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Research Article

When looks can be deceiving: a new cryptic land planarian species of *Choeradoplana* Graff, 1896 (Platyhelminthes: Geoplanidae) from Argentina

LISANDRO NEGRETE[1,2], AGUSTINA ZIVANO[1], MARINA LENGUAS FRANCAVILLA[1], CRISTINA DAMBORENEA[1,2] & FRANCISCO BRUSA[1,2]

[1]División Zoología Invertebrados, Facultad de Ciencias Naturales y Museo, UNLP, Boulevard 120 & 61, La Plata, Buenos Aires, B1900CHX, Argentina
[2]CONICET – Consejo Nacional de Investigaciones Científicas y Técnicas, La Plata, Buenos Aires, Argentina

A new species of the genus *Choeradoplana* found in the east-central region of Argentina is herein described, *Choeradoplana malaria* sp. nov. This new entity shows a striking similarity regarding the pigment pattern with other land planarian species of this genus, viz. *Choeradoplana langi*, *Choeradoplana gladismariae*, and *Choeradoplana minima*. Besides, the internal anatomy of *Ch. malaria* sp. nov. is remarkably similar to that of *Ch. gladismariae*, a species recorded in southeastern Brazil, some 2300 km distant from the new species. Although there are minimal differences in the colour pattern of the dorsum, both species can be differentiated by some anatomical and histological details, such as the thickness of the cutaneous musculature, the width of the creeping sole, and some features related to the reproductive system. This low morphological disparity between both species fits within the term 'cryptic species'. These subtle differences were also corroborated in a phylogenetic analysis using Cox-1 gene sequences. Likewise, species delimiters reinforced the identity of the new taxon herein proposed and allowed us to differentiate *Ch. malaria* sp. nov. from *Ch. gladismariae* at the genetic level.

http://zoobank.org/urn:lsid:zoobank.org:pub:DF432618-A85B-4C99-8E64-EC9C581B0714

Key words: cryptic species, Geoplaninae, land flatworms, Neotropical region

Introduction

The genus *Choeradoplana* Graff, 1896 encompasses 24 land planarian species which, with the exception of two species, have only been recorded in remnants of the Atlantic Forest, in south-eastern Brazil (Carbayo et al., 2018; Carbayo & Froehlich, 2012; du Bois-Reymond Marcus, 1951; Froehlich, 1954; Graff, 1899; Iturralde & Leal-Zanchet, 2019; Lago-Barcia et al., 2021; Lemos et al., 2014). Regarding the remaining two species, *Choeradoplana crassiphalla* Negrete & Brusa, 2012 was found in the Interior Atlantic Forest in Argentina (Negrete & Brusa, 2012), while *Choeradoplana langi* (Graff, 1894) was recorded in Argentina, Paraguay, and Brazil (du Bois-Reymond Marcus, 1951; Froehlich, 1954; Graff, 1894, 1897, 1899).

Species of *Choeradoplana* are characterized by the behaviour of curling their cephalic extremity backward mainly while they crawl. This peculiar feature is not exclusive to this genus since it is also observed in other Neotropical species, such as the representatives of the genus *Cephaloflexa* Carbayo & Leal-Zanchet, 2003. Although both genera share some other features (namely, lack of eyes and sensory pits at the anterior tip of the body, presence of a cephalic retractor muscle with fibres following sagittal planes, and a sub-neural parenchymatic muscle layer only present in the cephalic region), most species of *Choeradoplana* differentiate externally from those of *Cephaloflexa* by the usual presence of an expanded cephalic region, with two glandular cushions separated by a ventral median groove (Carbayo et al., 2013; Carbayo & Leal-Zanchet, 2003; Lago-Barcia et al., 2021). This feature was noted by Graff (1896), who gave the generic name.

Correspondence to: Lisandro Negrete. Email: lnegrete@fcnym.unlp.edu.ar

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In the last years, the intensive use of molecular markers has allowed, together with the re-evaluation of some morphological characters, the unravelling of many new species of land planarians that could hardly be differentiated due to their great morphological similarities. Small differences that were formerly assumed as population differences or phenotypic plasticity are now known to indicate different evolutionary lineages. In recent contributions, integrative taxonomic approaches revealed the presence of cryptic species belonging to different genera of the subfamily Geoplaninae, e.g., *Obama* Carbayo et al., 2013 (Álvarez-Presas et al., 2015; Carbayo et al., 2016), *Cratera* Carbayo et al., 2013 (Araujo et al., 2020), *Pasipha* Ogren & Kawakatsu, 1990 (Amaral et al., 2018), and *Choeradoplana* Graff, 1896 (Carbayo et al., 2018; Iturralde & Leal-Zanchet, 2019; Lemos et al., 2014). Indeed, *Choeradoplana* represents a particularly challenging genus because several species, many of them recently described, closely resemble each other, not only in their external appearance but also in their internal anatomy. This way, molecular markers have proven to be a useful tool to disentangle minor morphological differences and thus reveal the existence of species that would otherwise remain cryptic (Carbayo et al., 2018). Although Cox-1 as a molecular marker shows limitations in inferring phylogenetic relationships above species level, it is widely used to identify and delimit species (Carbayo et al., 2018; Lago-Barcia et al., 2021; Lemos et al., 2014).

In this paper, we describe a new species of *Choeradoplana* found in the east-central region of Argentina. At first sight, this new entity is hard to distinguish, regarding the external appearance, from *Ch. langi*, *Choeradoplana gladismariae* Carbayo & Froehlich, 2012, and *Choeradoplana minima* Lemos & Leal-Zanchet, 2014. Moreover, the new species exhibits a low morphological dissimilarity from *Ch. gladismariae*. Nevertheless, scrutiny of details of both the colour pattern and the internal anatomy, along with a molecular analysis using the Cox-1 gene, unveils the new species described herein.

Materials and methods

Study area and sampling

Land flatworms were manually collected in three nature reserves, Reserva Natural Punta Lara (34°46′53″S, 58°01′02″W), Monte Ribereño Isla Paulino, Isla Santiago (34°49′48″S, 57°52′31″W), and Reserva Natural Isla Martín García (34°10′49″S, 58°15′15″W), in the east-central region of Argentina (Buenos Aires province) (Fig. 1). Sampling was carried out during daylight and at night by searching beneath fallen logs and the leaf litter and by direct observation of the soil with the help of headlamps, respectively.

Morphological analysis

The animals were photographed and had their external morphology documented *in vivo*. Before fixation, the posterior tip of some specimens was cut off, fixed in 100% ethanol and preserved at −20 °C for molecular analysis. Then, the animals were killed using boiling water, fixed in 10% formaldehyde and preserved in 70% ethanol. Fragments of different parts of the body (i.e., cephalic region, anterior region at the level of the ovaries, pre-pharyngeal region, pharynx, and copulatory apparatus) were dehydrated in an ascending series of ethanol and, afterward, embedded in Paraplast©. Sagittal and transverse serial sections (6–8 µm thick) of these body fragments were cut with a microtome and stained with a modified version of Masson's trichrome method (Negrete et al., 2019). All photomicrographs of sagittal sections are oriented with the anterior body region to the left. The RAL colour standard chart (http://www.ralcolor.com) was used to describe the pigment pattern of the dorsal and ventral surfaces of the body in a precise way. Type material and additional specimens were deposited in the Invertebrate Collection at the Museo de La Plata (MLP), Argentina.

Molecular analysis

DNA extraction was carried out using Wizard© Genomic DNA Purification Kit (Promega). A portion of the mitochondrial gene Cox-1 was amplified by polymerase chain reaction (PCR) from the genomic isolate. The primers used were BarS (50-GTTATGCCTGTAATGATTG-3′0) (Álvarez-Presas et al., 2011) and COIR (50-CCWGTYARMCCHCCW-30) (Lázaro et al., 2009). PCR concentrations were 0.064 mM for dNTPs, 0.32 mM for each primer, and 0.024 U/µl for Taq polymerase (GoTaq®, Promega), adding 5 µl buffer 5X (Promega), 5 µl DNA template, and ultrapure water to make a final volume of 25 µl. For thermal cycling conditions we followed Álvarez-Presas et al. (2011). The PCR products were verified by agarose gel electrophoresis and then sent to be purified and sequenced by Macrogen Inc. (Seoul, South Korea). After removing the primers, the assembled sequences were compared with reference sequences from the NCBI database using BLAST.

Alongside the obtained sequences ($n = 2$), we added to the analyses sequences of eight species of the genus *Choeradoplana* ($n = 25$) downloaded from GenBank as

Fig. 1. Map with localities where *Choeradoplana malaria* sp. nov. was found (red squares) and known records (locality and publication year) of species with similar dorsal pigment pattern: *Choeradoplana gladismariae* (blue pentagon), *Choeradoplana langi* (yellow circle), and *Choeradoplana minima* (pink triangle); *Ch. langi* records with a question mark indicate that they are doubtful since the animals were only identified by external features.

part of the ingroup, and sequences of six species of close genera ($n = 9$) as outgroup (Table 1).

Sequence analysis and phylogenetic analyses

The sequences were aligned using MAFFT with default parameters on its online server (Katoh et al., 2019; Kuraku et al., 2013). The alignment and the reading frame were verified by checking the amino acid translation on MEGA-X (Kumar et al., 2018) according to the Flatworm Mitochondrial Code. Pairwise genetic distances (*p*-distance) were calculated, as well as mean distances within and between taxa, with 1000 bootstrap replicates for standard error estimates on MEGA-X.

