14 (61)	13 (73)	14 (74)
DG*	17 (74)	15 (83)	17 (89.5)
DMG*	3	2	3
DPVP*	0.5	0.17	-
Ovaries	5 (22)	5.4 (30)	-
Anteriormost testes	4.8 (21)	4.4 (24.5)	-
Posteriormost testes	11 (48)	10 (55.5)	-
Prostatic vesicle**	1.2	0.9	1.3
Penis papilla**	1	1	0.9
Male atrium**	0.9	0.7	0.9
Female atrium**	0.8	0.7	0.6

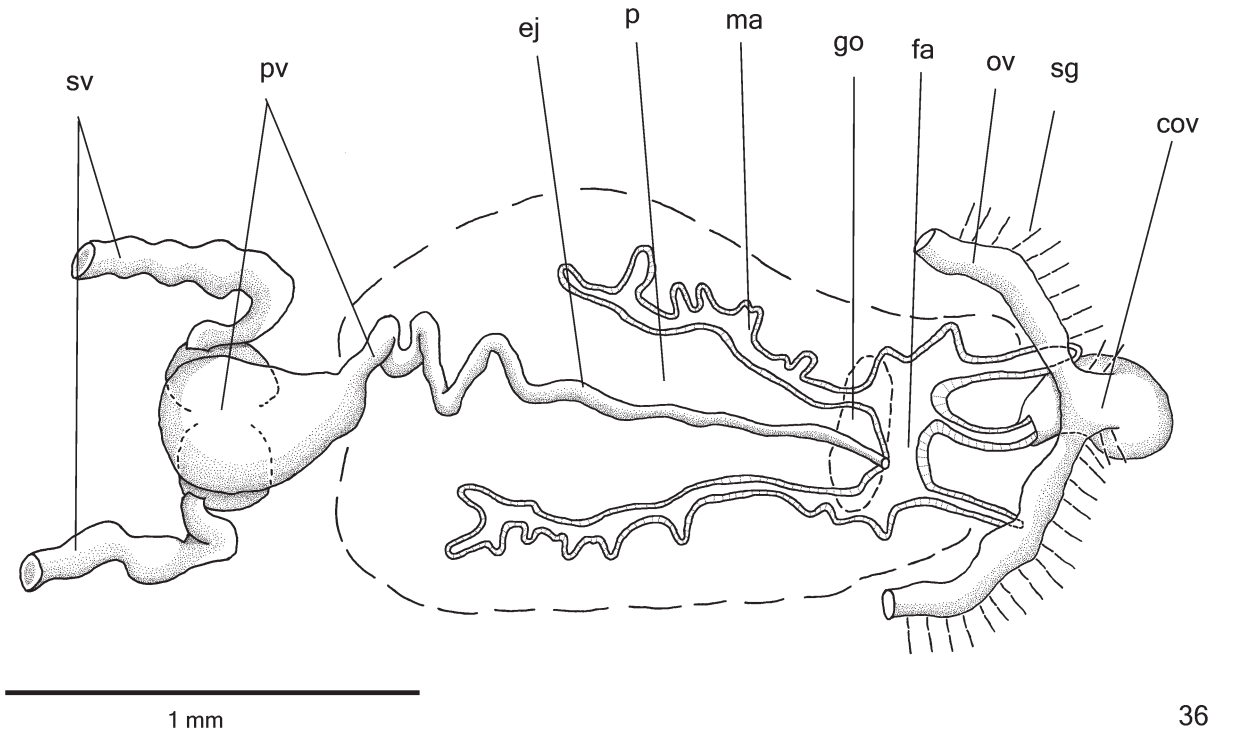
TABLE 4. Body height and cutaneous musculature in the median region of a transverse section of the pre-pharyngeal region, in micrometers, and ratio of the height of cutaneous musculature to the height of the body (mc:h index) of specimens of *Obama tribalis* **sp. nov.**

	Holotype	Paratype MZU PL. 00287
Dorsal cutaneous musculature	42	46
Ventral cutaneous musculature	51	58
Dorsal epidermis	20	11
Ventral epidermis	23	30
Body height	1219	1037
Mc:h (%)	8	10

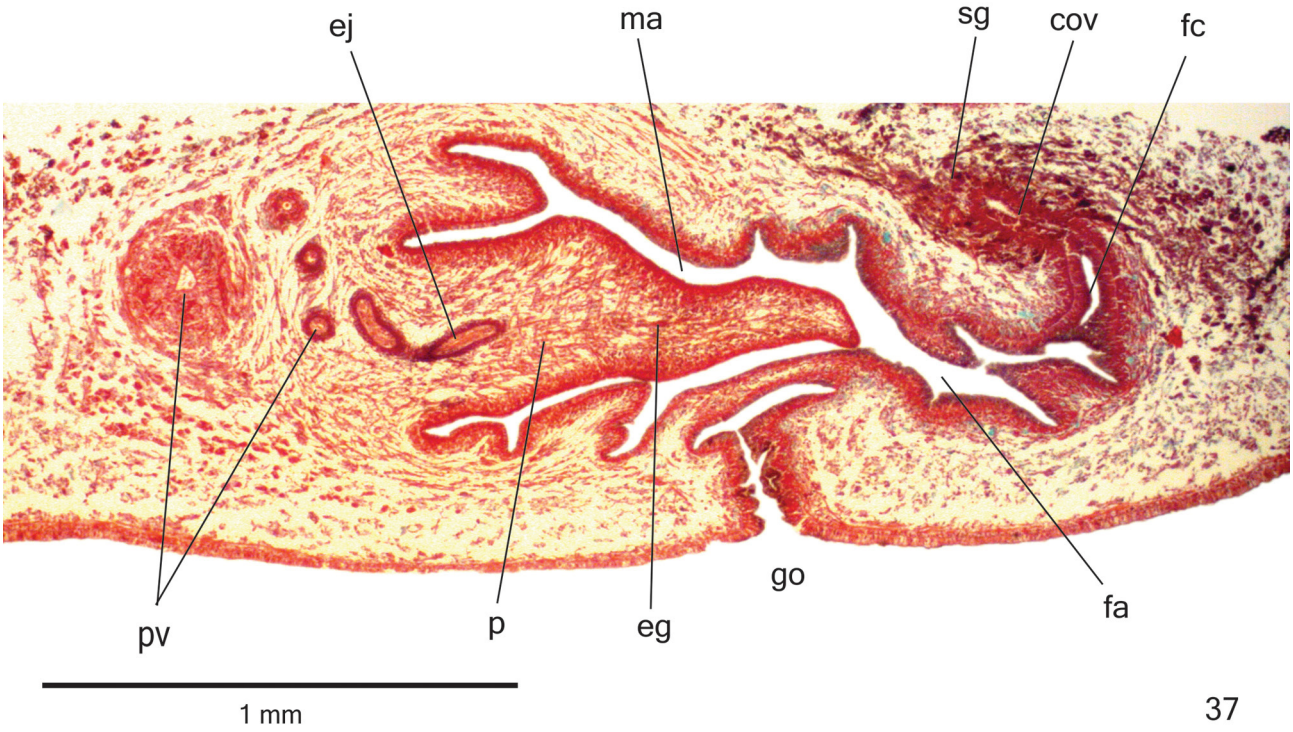
Reproductive organs. Testes in three irregular rows on either side of body, located beneath dorsal transverse mesenchymal muscles, between intestinal branches (Figs. 29, 31), begin slightly anteriorly to ovaries, in anterior third of body, and extend to near root of pharynx (Table 3). Sperm ducts dorsal to ovovitelline ducts, under or among fibres of sub-intestinal transverse mesenchymal musculature, in pre-pharyngeal region (Fig. 32). Spermiducal vesicles, located posteriorly to pharynx, enter laterally into proximal portion of prostatic vesicle (Figs. 35–36).