For phylogenetic inference analyses, the dataset was partitioned by codon position with PAUP v4 (Swofford, 2003) and a homogeneity test was performed on Tree Puzzle (Schmidt et al., 2002). Then, the substitution model for each subset was selected separately. The Maximum Likelihood analysis (ML) was conducted on the IQ-Tree online server (Nguyen et al., 2014; Trifinopoulos et al., 2016) with 10,000 Ultrafast Bootstrap (Hoang et al., 2017) and SH-aLRT replicates (Guindon et al., 2010). The selected models were TN + F+G4, HKY + F, and TIM2 + F + I + G4 (Felsenstein, 1981; Posada, 2003; Tajima & Nei, 1984) for first, second, and third codon position, respectively, and were determined by the ModelFinder function on the IQ-Tree website (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017). For Bayesian Inference (BI), the substitution models were selected under the Bayesian Information Criterion (BIC) using Partition

Table 1. List of specimens analysed genetically, including GenBank accession number of gen Cox-1, collection locality (country/state or province), and literature.

Ingroup	GenBank	Locality*	Literature
Choeradoplana abaiba	MF802631	Brazil/SC	Carbayo et al. (2018)
Choeradoplana abaiba	MF802632	Brazil/SC	Carbayo et al. (2018)
Choeradoplana abaiba	MF802633	Brazil/SC	Carbayo et al. (2018)
Choeradoplana abaiba	MF802634	Brazil/SC	Carbayo et al. (2018)
Choeradoplana agua	MF802635	Brazil/RJ	Carbayo et al. (2018)
Choeradoplana agua	MF802636	Brazil/RJ	Carbayo et al. (2018)
Choeradoplana agua	MF802637	Brazil/RJ	Carbayo et al. (2018)
Choeradoplana banga	KC608267	Brazil/SP	Carbayo et al. (2013)
Choeradoplana banga	KC608301	Brazil/SP	Carbayo et al. (2013)
Choeradoplana banga	MF802638	Brazil/SP	Carbayo et al. (2018)
Choeradoplana banga	MF802639	Brazil/SP	Carbayo et al. (2018)
Choeradoplana bocaina	KC608273	Brazil/SP	Carbayo et al. (2013)
Choeradoplana bocaina	KC608283	Brazil/SP	Carbayo et al. (2013)
Choeradoplana bocaina	KC608288	Brazil/SP	Carbayo et al. (2013)
Choeradoplana gladismariae	KC608306	Brazil/SP	Carbayo et al. (2013)
Choeradoplana gladismariae	KC608326	Brazil/SP	Carbayo et al. (2013)
Choeradoplana iheringi	MF802661	Brazil/RS	Carbayo et al. (2018)
Choeradoplana iheringi	MF802662	Brazil/RS	Carbayo et al. (2018)
Choeradoplana iheringi	MF802663	Brazil/RS	Carbayo et al. (2018)
Choeradoplana malaria	OR413631	Argentina/BA	This paper
Choeradoplana malaria	OR413632	Argentina/BA	This paper
Choeradoplana minima	KJ690051	Brazil/RS	Lemos et al. (2014)
Choeradoplana minima	KJ690052	Brazil/RS	Lemos et al. (2014)
Choeradoplana onae	MW127835	Brazil/SP	Lago-Barcia et al. (2021)
Choeradoplana onae	MW127836	Brazil/SP	Lago-Barcia et al. (2021)
Choeradoplana onae	MW127837	Brazil/SP	Lago-Barcia et al. (2021)
Choeradoplana onae	MW127838	Brazil/SP	Lago-Barcia et al. (2021)
Outgroup			
Cephaloflexa araucariana	KC608316	Brazil/RS	Carbayo et al. (2013)
Cephaloflexa araucariana	KC608319	Brazil/RS	Carbayo et al. (2013)
Cephaloflexa bergi	HQ026437	Brazil/SP	Álvarez-Presas et al. (2011)
Cephaloflexa bergi	HQ026438	Brazil/SP	Álvarez-Presas et al. (2011)
Cephaloflexa bergi	HQ026440	Brazil/SP	Álvarez-Presas et al. (2011)
Notogynaphallia parca	KC608298	Brazil/SP	Carbayo et al. (2013)
Notogynaphallia plumbea	KC608303	Brazil/SP	Carbayo et al. (2013)
Matuxia matuta	KC608277	Brazil/RJ	Carbayo et al. (2013)
Matuxia tuxaua	KC608253	Brazil/RJ	Carbayo et al. (2013)

*BA: Buenos Aires; RJ: Rio de Janeiro; RS: Rio Grande do Sul; SC: Santa Catarina; SP: São Paulo.

Finder 2.1.1 (Guindon et al., 2010; Lanfear et al., 2012, 2017). The analysis was run on MrBayes 3.2 (Ronquist et al., 2012) for 20 million generations, with a 25% burn-in. The models used were GTR + G, HKY + I, and GTR + G + I for each codon position respectively (Felsenstein, 1981; Tavaré, 1986). For BI, clade support was given by Bayesian posterior probability.

For species delimitation analyses, two approaches were considered, mPTP (Kapli et al., 2017; https://mptp.h-its.org), based on the trees resulting from BI and ML analyses, and ASAP (Puillandre et al., 2021; https://bio-info.mnhn.fr/abi/public/asap/), which is based on the alignment matrix. They were both conducted on their respective websites with default parameters.

Abbreviations in figures

(co) common muscle coat, (cod) common ovovitelline duct, (cs) creeping sole, (dc) dorsal cutaneous musculature, (de) dorsal epidermis, (di) dorsal insertion of the pharynx, (dlc) dorsal longitudinal cutaneous muscle layers, (dp) dorsal parenchymatic muscle layer, (dvp) dorsoventral parenchymatic musculature, (e) eye, (fa) female atrium, (fc) female canal, (go) gonopore, (i) intestine, (im) inner musculature of the pharynx, (lb) lateral band, (ma) male atrium, (mab) marginal band, (meb) median band, (mo) mouth, (ms) median stripe, (n) nerve plate, (od) ovovitelline duct, (om) outer musculature of the pharynx, (ov) ovary, (pg) prostatic glands, (ph) pharynx, (pl) pharyngeal lumen, (pms) para-marginal stripe, (pp) pharyngeal pouch, (pv1) prostatic vesicle (paired portion), (pv2) prostatic vesicle (unpaired portion), (r) retractor muscle, (rh) rhabditogen glands, (sbp) sub-intestinal parenchymatic muscle layer, (sd) sperm duct, (sg) shell glands, (sn) sub-neural muscle coat, (sp) sensory pit, (spp) supra-intestinal parenchymatic muscle layer, (t) testes, (ve) ventral epidermis, (vi) ventral insertion of the pharynx, (vlc) ventral longitudinal cutaneous muscle layers, (vt) vitellaria.

Results

Taxonomic account

Order Tricladida Lang, 1881
Family Geoplanidae Stimpson, 1858
Subfamily Geoplaninae Stimpson, 1858
Genus *Choeradoplana* Graff, 1896
Choeradoplana malaria Negrete & Brusa, sp. nov.

HOLOTYPE: MLP He-8037: Reserva Natural Punta Lara, Ensenada County, Buenos Aires province (Argentina), 18 November 2017, Negrete, L. coll.; cephalic region: transverse sections on 24 slides; anterior region at the level of ovaries: sagittal sections on 10 slides; pre-pharyngeal region: transverse sections on 5 slides; pharynx and copulatory apparatus: sagittal sections on 18 slides; GenBank accession number: OR413631. PARATYPE: MLP He-8038: Reserva Natural Punta Lara, Ensenada County, Buenos Aires province (Argentina), 18 November 2017, Brusa, F. coll.; cephalic region: transverse sections on 17 slides; anterior region at the level of ovaries: sagittal sections on 9 slides; pre-pharyngeal region: transverse sections on 7 slides; pharynx and copulatory apparatus: sagittal sections on 17 slides.

TYPE LOCALITY. Reserva Natural Punta Lara (34°46′53″S, 58°01′02″W), Ensenada County, Buenos Aires province (Argentina).

ADDITIONAL MATERIAL: MLP He-6227: Reserva Natural Punta Lara, Ensenada County, Buenos Aires province (Argentina), 3 June 2008, Negrete, L. coll.; cephalic region: transverse sections on 13 slides; anterior region at the level of ovaries: sagittal sections on 8 slides; pre-pharyngeal region: transverse sections on 4 slides; pharynx and copulatory apparatus: sagittal sections on 11 slides. MLP He-6228: Reserva Natural Punta Lara, Ensenada County, Buenos Aires province (Argentina), 28 October 2009, Negrete, L. coll.; pharynx and copulatory apparatus: sagittal sections on 15 slides. MLP He-8039: Isla Martín García, Buenos Aires province (Argentina), 26 January 2008, Negrete, L. coll.; cephalic region: transverse sections on 18 slides; anterior region at the level of ovaries and pre-pharyngeal region: transverse sections on 21 slides; pharynx and copulatory apparatus: sagittal sections on 10 slides. MLP He-6632-1: Isla Paulino, Berisso County, Buenos Aires province (Argentina), 6 November 2012, Merlo Álvarez, V. H. coll.; whole specimen in 70% ethanol. MLP He-6632-2: Isla Paulino, Berisso County, Buenos Aires province (Argentina), 6 November 2012, Merlo Álvarez, V. H. coll.; whole specimen in 70% ethanol. MLP He-8040: Reserva Natural Punta Lara, Ensenada County, Buenos Aires province (Argentina), 7 August 2018, Negrete, L. coll.; rest of specimen in 70% ethanol; GenBank accession number: OR413632.

DISTRIBUTION: Buenos Aires province, Argentina.

ETYMOLOGY: The specific name is a combination of a part of each of the specific names of three species of *Choeradoplana* which the new taxon externally resembles, viz. *Ch. minima* (*ma*), *Ch. langi* (*la*), and *Ch. gladismariae* (*ria*).

DIAGNOSIS: Species of *Choeradoplana* with a median band of zinc yellow longitudinally traversed by a median granite black stripe, two lateral bands of zinc yellow ground colour with numerous graphite black spots giving a marbled appearance, two jet black para-marginal stripes formed by the accumulation of the graphite black spots, and two marginal bands of sun yellow; cutaneous longitudinal muscle layer with fibres sunken into the parenchyma both dorsally and ventrally; prostatic vesicle with two portions, a proximal portion paired and tubular, and a distal portion with pleated walls giving a bellows-like appearance; penis papilla absent; female atrium lined with columnar epithelium of stratified appearance.

Description

External morphology

The body is elongate with parallel margins. The anterior tip is blunt, curved backward when animals are alive, and the posterior end is rounded (Fig. 2A–C). The dorsal pigment shows two variants: variant 1 (Fig. 2A, B; see also Fig. 12A) with a median band of zinc yellow (RAL 1018) (~20% of body width) longitudinally traversed by a median granite black (RAL 9011) stripe (~5% of body width); two broad lateral bands (each occupying ~25% of body width) consisting of zinc yellow ground colour with numerous graphite black spots of different sizes that give them a marbled appearance; two jet black (RAL 9005) para-marginal stripes (~5% of body width) formed by the accumulation of the graphite black spots; and two marginal bands (~10% of body width, each) of sun yellow (RAL 1037), whose pigment is a bit darker than the median band. Variant 2 (Fig. 2C; see also Fig. 12B) with a similar arrangement as variant 1 but with the median

Fig. 2. Dorsal view of live specimens of *Choeradoplana malaria* sp. nov. (cephalic region marked with an arrow); (A) holotype, (B) paratype, (C) MLP He-6228. Scale bars: (A, B) 5 mm, (C) 2.5 mm.

band oyster white (RAL 1013) (~15% of body width) longitudinally traversed by a median stripe (~8% of body width) formed by the accumulation of tiny black olive spots (RAL 6015); these spots, some of which spread toward the median band, separate forming two fine stripes at the posterior region of the body; the lateral bands (each occupying ~25% of body width) have, as variant 1, a marbled appearance by the irregular disposition of black olive spots, which accumulate outwards to conform fine para-marginal stripes (~2.5% of body width, each); the marginal bands (~15% of body width, each) are ivory pigmented (RAL 1014). Both variants have an oyster white ventral surface (RAL 1013).

The monolobulate eyes arise ~1 mm behind the anterior tip (Figs 2C, 3E, F). They are uniserial and marginal. Posteriorly, they extend bi- or tri-serially only on the body margins (Fig. 3I). At the level of the pre-pharyngeal region, the eyes are bi- or uniserial, somewhat more scattered, less numerous at the mouth level, and reach at the posterior end of the body uniserially. After fixation, the body length ranged between 21 and 29 mm, maximum width 1.8–2.4 mm, and maximum height 1.1–1.2 mm. The mouth was located at a distance of 61–63%, and the gonopore at 76–79% from the anterior tip.

Internal morphology

The cephalic region (Fig. 3) contains a musculo-glandular organ consisting of a retractor muscle and two glandular cushions on both sides of this muscle with abundant erythrophil secretion and rhabdites (Fig. 3B–H). The fibres of the retractor muscle concentrate in the median ventral region, and are triangle-shaped in cross section (Fig. 3E–H). They originate from the ventral cutaneous longitudinal muscle layer, detach from it, and sink into the parenchyma. The sub-neural parenchymatic muscle layer consists of transversal fibres above the retractor muscle, with some fibres interspersed with the retractor (Fig. 3D). Dorsally, the supra-intestinal parenchymatic muscle layer is formed by crisscrossed fibres (Fig. 3E, H). The creeping sole at the cephalic region occupies almost 25% of the body width (Fig. 3E, F). The sensory pits, as simple invaginations ranging from 20–25 µm deep, arise slightly behind (~250 µm) the cephalic tip and extend for 3–4 mm (14% of total body length) (Fig. 3G, I).

At the pre-pharyngeal region, three types of secretory cells discharge through the dorsal epidermis (20–25 µm height) (Fig. 4B): numerous rhabditogen cells with erythrophil secretion (rhammites), cells with fine granular erythrophil secretion, and scarce cells with amorphous cyanophil secretion. The glandular margin is absent (Fig. 4A). The ventral epidermis (25 µm height) (Fig. 4C) is ciliated on the creeping sole (~100% of body width). The same types of secretory cells that discharge their secretion through the dorsal epidermis are also present on the creeping sole. However, rhabditogen cells discharge small rhabdites, and the cells with fine

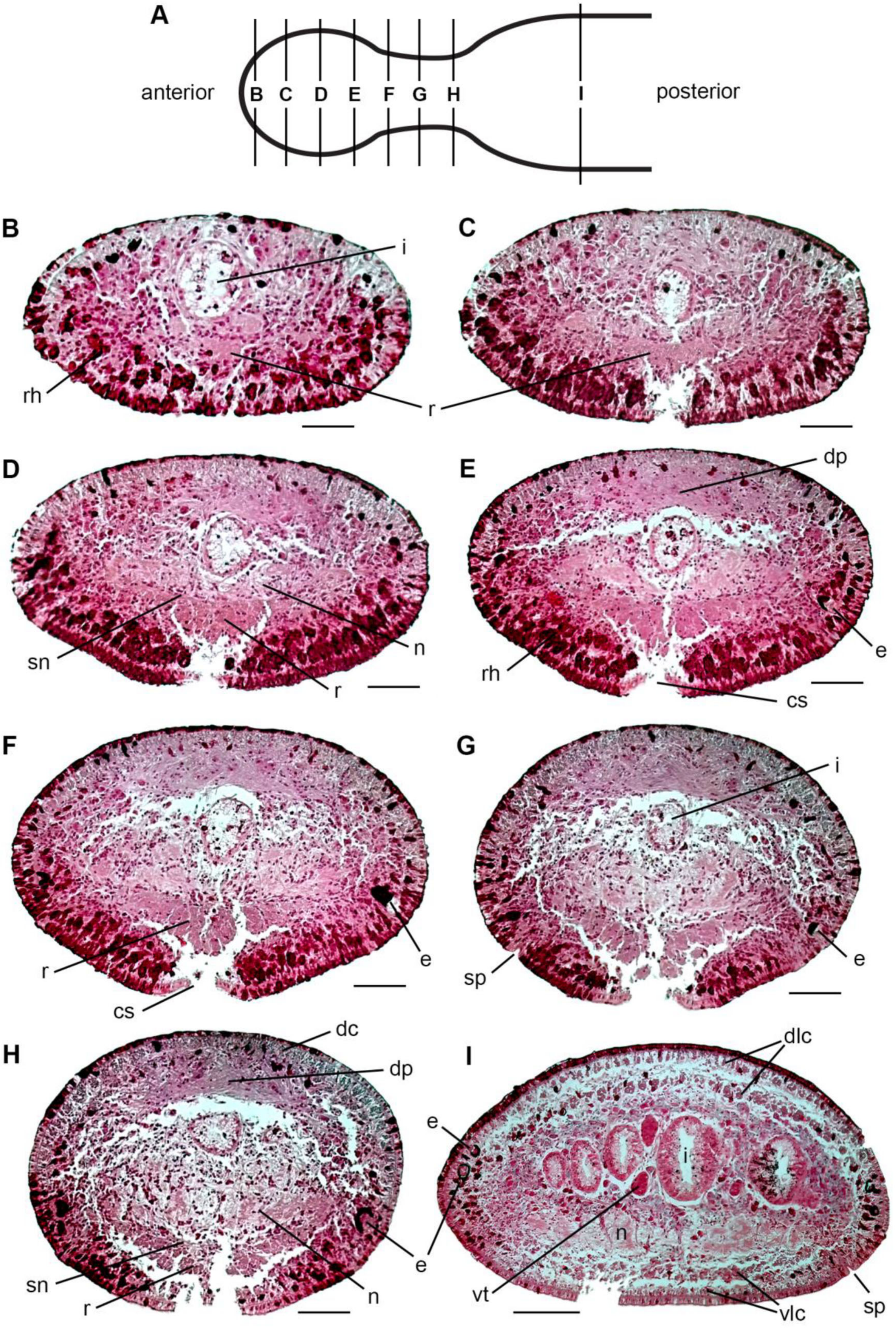


Fig. 3. Series of transverse sections of the cephalic region of *Choeradoplana malaria* sp. nov. (paratype). (A) Schematic drawing of the anterior body region with the location of the sections: (B) very near the apex, (C) 250 µm from the apex, (D) 500 µm from the apex, (E) 750 µm from the apex, (F) 1 mm from the apex, (G) 1.25 mm from the apex, (H) 1.5 mm from the apex, and (I) ~3 mm from the apex. Scale bars: (B–H) 100 µm, (I) 200 µm.