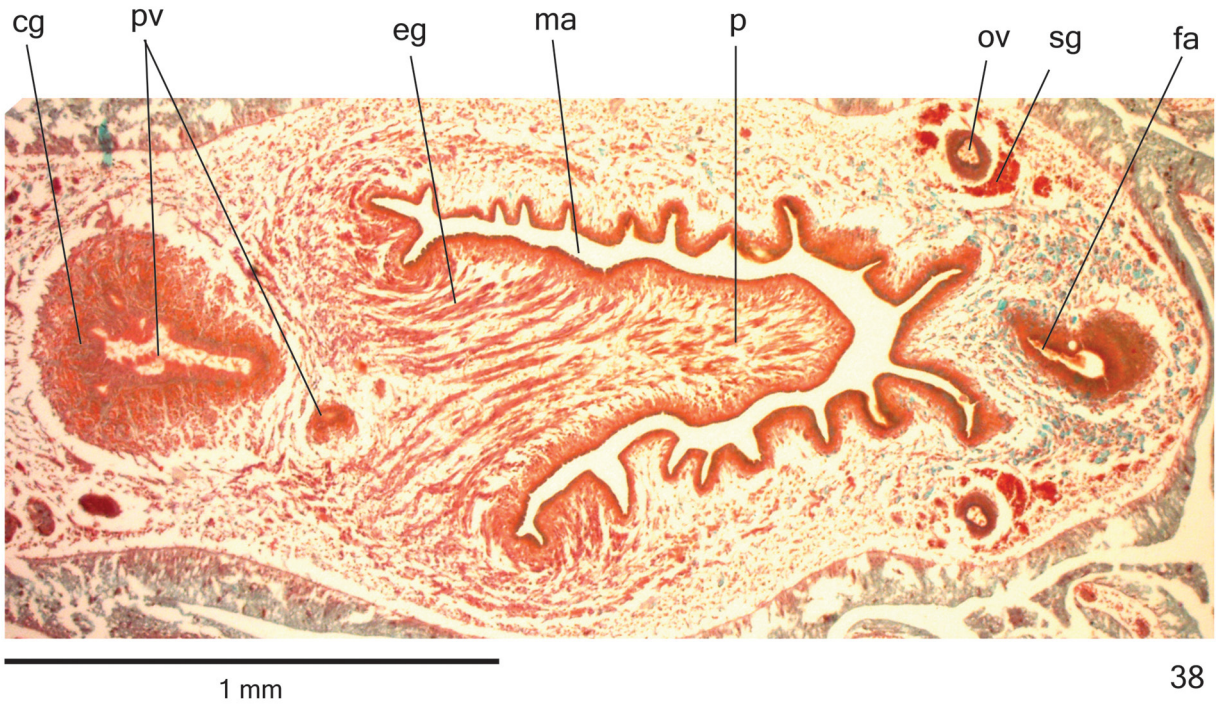
Prostatic vesicle, extrabulbar, located near common muscle coat, with two portions. Proximal portion forked and globose (Figs. 36, 38–39), being closer to ventral epidermis than to dorsal epidermis (holotype and paratype MZU PL. 00288) or equally distant to both epidermis (paratype MZU PL. 00287). Distal portion pear-shaped (Figs. 35–36). Ejaculatory duct almost straight, opening at tip of penis papilla (Figs. 35–36, 40). Male atrium with slightly folded walls. Penis papilla long (Table 3), conical and symmetrical, sometimes projecting into distal portion of female atrium (Figs. 35–38, 40).





FIGURES 35–36. *Obama tribalis* **sp. nov.**: (35) sagittal composite reconstruction of copulatory apparatus of the holotype; (36) horizontal composite reconstruction of copulatory apparatus of paratype MZU PL. 00288. Anterior tip to the left.





FIGURES 37–38. *Obama tribalis* **sp. nov.**, microphotographs of the copulatory apparatus: (37) paratype MZU PL. 00287 in sagittal section; (38) paratype MZU PL. 00288 in horizontal section. Anterior tip to the left.

Glands opening into the prostatic vesicle of two types: abundant glands with amorphous cyanophil secretion and scarcer glands with finely granular erythrophil secretion (Figs. 38–39); those opening into ejaculatory duct