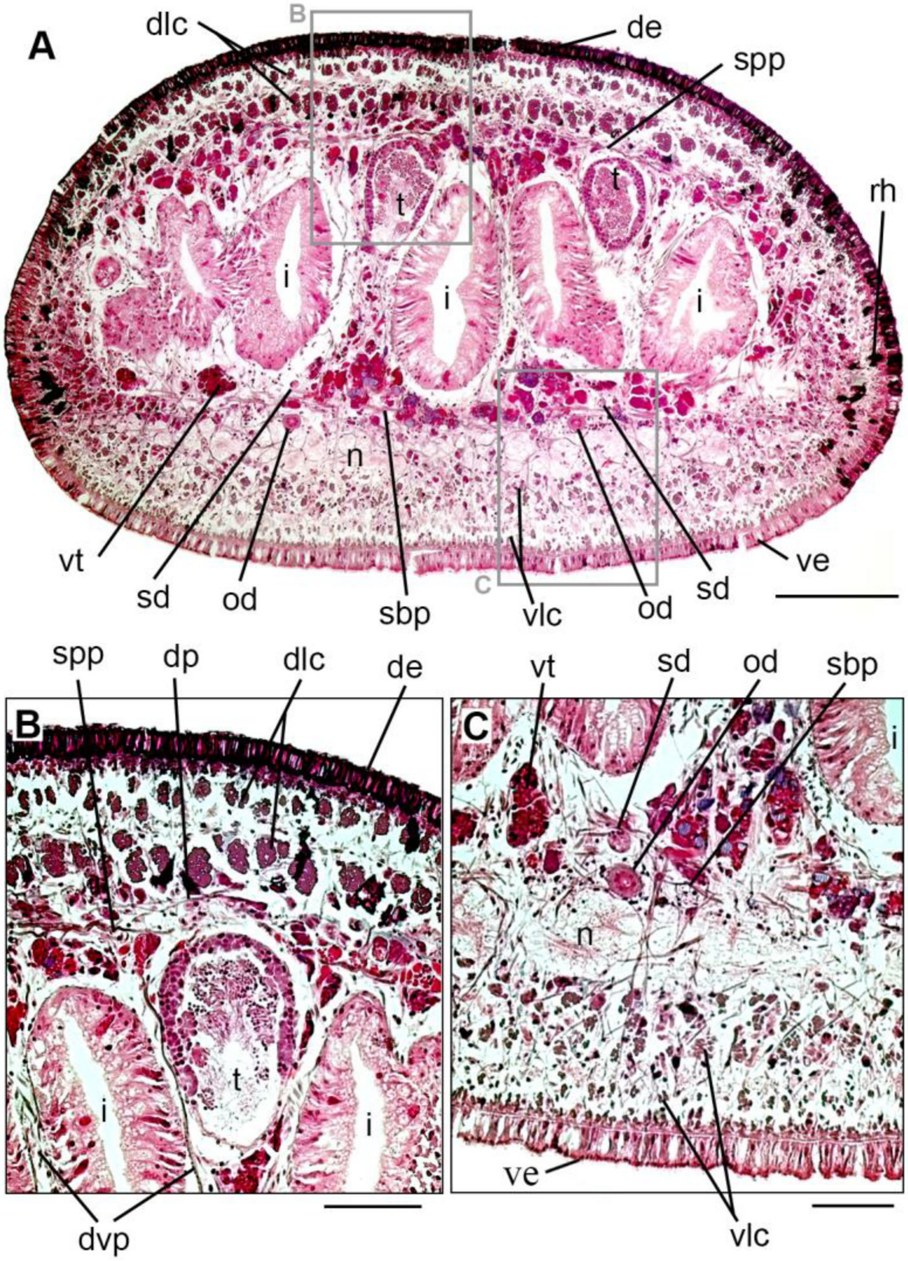


Fig. 4. Transverse sections of the pre-pharyngeal region of *Choeradoplana malaria* sp. nov. (holotype). (A) Total section; the insets show the details of the dorsal (B) and ventral (C) regions. Scale bars: (A) 250 μm; (B, C) 100 μm.

granular erythrophil and with amorphous cyanophil secretions are more abundant.

The cutaneous musculature consists of an external layer of circular muscle fibres (2.5–5 μm thick), a diagonal layer (5 μm thick), and an internal longitudinal muscle whose fibres are gathered, both dorsally and ventrally, in two groups of bundles: one subjacent to the diagonal layer (25–35 μm thick dorsally and 20–35 μm thick ventrally) and the other partially sunk into the parenchyma (40–50 μm thick dorsally and 35–60 μm thick ventrally) (Fig. 4B, C). The thickness of the cutaneous musculature represents 13–14% of the body height. The parenchymatic musculature (Fig. 4A, B) is mainly composed of three layers: dorsal subcutaneous, located close to the cutaneous longitudinal muscle bundles, with oblique decussate fibres (about 6–8 fibres thick), supra-intestinal transverse (about 4–6 fibres thick), and sub-intestinal transverse (~6 fibres thick). In addition, there are dorsoventral fibres that accommodate among the intestinal branches and oblique fibres crisscrossing the nervous plate.

The pharynx is bell-shaped, 1.3–1.7 mm long (5–6% of body length), with the dorsal insertion shifted posteriorly and located at mouth level in sagittal view (Fig. 5A). The mouth is placed approximately in the middle of the pharyngeal pouch (1.5–1.9 mm in length). The epithelial lining of the pharynx is cuboidal and ciliated with insunk nuclei, followed by a thin longitudinal subepithelial muscle layer (2.5–5 µm thick) and a subjacent circular layer (15–30 µm thick) (Fig. 5B). The pharyngeal lumen is lined by a ciliated columnar epithelium with insunk nuclei followed by a muscle coat comprised of circular fibres interspersed with some longitudinal fibres (50–75 µm thick) (Fig. 5C). The pharyngeal glands are constituted by two secretory cell types: abundant cells with fine granular erythrophil secretion and less abundant cells with fine granular cyanophil secretion. The cell bodies of the pharyngeal glands are mostly located in the surrounding parenchyma anterior to the pharynx. The oesophagus is absent.

The testes are dorsal, mature, and arranged in two irregular rows on each side of the body (Fig. 4A, B). They are located below the supra-intestinal parenchymatic muscle layer. The anteriormost testes lie a bit before the ovaries and the posteriormost nearly reach the ventral root of the pharynx (between 25–28% and 56–62% of body length, respectively). The sperm ducts are located among the muscle fibres of the sub-intestinal parenchymal layer, dorsal or dorsolateral to the ovovitelline ducts in the prepharyngeal region (Fig. 4A, C). Near the copulatory apparatus, the lumen of the sperm ducts is dilated and full of spermatozoa (Fig. 9A), but their distal paths become narrow and devoid of sperm (Fig. 6), opening into the paired portion of the extrabulbar prostatic vesicle. This paired portion consists of two short 'arms' (∼100 µm in length) that traverse the common muscle coat to open ventrolaterally into the unpaired intrabulbar portion of the vesicle (Figs 6, 7A, B, 8A–D). The latter, which is the main part of the prostatic vesicle, comprises a cavity with an irregular contour with numerous folds that radiate from the bottom and the dorsoposterior wall and merge, giving it a fenestrated or bellows-like appearance (Figs 6, 7A, B, 8A–D). The prostatic vesicle communicates with the male atrium through a narrow canal that arises from the posterior-dorsal wall of the vesicle (Figs 6, 7A, 8D). The male atrium (900 and 500 µm long in the holotype and paratype, respectively) (Figs 6, 7A, 8D) is a narrow and highly

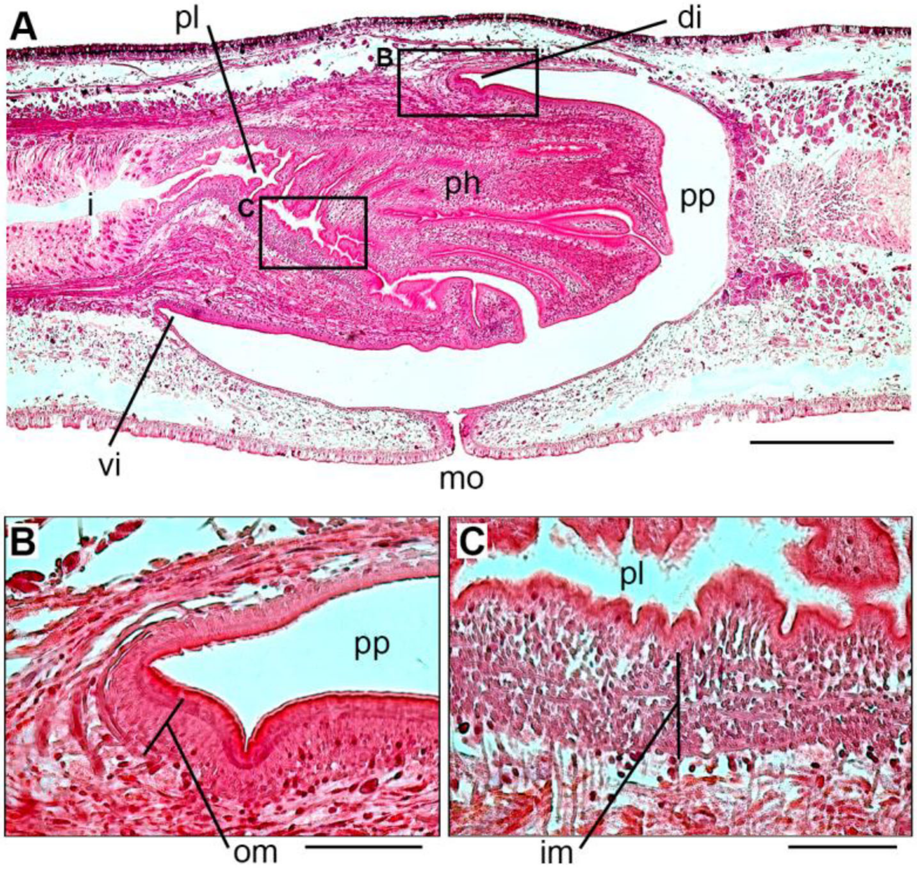


Fig. 5. Sagittal section of the pharynx of *Choeradoplana malaria* sp. nov. (holotype) (A), with details of the outer (B) and inner (C) musculatures. Scale bars: (A) 500 µm; (B, C) 50 µm.

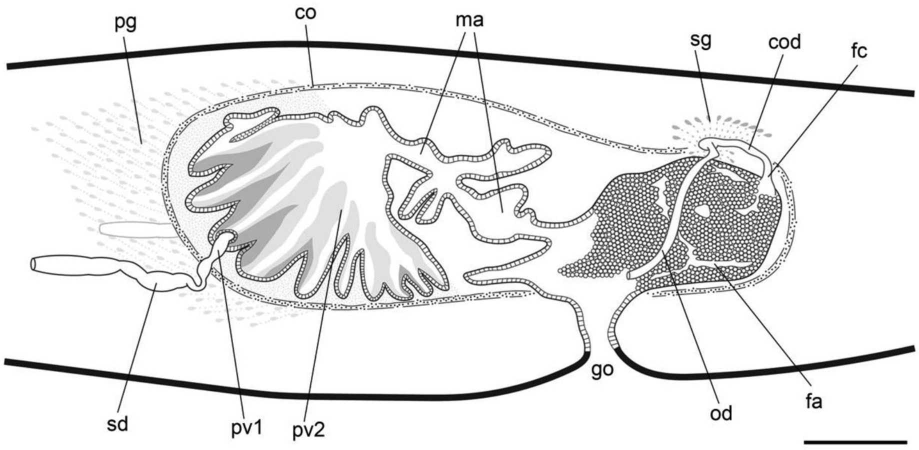


Fig. 6. Schematic reconstruction of the copulatory apparatus of *Choeradoplana malaria* sp. nov. (holotype). Scale bar: 500 µm.

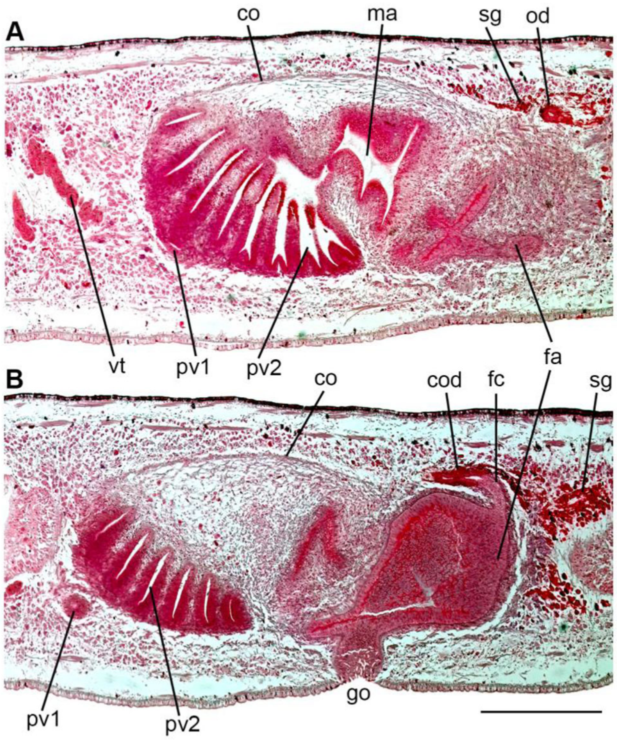


Fig. 7. Sagittal sections of the copulatory apparatus of *Choeradoplana malaria* sp. nov. (paratype). Scale bar (A, B): 500 µm.

plicate compartment that runs obliquely to the ventral side to open, together with the female atrium, into the gonopore canal. There is no penis papilla.

The sperm ducts are lined with ciliated cuboidal epithelium, without any musculature subjacent at the pre-pharyngeal region (Fig. 4C). Near the copulatory apparatus, the sperm ducts are lined with a circular muscle layer (~10 µm thick). The paired portion of the prostatic vesicle, both the extrabulbar and the intrabulbar tract, is lined with ciliated columnar epithelium pierced by fine erythrophil secretion and coated by circular muscle fibres intermingled with longitudinal fibres (5–15 µm thick) (Fig. 9B). The intrabulbar unpaired portion of the prostatic vesicle, lined with ciliated columnar epithelium, receives the same secretion as the paired portion (Fig. 9B–D) as well as scarce fine cyanophil granules. Also, the epithelium of the folds of the prostatic vesicle is filled with coarse granular erythrophil secretion (Fig. 9C, D). The muscular coat of the unpaired portion of the vesicle consists of circular and longitudinal fibres (10–15 µm thick). The male atrium is lined with a columnar epithelium with abundant fine granular erythrophil secretion (Fig. 9D). It is ciliated only in its proximal portion just at the opening of the prostatic vesicle (Fig. 9D). The male atrium is coated by a decussate muscle layer consisting of circular, longitudinal and oblique fibres (20–30 µm thick). The common muscle coat consists mainly of longitudinal fibres intermingled with some circular fibres (15–30 µm thick).

The ovaries, roughly ovoid or ellipsoid in shape (Fig. 10A, B), measure, in sagittal section, 500–550 µm in length and 160–200 µm in height. They are located at 29–31% of the body length and lie between the sub-intestinal parenchymal muscle layer and the nerve plate (Fig. 10A, B). The ovovitelline ducts emerge dorsally from the median third of the ovaries (Fig. 10A) and lie just below the sub-intestinal parenchymal muscle layer in the pre-pharyngeal region (Fig. 4A, C). Immediately behind the gonopore, the ovovitelline ducts ascend and progressively turn to the sagittal plane (Fig. 6). They converge dorsally, above the median third of the female atrium, to continue as a common ovovitelline duct (150–200 µm in length), which runs posteriorly almost horizontally to communicate with the female canal (~200 µm in length) (Figs 6, 7B, 10D). It curves ventrally to open into the posterior-dorsal part of the female atrium (Figs 6, 7B, 10D). The

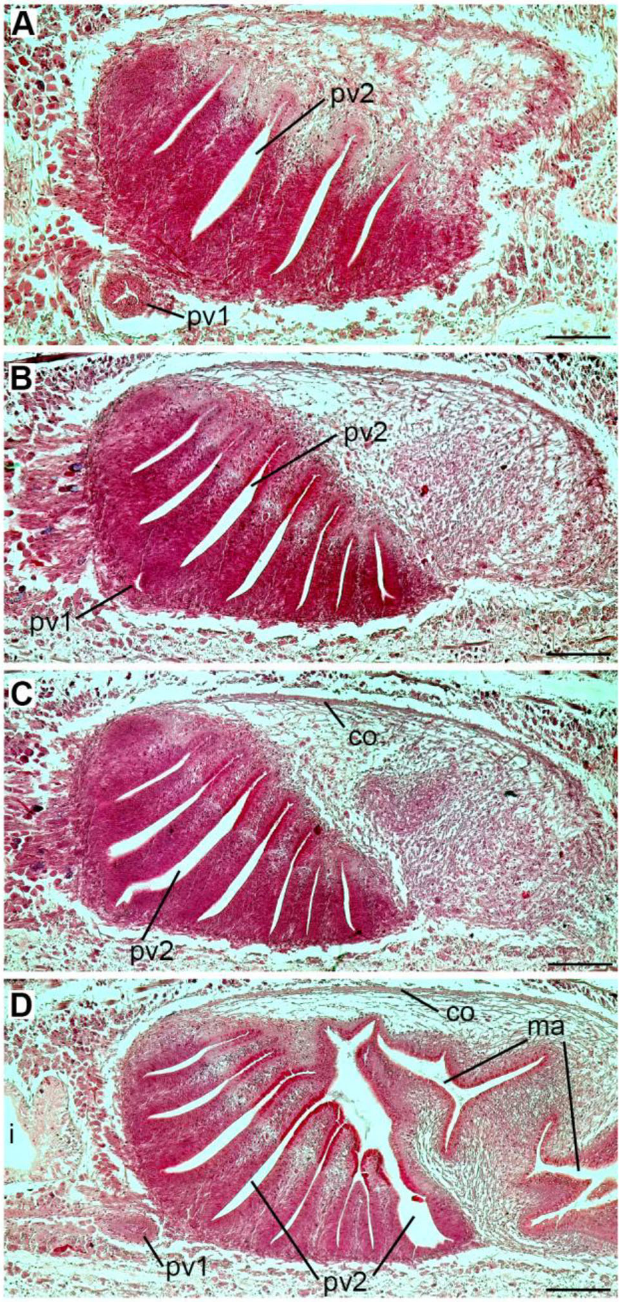


Fig. 8. Sagittal sections of the male reproductive system of *Choeradoplana malaria* sp. nov. (holotype). Scale bars: 250 µm.

female atrium is ovoid or pyriform with its lumen highly narrowed (Figs 6, 7B, 10D). It is a bit shorter (800 µm) than the male atrium in the holotype, but longer in the paratype (700 µm). The gonopore canal is straight, lined with ciliated columnar epithelium.

The ovovitelline ducts are lined with a ciliated cuboidal epithelium, surrounded by intermingled circular and longitudinal fibres (5 µm thick) in the pre-pharyngeal region (Fig. 4C). Their distal ascending portion receives the secretion from shell glands consisting of coarse erythrophil granules (Fig. 10C). The epithelial lining of the common ovovitelline duct is columnar and ciliated, pierced by abundant secretion from shell glands whose cell bodies locate dorsally, behind the female atrium (Fig. 10D). The common ovovitelline duct is coated by a decussate muscle layer with circular and longitudinal fibres (10–15 µm thick). The female genital canal is lined with ciliated columnar epithelium, filled with fine granular erythrophil secretion, and coated by a decussate muscle layer (10–20 µm thick) (Fig. 10D). The female atrium is lined by a non-ciliated columnar epithelium (50–60 µm in height) with a stratified appearance, leaving a very narrow lumen (Figs 6, 7B, 10D). This epithelium receives abundant fine granular erythrophil secretion and less abundant fine granular cyanophil secretion. The muscle coat of the female atrium is composed of circular fibres mixed with longitudinal (20–30 µm thick). The common muscle coat enfolding the female atrium is as that in the male portion but a bit thicker (25–50 µm thick). The vitellaria are scarce in the holotype but abundant in the paratype (Fig. 10A, B). The vitelline follicles are located among intestinal branches in the anterior and pre-pharyngeal body regions.