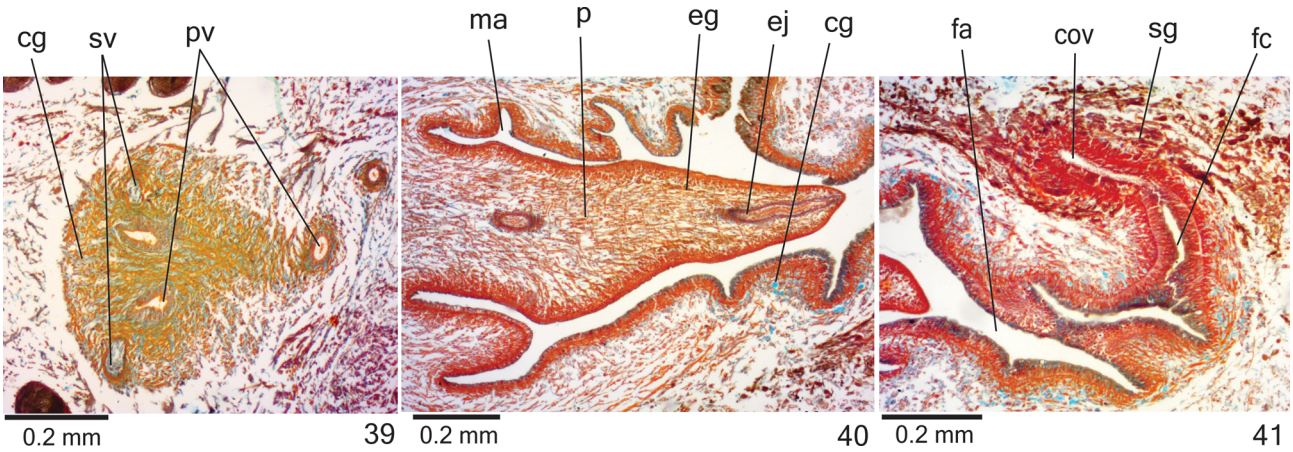
with amorphous cyanophil secretion. Glands opening through the penis papilla and epithelial lining of male atrium of three types. Abundant glands with finely granular erythrophil secretion, as well as glands with amorphous cyanophil secretion (Figs. 37–40), besides a third type of gland with xanthophil secretion. The latter usually is finely granular, but changes to a coarse granular secretion when opens close to the dorsal insertion of penis papilla. Muscularis of the penis papilla and male atrium mainly comprised of circular fibres mixed with some longitudinal fibres.

Vitelline follicles (Figs. 29, 31–32, 34) well developed in all specimens analysed, situated between intestinal branches. Ovaries oval-elongate, approximately three times longer than wide (0.2 mm in its lateral axis), dorsal to ventral nerve plate, in anterior fourth of body (Fig. 34, Table 3). Ovovitelline ducts emerge dorsally from median third of ovaries (Fig. 34) and run posteriorly above nerve plate, among fibres of sub-intestinal transverse mesenchymal musculature (Fig. 32). Ascending portion of ovovitelline ducts located lateral to female atrium (Fig. 35). Common glandular ovovitelline duct short, located dorsally to posterior third of female atrium (Figs. 35–37, 41). Female genital duct dorso-anteriorly curved. Female atrium ovoid, showing folds that narrow its lumen (Figs. 37, 41), almost as long as male atrium, excepting in paratype MZU PL. 00288 (Table 3).

Common muscle coat poorly developed, with circular, longitudinal and oblique fibres, thicker around male atrium than around female atrium. Male and female atria with ample communication, without separating folds (Figs. 35–38). Gonoduct vertical at sagittal plane (Figs. 35, 37).

Molecular results and phylogenetic analyses. The results from the comparison of species of *Obama*, including sequences downloaded from NCBI, showed that the genetic distances are highly divergent among specimens. In general, the mean intraspecific variations ranged from 0% to 2.1%, except for *O. braunsi* (2.8%), *O. baptistae* (4.3%), *O. carinata* (6.5%) and *O. eudoximariae* (8.7%). Meanwhile, the mean interspecific divergences vary between > 6% to < 17% (Table 5). The results showed that the divergence between the new species herein described (*Obama allandra* vs. *Obama tribalis*) was larger than 7% (mean 7.6%), as high as the other interspecific divergences.

Maximum likelihood (RAxML) and Bayesian (MrBayes) phylogenetic trees, which were constructed based on the partial sequences of the COI gene, show a comparable topology (Figs. 42, 43). Both analyses resulted in similar, highly supported trees (bootstrap> 90% and P> 0.95), recovering the monophyly for the currently recognized species of *Obama* herein analysed (Figs. 42, 43). All phylogenetic analyses showed a sister relationship between both new species herein described and *O. maculipunctata*, which occurs in areas of *Araucaria* Forest in southern Brazil (Rossi *et al.* 2015).



FIGURES 39–41. *Obama tribalis* **sp. nov.**, microphotographs of the copulatory apparatus: (39) detail of prostatic vesicle of paratype MZU PL. 00288 in horizontal section; (40) detail of penis papilla and ejaculatory duct of paratype MZU PL. 00287 in sagittal section; (41) detail of female genital duct and common ovovitelline duct of paratype MZU PL. 00287 in sagittal section. Anterior tip to the left.

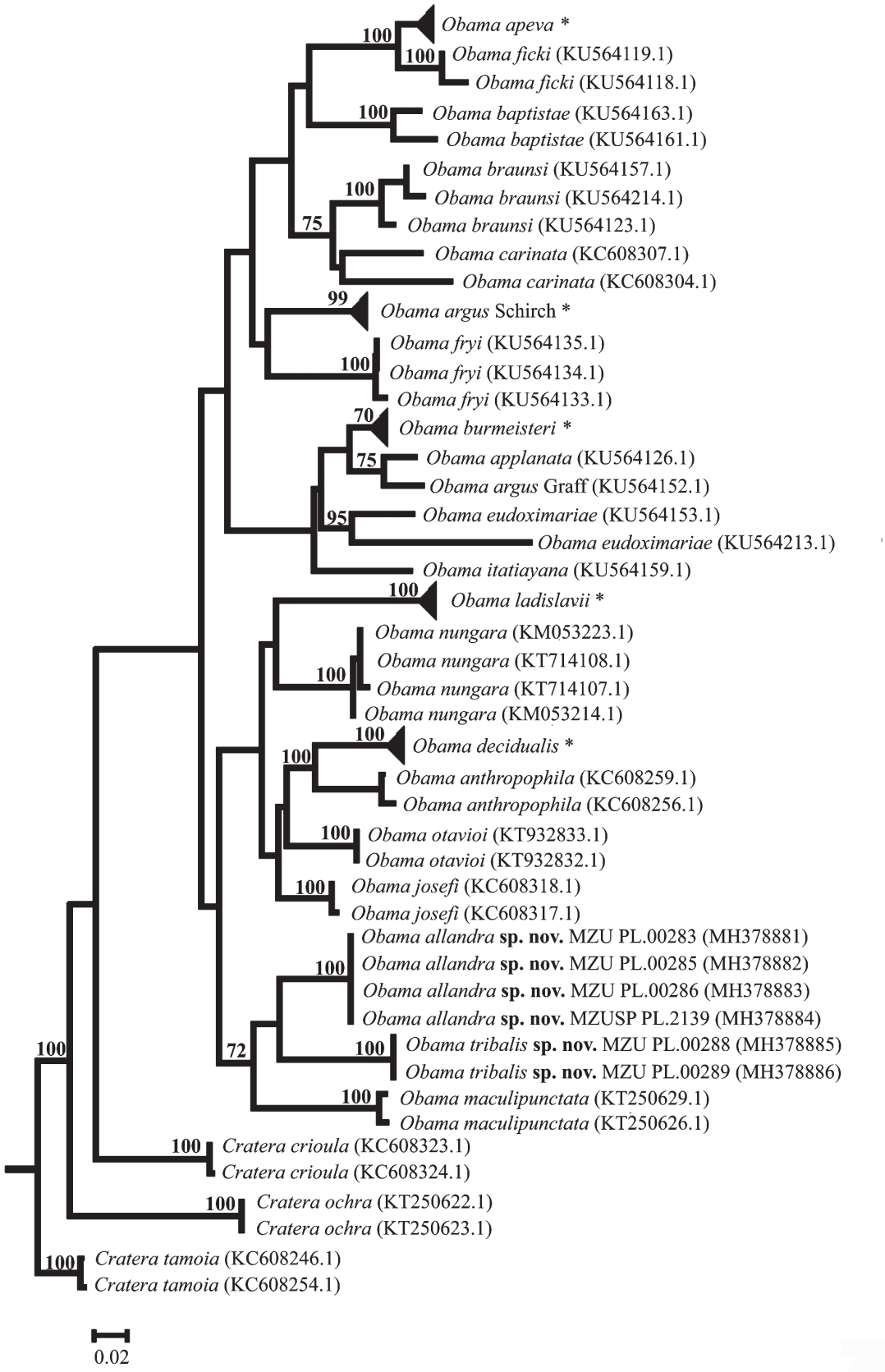


FIGURE 42. Phylogenetic tree inferred from maximum likelihood (GTR-GAMMA model), based on 653 bp of cytochrome c oxidase subunit I gene, performed by using RAxML. Specific values for those nodes that were weakly supported in the analyses (bootstrap support<70%) are not reported in the tree. Scale bar represents substitutions per nucleotide site. GenBank accession numbers are in parentheses, excepting accession numbers of the compressed specimens, which were indicated by an asterisk in this figure and detailed in Appendix S1.

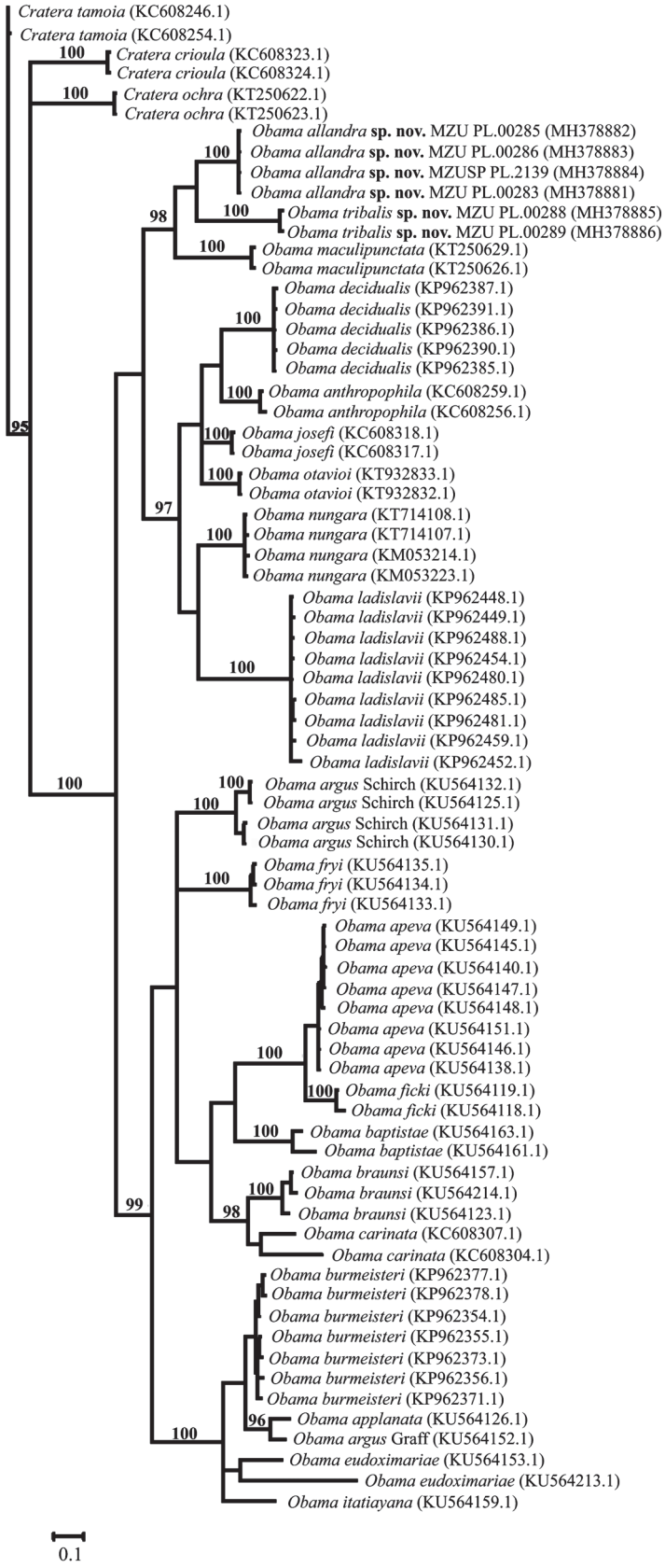


FIGURE 43. Bayesian inference tree topology based on 653 bp of cytochrome c oxidase subunit I gene. The Bayesian phylogeny was constructed under the GTR+I+G substitution model. Numbers on nodes indicate posterior probabilities of the Bayesian method. Scale bar represents substitutions per nucleotide site. GenBank accession numbers are in parentheses.

TABLE 5. Intraspecific (highlighted in gray) and interspecific nucleotide substitution rates expressed as a percentage (%) estimated for COI sequences by Kimura 2-parameter (Kimura 1980) and the same database used in the phylogenetic analyses.