Molecular results and phylogenetic analyses

The sequences of the Cox-1 gene included in our analyses are listed in Table 1. The dataset comprised 27 ingroup sequences (nine species of *Choeradoplana*) and nine outgroup sequences (six species), all belonging to the subfamily Geoplaninae. The final length of the alignment was 822 bp. The phylogeny inferred from the BI analysis (Fig. 11) places *Choeradoplana malaria* sp. nov. within the genus *Choeradoplana*, which is monophyletic although with low support. The sister group of the new species here described is *Ch. gladismariae*, both conforming a well-supported clade. This clade is sister of (*Ch. minima* (*Ch. banga* (*Ch. iheringi* + *Ch. abaiba*))). The other group is made up of *Ch. agua*, *Ch. onae*, and *Ch. bocaina*. The sister group of the genus *Choeradoplana* is *Cephaloflexa araucariana* Carbayo & Leal-Zanchet, 2003, whose genus is paraphyletic in this analysis. The topology of the ML tree showed very similar groupings as BI, also retrieving *Ch. gladismariae* as the sister group of *Ch. malaria* sp. nov. (Supplemental Fig. S1).

Considering only *Choeradoplana* species, the mPTP analysis based on the BI tree indicated a total of 10 clusters (= entities) (Fig. 11). Eight of these groups match the nominal species already known, except for *Ch. bocaina* which appears as two separate entities. The new species described in this study, *Ch. malaria* sp. nov.,

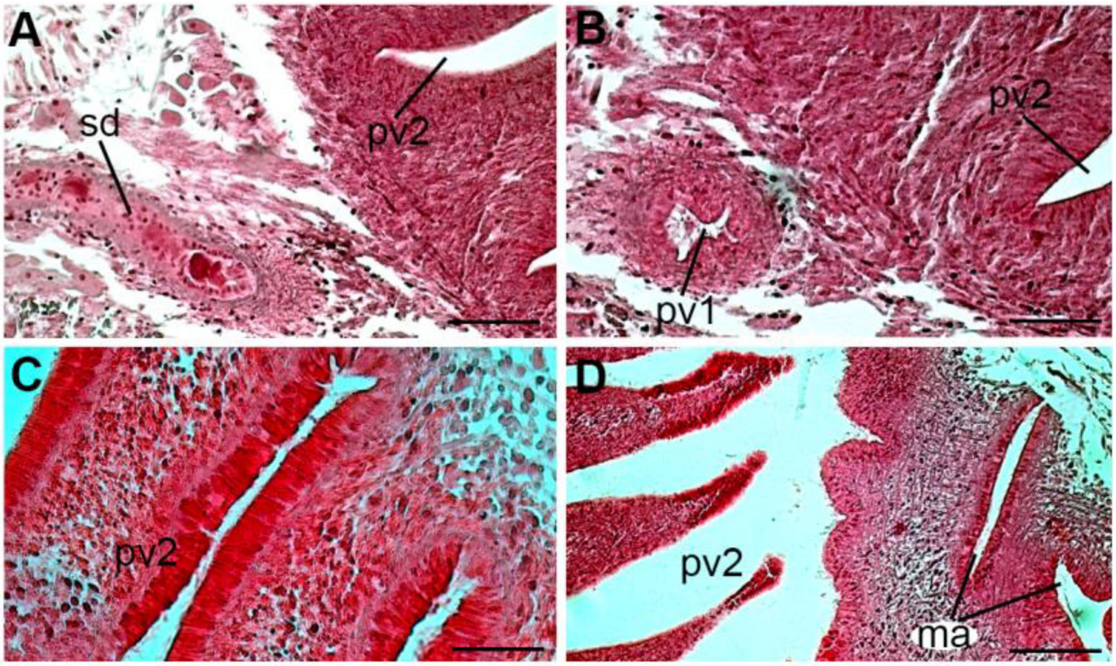


Fig. 9. Sagittal sections showing details of the male reproductive system of *Choeradoplana malaria* sp. nov. (holotype). (A) Distal region of the sperm ducts; (B) extrabulbar prostatic vesicle; (C) intrabulbar unpaired portion of the prostatic vesicle; (D) transition between the intrabulbar prostatic vesicle and the male atrium. Scale bars: 50 µm.

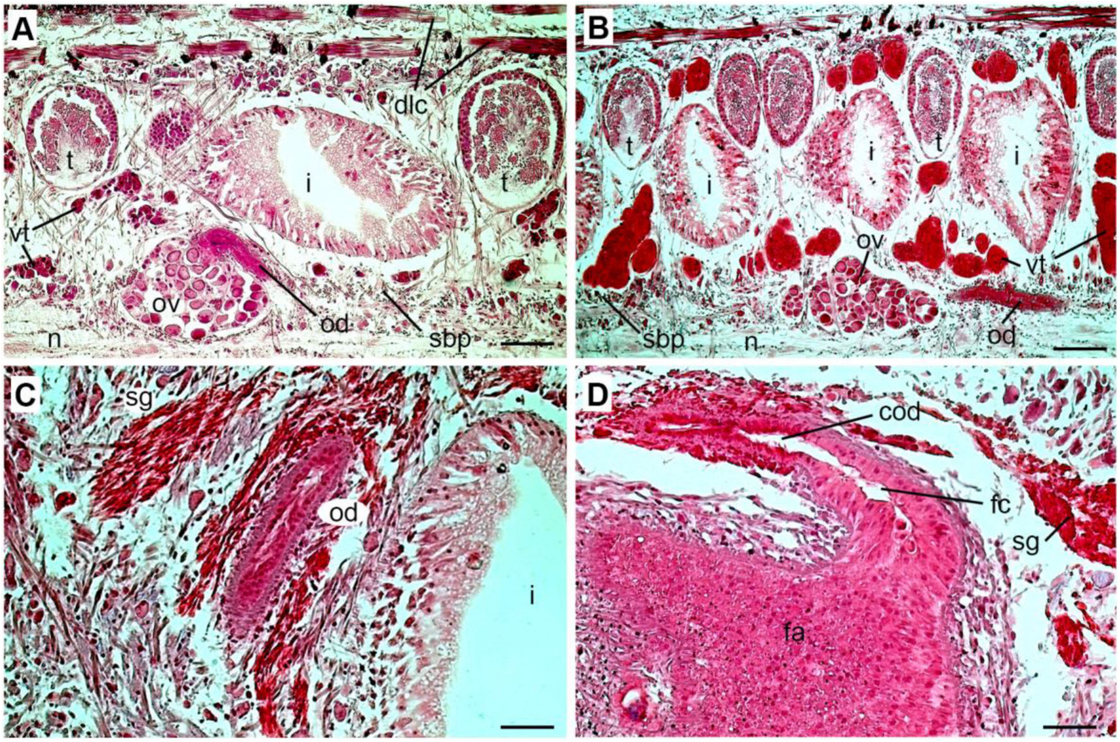


Fig. 10. Sagittal sections of the female reproductive system of *Choeradoplana malaria* sp. nov. (A, C, D, holotype; B, paratype). (A, B) At the level of the ovaries; (C) ascending portion of the ovovitelline ducts; (D) female atrium. Scale bars: (A, B) 100 µm, (C, D) 50 µm.

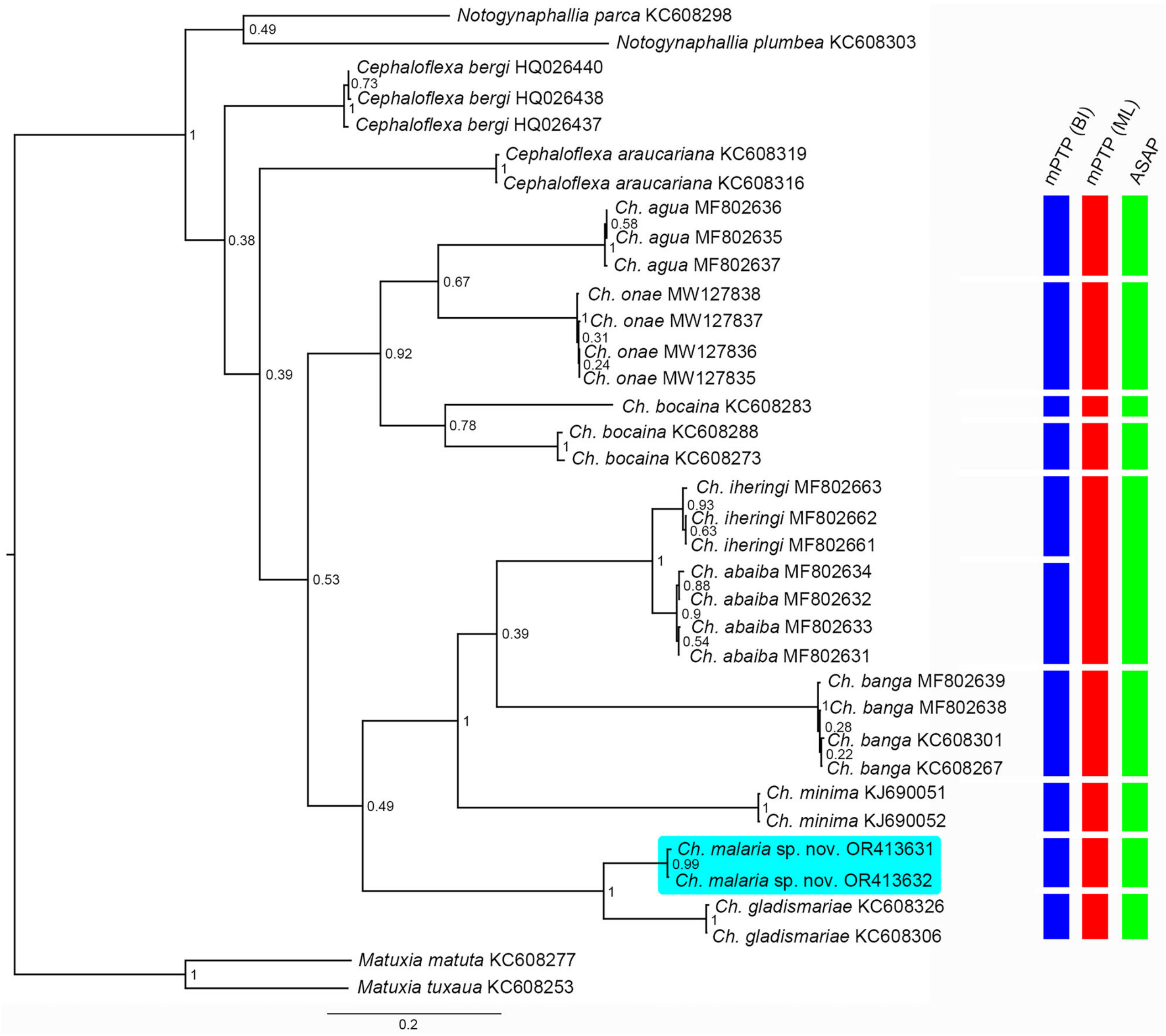


Fig. 11. Phylogenetic tree obtained by Bayesian inference showing the relationships, based on the Cox-1 dataset, of *Choeradoplana malaria* sp. nov. (highlighted in turquoise) with other representatives of Geoplanidae (with their GenBank accession numbers). Support values on the nodes correspond to posterior probability. The scale bar indicates substitutions per site. Coloured bars to the right of the tree represent grouping according to different species delimitation approaches for the genus *Choeradoplana.*