		1[A]	2[B]	3[C]	4[D]	5[E]	6[F]	7[G]	8[H]	9[I]	10[J]	11[L]	12[M]	13[N]	14[O]	15[P]	16[Q]	17[R]	18[S]	19[T]	20[U]	21[V]	22[W]	23[X]	24[V]
1	*O. allandra* **sp. nov.**	**0.0**																							
2	*O. tribalis* **sp. nov.**	7.6	**0.0**																						
3	*O. ladislavii*	9.5	16.8	**0.3**																					
4	*O. burmeisteri*	8.8	9.9	6.0	**0.8**																				
5	*C. ochra*	10.1	11.1	14.8	9.4	**0.0**																			
6	*O. fryi*	9.5	2.4	8.0	13.0	11.5	**0.7**																		
7	*O. braunsi*	13.5	9.1	12.3	9.4	16.5	11.9	**2.8**																	
8	*O. baptistae*	14.2	7.0	9.9	7.1	15.5	9.7	10.0	**4.3**																
9	*O. apeva*	14.8	8.8	9.9	6.8	18.6	9.1	7.6	6.5	**0.1**															
10	*O. ficki*	16.1	7.6	8.7	7.9	20.0	8.0	8.0	7.6	1.0	**0.1**														
11	*O. argus* Schirch	11.1	7.0	3.7	10.0	14.8	8.6	14.3	9.4	9.3	8.2	**2.1**													
12	*O. argus* Graff	12.3	8.7	7.6	3.8	17.4	9.1	10.3	9.3	9.9	9.9	11.7	*												
13	*O. applanata*	11.1	11.1	9.9	4.9	13.6	11.5	10.0	10.5	11.1	12.3	13.5	3.2	*											
14	*O. eudoximariae*	14.8	9.9	11.1	8.1	14.8	10.3	14.4	9.9	10.0	10.0	11.7	8.2	7.6	**8.7**										
15	*O. itaiayana*	13.5	6.5	7.6	4.9	16.1	6.8	11.5	8.2	8.8	7.6	9.3	2.1	4.2	6.5	*									
16	*O. otavioi*	13.5	8.7	12.3	16.8	16.1	11.1	15.3	14.2	11.1	9.9	11.1	16.0	16.1	14.8	13.5	**0.0**								
17	*O. josefi*	9.9	7.6	11.1	10.6	9.9	7.6	13.5	10.5	12.3	13.5	11.1	11.1	7.6	11.1	9.9	8.8	**0.0**							
18	*O. anthropophila*	7.6	6.5	7.6	14.2	12.4	6.5	14.8	12.9	13.6	12.3	7.6	13.5	14.8	13.5	11.1	7.6	7.6	**0.0**						
19	*O. decidualis*	9.9	6.5	9.9	14.2	12.4	6.8	14.8	12.9	13.5	12.3	9.9	13.5	13.5	12.9	11.1	11.1	9.9	7.6	**0.0**					
20	*C. tamoia*	11.7	4.8	9.3	11.2	11.7	5.2	14.6	11.1	12.9	11.7	9.9	8.2	10.5	12.9	5.9	12.9	9.3	9.3	9.4	**1.1**				
21	*C. crioula*	7.6	4.2	7.6	7.1	8.8	4.6	7.6	9.3	11.1	12.3	10.5	7.6	6.5	12.3	8.7	13.5	5.3	8.7	8.8	7.1	**0.0**			
22	*O. nungara*	5.3	6.5	5.3	9.4	8.8	6.9	13.1	8.2	8.8	9.9	5.3	9.9	8.7	8.7	8.8	8.7	5.3	5.3	6.5	8.2	6.5	**0.0**		
23	*O. carinata*	9.4	6.5	5.9	8.9	14.8	8.0	9.2	9.7	9.3	9.4	8.2	8.2	8.	13.5	8.8	9.9	8.7	9.9	9.9	8.2	5.4	6.5	**6.5**	
24	*O. maculipunctata*	11.1	7.6	4.3	10.6	12.3	8.0	13.5	11.7	12.3	11.1	5.9	9.9	9.9	11.1	7.6	12.3	8.7	7.6	9.9	9.3	7.6	5.3	7.6	**0.0**

Accession numbers in GenBank: [A] (MH378881, MH378882, MH378883, MH37888); [B] (MH378885, MH378886); [C](KP962485.1, KP962481.1, KP962488.1, KP962480.1, KP962454.1, KP962459.1, KP962449.1, KP962448.1, KP962452.1); [D](KP962377.1, KP962378.1, KP962354.1, KP962356.1, KP962355.1, KP962373.1, KP962371.1);); [E](KT250622.1, KT250623.1); [F](KU564135.1, KU564134.1, KU564133.1); [G](KU564157.1, KU564214.1); [H](KU564163.1, KU564161.1); [I](KU564149.1, KU564147.1, KU564145.1, KU564140.1, KU564148.1, KU564138.1, KU564146.1, KU564151.1); [J](KU564119.1, KU564118.1); [L](KU56413.1, KU56413.1, KU56413.1, KU56412.1); [M](KU564152.1); [N](KU564126.1); [O](KU56415.1, KU564213.1); [P](KU564159.1); [Q](KT932833.1, KT932832.1); [R](KC608318.1, KC608317.1); [S](KC60825.1, KC60826.1); [T](KP962391.1, KP962387.1, KP962386.1, KP962385.1, KP962390.1); [U](KC608246.1, KC608254.1); [V](KC608323.1, KC608324.1); [W](KM053223.1, KM053214.1; KT714108.1, KT714107.1); [X](KC608307.1, KC608304.1); [Y](KT250629.1, KT250626.1). * Only one specimen.

Notes on ecology and distribution. During inventories of land flatworms in areas of *Araucaria* moist forest located in the states of Paraná and Santa Catarina, in south Brazil, *Obama allandra* was found only in its type-locality, the *Araucaria* Natural Heritage Private Reserve, state of Paraná, in a site showing an initial stage of regeneration with poorly developed understorey (Rossi & Leal-Zanchet 2017). The species showed moderate abundance during both day and night samplings between May 2014 and July 2015. *Obama tribalis* occurs in an unprotected, private area belonging to an urban environment, located about 500km south from the type-locality of *O. allandra*, at the northeast hillside of Rio Grande do Sul (southern Brazil). Specimens of *O. tribalis* were collected during occasional samplings, without standardized effort. The vegetation of the type-locality of *O. tribalis* is a secondary forest that represents a remnant of the original forest (semi-deciduous forest), which covered the metropolitan region of Porto Alegre.

Discussion

Both new species herein described can be easily assigned to the genus *Obama* Carbayo *et al*., 2013, by presenting its diagnostic features, such as body broad and flat, eyes mono- and trilobated, prostatic vesicle extrabulbar and with short anterior branches, and female canal dorso-anteriorly flexed (Carbayo *et al*. 2013). Comparisons of both new species with their congeners using intraspecific and interspecific variations of the COI gene, as well as the phylogenetical analyses, support the results of the morphological analyses.