appears as a unique entity. The mPTP based on the tree of the ML analysis and the ASAP analysis yielded the same results (Fig. 11). They delimited nine clusters. From them, *Ch. malaria* sp. nov. is retrieved as a unique entity and *Ch. bocaina* is separated as two entities (as in the mPTP analysis based on the BI tree), while *Ch. iheringi* and *Ch. abaiba* collapse in a single cluster.

The two specimens of *Ch. malaria* sp. nov. showed a divergence of 0.27% (± 0.19%) (Supplemental Table S1). *Choeradoplana malaria* sp. nov. showed the lowest genetic distance with *Ch. gladismariae* (6%) (Supplemental Table S2), which also represented the lowest distance between species of *Choeradoplana* herein analysed, except between *Ch. iheringi* and *Ch. abaiba* (~3%). The new species showed the largest genetic distance (~12%) with *Ch. banga*, *Ch. bocaina*, *Ch. minima*, and *Ch. onae*. Only considering the genus *Choeradoplana*, the highest percentage of genetic distance was 13% (average: 11% ± 1.8%) (Supplemental Table S2).

Discussion

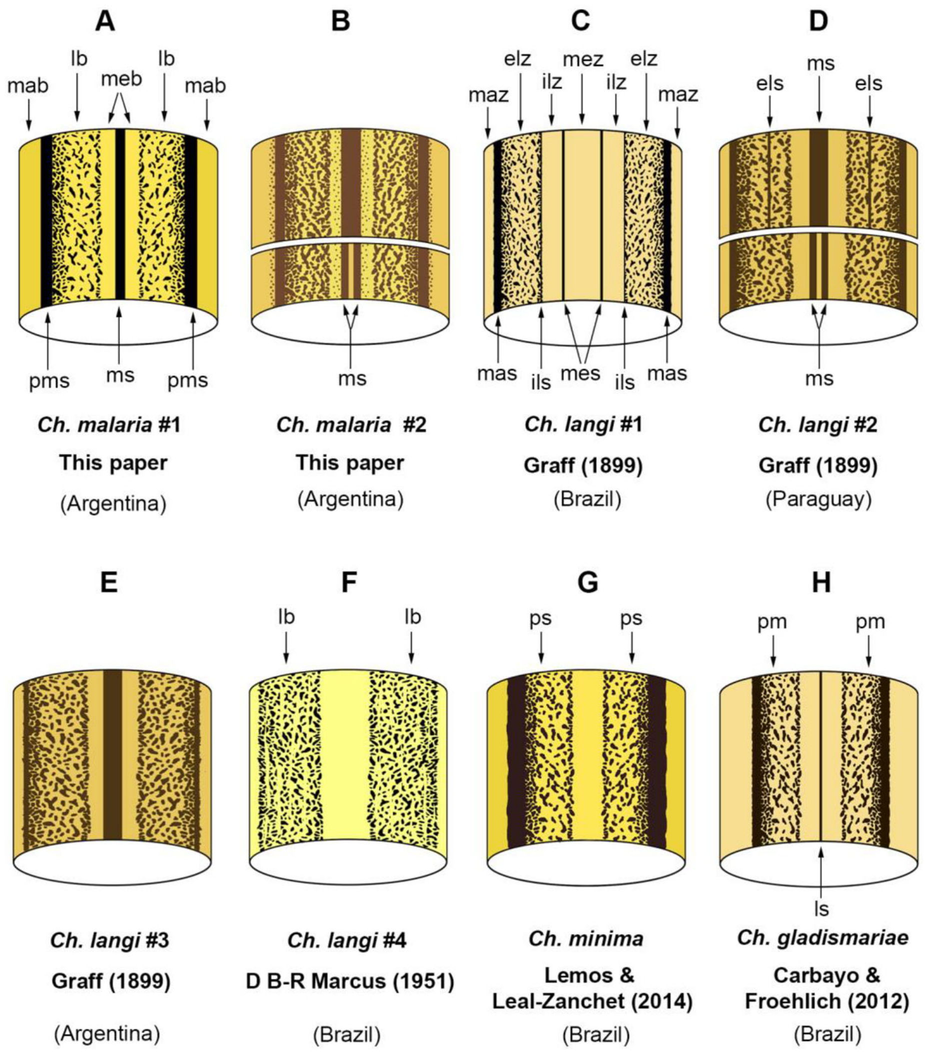
The new species herein described, *Choeradoplana malaria* sp. nov., meets the diagnostic characters of the genus *Choeradoplana* Graff, 1896, namely: cephalic region provided with a musculo-glandular organ consisting of (1) a cephalic retractor muscle, in the shape of an inverted triangle, arisen of sunk muscle fibres of the ventral cutaneous longitudinal musculature, and (2) two glandular cushions; strong cutaneous longitudinal musculature, dorsally and ventrally sunk into the parenchyma, like *Ch. gladismariae*; and cephalic apex devoid of eyes and sensory pits (Carbayo et al., 2013; Carbayo & Leal-Zanchet, 2003; Froehlich, 1954). This new taxon resembles, at least in the general arrangement of the dorsal pigment pattern, three other species of the genus, viz. *Ch. langi*, *Ch. gladismariae*, and *Ch. minima*. Moreover, *Ch. gladismariae* is very similar to *Ch. malaria* sp. nov. in the internal anatomy, mainly the pharynx and the copulatory apparatus, so both species can be considered cryptic species (see below).

The description of cryptic land planarian species has increased in recent years due to, in part, the renewed interest in their diversity (mainly in the Neotropics) and the use of molecular tools (Álvarez-Presas et al., 2015; Amaral et al., 2018; Araujo et al., 2020; Carbayo et al., 2016, 2018; Iturralde & Leal-Zanchet, 2019; Lemos et al., 2014). The genus *Choeradoplana* is an interesting example because several cryptic species have recently been identified. For instance, three species described by Carbayo et al. (2018), namely, *Choeradoplana pucupucu* Carbayo et al., 2018, *Choeradoplana agua* Carbayo et al., 2018, and *Choeradoplana abaiba* Carbayo et al., 2018 are very similar in their dorsal colour pattern so it is difficult to discriminate among them and even from *Choeradoplana iheringi* Graff, 1899, the type species of the genus. These last two species, *Ch. abaiba* and *Ch. iheringi*, are even so similar in their internal anatomy that without molecular support they would be considered a single species with intrapopulation variation (Carbayo et al., 2018). In this sense, some specimens considered by Leal-Zanchet and de Souza (2003) as *Ch. iheringi* were later transferred to *Ch. abaiba* by Carbayo et al. (2018). Three other species, *Choeradoplana benyai* Lemos & Leal-Zanchet, 2014, *Choeradoplana longivesicula* Iturralde & Leal-Zanchet, 2019, and *Choeradoplana cyanoatria* Iturralde & Leal-Zanchet, 2019 externally resemble the type species of the genus but are easily distinguished from *Ch. iheringi* by the presence of a large penis papilla (Iturralde & Leal-Zanchet, 2019; Lemos et al., 2014), which is absent in *Ch. iheringi*. Although they are not cryptic species in the strict sense, their remarkable similarity in external appearance forces taxonomists to observe the internal anatomy to discern them. Another example is *Choeradoplana crassiphalla*, which also resembles *Ch. iheringi* externally, but the anatomy of the copulatory apparatus distinguishes it well from the latter (Negrete & Brusa, 2012). Similarly, *Ch. banga* Carbayo & Froehlich, 2012 is difficult to differentiate from *Ch. iheringi* by their external appearance only (Carbayo & Froehlich, 2012).

In the following sections, we will compare separately the new species herein described, *Ch. malaria* sp. nov., with *Ch. langi*, *Ch. minima*, and *Ch. gladismariae* considering (1) differences, in some cases subtle, regarding the dorsal pigment pattern (Fig. 12) and the internal anatomy, and (2) a molecular analysis using the Cox-1 gene (without *Ch. langi* for lacking sequences) which supports the erecting of the new species.

Choeradoplana langi vs. *Choeradoplana malaria* sp. nov.