Considering general external features and characteristics of the pharynx and copulatory apparatus, *O. allandra* and *O. tribalis* share similar characteristics with *O. argus* (Graff, 1899), *O. burmeisteri* (Schultze & Muller, 1857), *O. evelinae* (Marcus, 1951), *O. itatiayana* (Schirch, 1929), *O. riesteri* (CG Froehlich, 1955) and *O. polyophthalma* (Graff, 1899). These species show cylindrical pharynx, symmetrical penis papilla and prostatic vesicle with forked proximal portion globose (Graff 1899; Riester 1938; Marcus 1951; Froehlich 1959). Hence, in the following comparative discussion we discuss *O. allandra* and *O. tribalis* in relation to these species.

Obama allandra sp. nov.

By presenting a median band yellowish and lighter on the anterior region of body, *O. allandra* can be distinguished from *O. argus*, *O. burmeisteri*, *O. evelinae,* and *O. riesteri,* which show a stripped pattern along the entire body (Graff 1899; Schirch 1929; Riester 1938; Marcus 1951; CG Froehlich 1955), and from *O. polyophthalma*, which has irregular flecks without forming stripes or bands. In addition, *O. polyophthalma* shows conspicuous clear halos surrounding the eyes (Graff 1899; Froehlich 1956), whereas in *O. allandra*, clear halos are inconspicuous.

With respect to the copulatory organs, by presenting male atrium with folded walls, *O. allandra* differs from these six species, which show male atrium unfolded, but resembles *O. polyophthalma* (Graff 1899; Riester 1938; Marcus 1951; CG Froehlich 1955). *Obama allandra* and *O. polyophthalma* show similar overall characteristics in their copulatory organs, with high variability regarding male atrium and penis papilla shape. Besides the external features commented above, histological details of the copulatory organs can be used to distinguish both species. In *O. allandra* there is an invagination of the dorsolateral wall of the male atrium, which receives numerous openings of glands containing a predominantly cyanophil secretion, (mixed secretion with a cyanophil peripheral part and an erythrophil core). In contrast, in *O. polyophthalma*, the dorsal wall shows no invagination and therefore erythrophil glands open directly through the dorsal wall of the male atrium (CG Froehlich 1956).

Obama tribalis sp. nov.

By having yellowish dorsal colour covered by irregular flecks, *O. tribalis* differs from *O. argus*, *O. burmeisteri*, *O. evelinae* and *O. riesteri* which show a stripped pattern (Graff 1899; Schirch 1929; Riester 1938; Marcus 1951). *Obama tribalis,* showing a greyish ventral colour, can be distinguished from *O. itatiayana,* which has reddish ventral colour. In addition, *O. tribalis* shows inconspicuous clear halos surrounding the eyes, whereas in *O. polyophthalma* clear halos are conspicuous, as stated above (Schirch 1929; EM Froehlich 1955; Froehlich 1959).

Regarding the internal anatomy, in *O. tribalis* the penis papilla occurs only inside the male atrium, thus differing from *O. burmeisteri* and *O. riesteri*, in which the penis papilla is longer, occupying almost the entire female atrium length (Riester 1938; Marcus 1951). In addition, *O. riesteri* shows a short male atrium, with the gonoduct closer to the insertions of the penis papilla (Riester 1938), whereas in *O. tribalis* the male atrium length is similar to that of the female atrium. By having an ovoid and folded female atrium, *O. tribalis* can be distinguished from *O. argus* and *O. itatiayana*, in which the female atrium is funnel-shaped and unfolded (Graff 1899; Schirch 1929; Riester 1938; Froehlich 1959) and *O. polyophthalma*, in which the female atrium has an ample cavity (Froehlich 1956). Since *O. tribalis* has a straight ejaculatory duct, it differs from *O. evelinae*, which shows a sinuous ejaculatory duct (Marcus 1951).

Obama tribalis differs from *O. allandra* by its external features and details of the copulatory organs. Although both species exhibit part of the dorsal surface covered by irregular flecks, *O. allandra*, by showing a yellowish median band, which is lighter on anterior region of body, can be distinguished from *O. tribalis*, which shows a homogeneously yellowish dorsal colour. With respect to the copulatory apparatus, both species present a prostatic vesicle with forked proximal portion, but the distal portion is pear-shaped in *O. tribalis*, whereas it is wide and inverted-U shaped in *O. allandra*. Some details of the copulatory apparatus, such as the occurrence of glands of the mixed type, opening into an invagination of the dorsolateral wall of the male atrium in *O. allandra*, are absent in *O. tribalis*.

In addition, we extend our comparative analysis of *O. allandra* and *O. tribalis* to species of *Geoplana* incertae sedis, since some of them have general characteristics similar to species of *Obama*. Among the 47 species indicated by Carbayo *et al*. (2013) as *Geoplana* incertae sedis, only four species show characteristics of the pharynx and copulatory apparatus similar to those of *O. allandra* and *O. tribalis*. They are *Geoplana beckeri* (Froehlich, 1959), *Geoplana caya* (du Bois-Reymond Marcus, 1951), *G. hina* (Marcus, 1951) and *G. toriba* (Froehlich, 1958). By presenting a median band only in the anterior region of the body, *O. allandra* differs from *G. hina* and *G. toriba,* which show a stripped pattern along the entire body length (Marcus 1951; Froehlich 1958). *Obama allandra* shows initially marginal eyes, which become dorsal posteriorly, differing from *G. beckeri,* which has dorsal eyes on the anterior tip. It also differs from *G. caya*, which has only marginal eyes (Froehlich 1959; du Bois-Reymond Marcus 1951). By having a yellowish dorsal colour without stripes or bands, *O. tribalis* differs from all these species of *Geoplana* incertae sedis, which have striped patterns. With respect to the copulatory organs, *O. allandra* presents a distal portion of prostatic vesicle inverted-U shaped and wide, differing from these four species, which have the distal portion of prostatic vesicle tubular in shape (du Bois-Reymond Marcus 1951; Marcus 1951; du Bois-Reymond Marcus 1958; Froehlich 1958). *Obama tribalis*, by having the unforked portion of the prostatic vesicle pear-shaped, can be distinguished from these four species, which have the prostatic vesicle tubular shaped and sinuous (du Bois-Reymond Marcus 1951; Marcus 1951; du Bois-Reymond Marcus 1958; Froehlich 1958).

Regarding the molecular phylogeny herein presented, based on the mitochondrial marker cytochrome *c* oxidase subunit I gene (COI), likelihood and Bayesian analyses invariably recovered the terminal clades with moderate and good support values. However, the segment of the COI gene used in the analyses demonstrated a lack of sufficient information to solve most relationships between the closely related congeneric species. This result was expected, since the COI gene does not show good resolution at higher taxonomic levels (Luo *et al.* 2011; Huang *et al.* 2013). Our results indicate the need to use at least 600 bp of the gene COI in the definitions of interspecific divergences and for species delineation, at least for the genus *Obama*. Considering length limitation to determine the threshold taxonomic, the short sequences of *O. marmorata* available on GenBank (297 pb) were insufficient to determine the taxonomic divergence with *O. nungara*, closely related species.

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APPENDIX S1. Specimens used in the study, their sampling locality and the corresponding GenBank accession numbers for the gene studied. –: unavailable information; MG: state of Minas Gerais; PR: state of Paraná; RJ: state of Rio de Janeiro; RS: state of Rio Grande do Sul; SC: state of Santa Catarina; SP: state of São Paulo.

Species	Specimen's accession number	Sampling locality	Latitude	Longitude	Identification	COI	References
Obama allandra **sp. nov.**	MZUSP PL.2139	General Carneiro, PR (Brazil)	-26.396	-51.405	Holotype	MH378884	In this study
	MZU PL. 00283	General Carneiro, PR (Brazil)	-26.396	-51.405	Paratype	MH378881	In this study
	MZU PL. 00285	General Carneiro, PR (Brazil)	-26.396	-51.405	Paratype	MH378882	In this study
	MZU PL. 00286	General Carneiro, PR (Brazil)	-26.41	-51.356	Paratype	MH378883	In this study
Obama tribalis **sp. nov.**	MZU PL. 00288	Gravataí, RS (Brazil)	-29.803	-50.931	Paratype	MH378885	In this study
	MZU PL. 00289	Gravataí, RS (Brazil)	-29.803	-50.931	Paratype	MH378886	In this study
Obama anthropophila Amaral, Leal-Zanchet & Carbayo, 2015	MZUSP PL. 1007	Parque Nacional da Serra de Itajaí, SC (Brazil)	-27.048	-49.092	GenBank	KC608256.1	Carbayo *et al*. 2013
	MZUSP PL. 698	Parque Nacional da Serra de Itajaí, SC (Brazil)	-27.048	-49.092	GenBank	KC608259.1	Carbayo *et al*. 2013
Obama apeva (Froehlich, 1959)	MZUSP PL. 1610	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KU564149.1	Carbayo *et al*. 2016b
	MZUSP PL. 1615	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KU564147.1	Carbayo *et al*. 2016b
	MZUSP PL. 1614	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KU564145.1	Carbayo *et al*. 2016b
	MZUSP PL. 1613	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KU564140.1	Carbayo *et al*. 2016b
	MZUSP PL. 1610	São Bonifácio, SC (Brazil)	–	–	GenBank	KU564148.1	Carbayo *et al*. 2016b
	MZUSP PL. 1609	São Bonifácio, SC (Brazil)	–	–	GenBank	KU564138.1	Carbayo *et al*. 2016b
	MZUSP PL. 1607	São Bonifácio, SC (Brazil)	–	–	GenBank	KU564146.1	Carbayo *et al*. 2016b
	MZUSP PL. 1608	Paulo Lopes, SC (Brazil)	–	–	GenBank	KU564151.1	Carbayo *et al*. 2016b
Obama applanata (Graff, 1899)	MZUSP PL. 1616	Teresópolis, RJ (Brazil)	–	–	GenBank	KU564126.1	Carbayo *et al*. 2016b
Obama argus (Graff, 1899)	MZUSP PL. 1617	Santa Maria Madalena, RJ (Brazil)	–	–	GenBank	KU564152.1	Carbayo *et al*. 2016b
Obama argus sensu Schirch (1929)	MZUSP PL. 1618	Santa Maria Madalena, RJ (Brazil)	–	–	GenBank	KU564130.1	Carbayo *et al*. 2016b
	MZUSP PL. 1619	Santa Maria Madalena, RJ (Brazil)	–	–	GenBank	KU564131.1	Carbayo *et al*. 2016b

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APPENDIX S1.(Continued)

Species	Specimen's accession number	Sampling locality	Latitude	Longitude	Identification	COI	References
	MZUSP PL. 1622	Teresópolis, RJ (Brazil)	–	–	GenBank	KU564132.1	Carbayo *et al.* 2016b
	MZUSP PL. 1621	Teresópolis, RJ (Brazil)	–	–	GenBank	KU564125.1	Carbayo *et al.* 2016b
Obama baptistae (Leal-Zanchet & Oliveira, 2012)	MZUSP PL. 1624	Toledo, PR (Brazil) (Brazil)	–	–	GenBank	KU564163.1	Carbayo *et al.* 2016b
	MZUSP PL. 1625	Machadinho, RS (Brazil)	–	–	GenBank	KU564161.1	Carbayo *et al.* 2016b
Obama braunsi (Graff, 1899)	MZUSP PL. 1626	Camanducaia, MG (Brazil)	–	–	GenBank	KU564214.1	Carbayo *et al.* 2016b
	MZUSP PL. 1627	Camanducaia, MG (Brazil)	–	–	GenBank	KU564157.1	Carbayo *et al.* 2016b
	MZUSP PL. 1628	Mogi das Cruzes, SP (Brazil)	–	–	GenBank	KU564123.1	Carbayo *et al.* 2016b
Obama burmeisteri (Schultze & Müller, 1857)	–	–	–	–	GenBank	KP262354.1	Álvarez-Presas *et al.* 2015
	–	–	–	–	GenBank	KP262355.1	Álvarez-Presas *et al.* 2015
	–	–	–	–	GenBank	KP262356.1	Álvarez-Presas *et al.* 2015
	–	–	–	–	GenBank	KP262373.1	Álvarez-Presas *et al.* 2015
	–	–	–	–	GenBank	KP262371.1	Álvarez-Presas *et al.* 2015
	–	–	–	–	GenBank	KP262377.1	Álvarez-Presas *et al.* 2015
	–	–	–	–	GenBank	KP262378.1	Álvarez-Presas *et al.* 2015
Obama carinata (Riester, 1938)	MZUSP PL 1062	Parque Estadual Intervales, SP (Brazil)	-24.276	-48.415	GenBank	KC608304.1	Carbayo *et al.* 2013
	MZUSP PL 1064	Parque Estadual Intervales, SP (Brazil)	-24.276	-48.415	GenBank	KC608307.1	Carbayo *et al.* 2013
Obama decidualis Amaral & Leal-Zanchet	MZUSP PL. 1210	Santa Maria, RS (Brazil)	-29.778	-53.867	GenBank	KP962385.1	Álvarez-Presas *et al.* 2015
	MZUSP PL. 1215	Santa Maria, RS (Brazil)	–	–	GenBank	KP962386.1	Álvarez-Presas *et al.* 2015

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APPENDIX S1.(Continued)

Species	Specimen's accession number	Sampling locality	Latitude	Longitude	Identification	COI	References
	MZUSP PL. 1216	Santa Maria, RS (Brazil)	–	–	GenBank	KP962387.1	Álvarez-Presas *et al.* 2015
	MZUSP PL. 1219	Santa Maria, RS (Brazil)	-29.742	-53.844	GenBank	KP962390.1	Álvarez-Presas *et al.* 2015
	MZUSP PL. 1220	Santa Maria, RS (Brazil)	–	–	GenBank	KP962391.1	Álvarez-Presas *et al.* 2015
*Obama eudoximariae (*Ogren & Kawakausu, 1990)	MZUSP PL. 1636	Teresópolis, RJ (Brazil)	–	–	GenBank	KU564213.1	Carbayo *et al.* 2016b
	MZUSP PL. 1637	Santa Maria Madalena, RJ (Brazil)	–	–	GenBank	KU564153.1	Carbayo *et al.* 2016b
Obama ficki (Amaral & Leal-Zanchet, 2012)	MZUSP PL. 1146	São Francisco de Paula, RS (Brazil)	–	–	GenBank	KU564118.1	Carbayo *et al.* 2016b
	MZU PL. 00104	São Francisco de Paula, RS (Brazil)	–	–	GenBank	KU564119.1	Carbayo *et al.* 2016b
Obama fryi (Graff, 1899)	MZUSP PL. 1638	Teresópolis, RJ (Brazil)	–	–	GenBank	KU564133.1	Carbayo *et al.* 2016b
	MZUSP PL. 1639	Teresópolis, RJ (Brazil)	–	–	GenBank	KU564134.1	Carbayo *et al.* 2016b
	MZUSP PL. 1640	Teresópolis, RJ (Brazil)	–	–	GenBank	KU564135.1	Carbayo *et al.* 2016b
Obama itatiayana (Schirch, 1929)	MZUSP PL. 1641	Resende, RJ (Brazil)	–	–	GenBank	KU564159.1	Carbayo *et al.* 2016b
Obama josefi (Carbayo & Leal-Zanchet, 2001)	MZUSP PL. 1074	São Francisco de Paula, RS (Brazil)	–	–	GenBank	KC608317.1	Álvarez-Presas *et al.* 2015
	MZUSP PL. 1075	São Francisco de Paula, RS (Brazil)	–	–	GenBank	KC608318	Álvarez-Presas *et al.* 2015
Obama ladislavii (Graff, 1899)	MZUSP PL. 736	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962448.1	Álvarez-Presas *et al.* 2015
	MZUSP PL. 737	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962449.1	Álvarez-Presas *et al.* 2015
	MZUSP PL. 742	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962452.1	Álvarez-Presas *et al.* 2015
	MZUSP PL. 744	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962454.1	Álvarez-Presas *et al.* 2015
	MZUSP PL 749	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962459.1	Álvarez-Presas *et al.* 2015

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APPENDIX S1.(Continued)

Species	Specimen's accession number	Sampling locality	Latitude	Longitude	Identification	COI	References
	MZUSP PL 792	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962480.1	Álvarez-Presas *et al.* 2015
	MZUSP PL 793	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962481.1	Álvarez-Presas *et al.* 2015
	MZUSP PL 797	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962485.1	Álvarez-Presas *et al.* 2015
	MZUSP PL 800	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	–	–	GenBank	KP962488.1	Álvarez-Presas *et al.* 2015
Obama maculipunctata Rossi *et al.*, 2015	MZU PL. 00201	São Francisco de Paula, RS (Brazil)	-29.423	- 50.387	GenBank	KT250629.1	Rossi *et al.* 2015
	MZU PL. 00198	São Francisco de Paula, RS (Brazil)	-29.424	-50.386	GenBank	KT250626.1	Rossi *et al.* 2015
Obama nungara Carbayo, Alvarez-Presas, Jones & Riutort, 2016	–	Greenhouse Ferrari, Buenos Aires (Argentina)	–	–	GenBank	KM053223.1	Lago-Barcia *et al.* 2015
	–	Reborio, Muros de Nalón, Asturias (Spain)	–	–	GenBank	KM053214.1	Lago-Barcia *et al.* 2015
	–	Torroella de Fluvià, Girona (Spain)	42.175	-3.045	GenBank	KT714107.1	Carbayo *et al.* 2016a
	MZUSP PL. 1599	Torroella de Fluvià, Girona (Spain)	42.175	-3.045	GenBank	KT714108.1	Carbayo *et al.* 2016a
Cratera ochra Rossi *et al.*, 2015	MZUSP PL. 1564	São Francisco de Paula, RS (Brazil)	-29.427	-50.387	GenBank	KT250622.1	Rossi *et al.* 2015
	MZU PL. 00191	São Francisco de Paula, RS (Brazil)	-29.410	-50.388	GenBank	KT250623.1	Rossi *et al.* 2015
Obama otavioi Carbayo, 2016	MZUSP PL. 1574	Santo André, SP (Brazil)	-23.777	-46.312	GenBank	KT932833.1	Carbayo *et al.* 2016a
	MZUSP PL. 1573	Santo André, SP (Brazil)	-23.777	-46.312	GenBank	KT932832.1	Carbayo *et al.* 2016a
Cratera crioula (E. M. Froehlich, 1955)	MZUSP PL. 1078	Parque Estadual da Cantareira, SP (Brazil)	-23.431	-46.634	GenBank	KC608323.1	Carbayo *et al.* 2013
	MZUSP PL. 1079	Parque Estadual da Cantareira, SP (Brazil)	-23.431	-46.634	GenBank	KC608324.1	Carbayo *et al.* 2013
Cratera tamoia (E. M. Froehlich, 1955)	MZUSP PL. 665	Parque Nacional da Serra dos Órgãos, RJ (Brazil)	-22.455	-42.997	GenBank	KC608246.1	Carbayo *et al.* 2013
	MZUSP PL. 672	Parque Nacional da Serra dos Órgãos, RJ (Brazil)	-22.499	-42.996	GenBank	KC608254.1	Carbayo *et al.* 2013