Choeradoplana langi was described by Graff (1894) originally as *Geoplana langi* from specimens collected in Brazil (without a specific locality), and Paraguay (near Asunción). In his publication, Graff gave a brief comment about the pigment pattern. Later, Graff (1897) transferred this species to the genus *Choeradoplana*, including a mention of one specimen found in Buenos Aires (Argentina). In his great contribution to land planarians of the world, Graff (1899) expanded the description of *Ch. langi* not only regarding the external appearance but also by describing the internal anatomy for the first time. Regarding the dorsal pigment pattern, Graff took specimens from Brazil (Blumenau, state of Santa Catarina) as reference for his description (Graff, 1899, plate 7, figs 6–8) (see also Fig. 12C, Table 2). He described *Ch. langi* as having a dark yellow ground colour ('ochraceus—ferrugineus') with two thin dark median stripes ('Medialstreif') separated by a yellow median zone ('Medianzone'), and flanked, on each side, by an internal lateral zone ('innere Lateralzone') also yellow; besides, the latter bordered by lateral bands ('äussere Lateralzone') consisting of numerous tiny dark spots which are closely packed on the external margins to conform a marginal stripe ('Marginalstreif'); and finally, the body margins with the same ground colour forming a marginal zone ('Marginalzone'). Graff (1899) also mentioned that the lateral bands set off an inner stripe ('inneren Lateralstreifen') on their internal margins by an accumulation of minute dark spots, which can, however, show interruptions. A variant of this general pattern was observed in one specimen from Paraguay (near Asunción), in which the median stripes merge into a single one ('Medianstreifen') along the body but separating in the posterior region, whereby a clear median zone is formed, as in the Brazilian specimens (Fig. 12D, Table 2). Additionally, a new stripe also appears in the middle of each lateral band formed



***Ch. malaria* #1**
This paper
(Argentina)

***Ch. malaria* #2**
This paper
(Argentina)

***Ch. langi* #1**
Graff (1899)
(Brazil)

***Ch. langi* #2**
Graff (1899)
(Paraguay)

***Ch. langi* #3**
Graff (1899)
(Argentina)

***Ch. langi* #4**
D B-R Marcus (1951)
(Brazil)

Ch. minima
Lemos & Leal-Zanchet (2014)
(Brazil)

Ch. gladismariae
Carbayo & Froehlich (2012)
(Brazil)

Fig. 12. Schematic drawing showing the pigment pattern of the dorsal surface of *Choeradoplana malaria* sp. nov. (with two variants), and of species with similar patterns (based on the original descriptions): *Choeradoplana langi* (with four variants), *Choeradoplana minima*, and *Choeradoplana gladismariae*. In all species (with their variants) are included: the source (author/s and year of publication), the country where each species (or variant) was found, and abbreviations of the terminology used for the pigment pattern (see Table 2).

Table 2. List of the abbreviations of the terminology used by different authors for the pigment pattern of the species of *Choeradoplana* schematized in Fig. 12.

Author/s	Abbreviations
Graff (1899)	**els**: external lateral stripe (äusseren Lateralstreifen); **elz**: external lateral zone (äussere Lateralzone); **ils**: inner lateral stripe (inneren Lateralstreifen); **ilz**: inner lateral zone (innere Lateralzone); **mas**: marginal stripe (Marginalstreif); **maz**: marginal zone (Marginalzone); **mes**: median stripes (Medialstreif); **mez**: median zone (Medianzone); **ms**: median stripe (Medianstreifen).
du Bois Reymond-Marcus (1951)	**lb**: lateral band.
Carbayo and Froehlich (2012)	**ls**: longitudinal mid-stripe; **pm**: para-median band.
Lemos et al. (2014)	**ps**: para-median stripe.
This paper	**lb**: lateral band (= elz in Graff); **mab**: marginal band (= maz in Graff); **meb**: median band (mez + ilz, in Graff); **ms**: median stripe (= ms in Graff); **pms**: para-marginal stripe (= mas in Graff).

by the accumulation of tiny dark spots (named by Graff as 'äusseren Lateralstreifen'); and the marginal stripes are very thin. Besides, Graff (1899) described one specimen from Argentina (Tigre city, Buenos Aires province) which maintains, in general, the same pattern as the specimen from Paraguay but presents a thickening of the lateral bands (formed by numerous tiny spots) that precludes discrimination between the marginal and inner stripes (Fig. 12E, Table 2).

Later, du Bois Reymond-Marcus (1951) published new observations on *Ch. langi* from material found in Brazil (Brusque, state of Santa Catarina), distant about 30 km from the locality of the specimens studied by Graff (1899) (Blumenau, state of Santa Catarina). She commented on the existence of a wide range of variations in the colour pattern, mainly regarding the lateral bands with dark brown or black pigment. However, according to her drawings, the main difference between this material and the specimens from Blumenau arises from the absence, in the former, of the median stripes (Fig. 12F, Table 2).

From all this background, it is possible to expect that more than one species resides under the name *Ch. langi*, especially if we consider the geographic distance (including important barriers to dispersion as numerous streams and rivers) between the localities of the material studied by Graff and du Bois Reymond-Marcus. For instance, Blumenau or Brusque (Brazil) are separated from Asunción (Paraguay) by about 1100 km, a similar distance as between Asunción and Buenos Aires (Argentina); and from Blumenau to Buenos Aires there are about 1700 km. Species with a wide distribution have been described, such as *Winsoria bipatria*, with disjunct populations separated by about 800 km. However, they were specimens with identical external appearance and very similar anatomical features, in addition to showing very low genetic divergence (Negrete et al., 2020). Here, we cannot verify differences at the molecular level between *Ch. malaria* sp. nov. and *Ch. langi* because there are no available sequences of *Ch. langi*, but they can be distinguished by details of the pigmentation pattern of the dorsum and more clearly by their internal anatomy.

The main difference between *Ch. langi* from Blumenau (Brazil) and *Ch. malaria* sp. nov. is the presence, in the former, of two thin median stripes (named as 'Medialstreif' by Graff) versus the only stripe observed in the new species, which is also much wider than each stripe observed in *Ch. langi* (Fig. 12A–C). The specimens assigned by du Bois Reymond-Marcus (1951) as *Ch. langi* are further differentiated by the absence of a median stripe (Fig. 12F). On the other hand, the material from Paraguay and Argentina studied by Graff seems to match the pigment pattern of *Ch. malaria* sp. nov. (Fig. 12D, E). Even the presence, in at least one specimen from Argentina studied by us (named variant 2 in the description), of a unique median stripe arranged along the body but separating into two fine stripes toward the posterior region is in agreement with the observation given by Graff (1899) for the specimen from Paraguay (Fig. 12B, D).

Regarding the internal anatomy, we assume that the first description of *Ch. langi* was based on specimens from Brazil (Blumenau), because Graff (1899) only studied one specimen from Paraguay, from which he only referred to external features, and, according to him, the material from Argentina was badly preserved. Regarding the male system, he remarked on the presence of a highly muscular male atrium 'with a penis still hinted', and a penis sheath formed by a circular fold located close to the gonopore. Graff also described a glandular part of the ejaculatory duct with the same features and glandular secretions as the prostatic vesicle, and the distal sections of sperm ducts being intrabulbar. Regarding the female system, the female atrium was described as being remarkably small and tubular-shaped, obliquely ascending to open into the female canal and then into the common ovovitelline duct (Graff, 1899, Fig. 46). From a sagittal section of the pharynx (Graff, 1899, plate 29, fig. 11), although it seems somewhat contracted by the fixation, it appears to be cylindrical with the dorsal insertion a bit posteriorly displaced. An interesting feature of *Ch. langi* underlies on Graff's drawing of a cross section at the level of the ovaries (Graff, 1899, plate 30, fig. 1), showing the ventral cutaneous longitudinal muscle fibre bundles divided into two layers, one underlying the diagonal layer (typical arrangement) and another sunken in the parenchyma. In this figure, the dorsal longitudinal muscle bundles are not shown as subdivided, i.e., they lack a sunken portion into the parenchyma.

According to du Bois Reymond-Marcus (1951), the specimens from Brusque (Brazil) were 'in complete agreement' with the material studied by Graff, showing a muscularized male atrium, absence of a penis papilla, an intrabulbar prostatic vesicle receiving the same granular secretion as the ejaculatory duct, a female system with a narrow tube-shaped female atrium, oriented obliquely backward, and an also narrow female canal which continues into a short common ovovitelline duct. The pharynx also appears to be cylindrical, with the dorsal insertion a bit posteriorly displaced. Froehlich (1954) made some observations on *Ch. langi* regarding features of the cephalic and pre-pharyngeal body regions. Although Froehlich did not specify the locality, the number of specimens studied, and if it was new material, we assume they come from southern Brazil. He mentioned that the longitudinal cutaneous musculature is ventrally sunk, separated from the ‘normal’ cutaneous layer, as in *Ch. iheringi*. This latter feature agrees with the schematic drawing of Graff (1899), as mentioned above.

All these clues lead us to speculate that the specimens from southern Brazil, namely, those named as *Ch. langi* by Graff (1897) from Blumenau together with the animals identified by du Bois Reymond-Marcus (1951) from Brusque, and the material studied by Froehlich (1954), belong to the same species: *Ch. langi*. These specimens are not only very similar in their external features but also share the anatomy of the copulatory apparatus (highly muscularized male part and tubular female atrium), the cylindrical pharynx, and the arrangement of the longitudinal cutaneous musculature (only ventrally sunk into the parenchyma). These characteristics differentiate this species from *Ch. malaria* sp. nov. In this way, *Ch. langi* would have its geographic distribution limited to southern Brazil. On the other hand, the specimens mentioned by Graff (1897, 1899) from Argentina (Tigre city in Buenos Aires province) could most likely belong to *Ch. malaria* sp. nov. because, as stated above, their pigmentation pattern is essentially the same as that of *Ch. malaria* sp. nov. (Fig. 12B, E, Table 2) added to the fact that their geographic location is rather closer (∼90 km) to the type-locality of the new species herein described in comparison to the Brazilian specimens. The specimen from Paraguay, which externally resembles variant 2 of *Ch. malaria* sp. nov. so closely, represents an enigma (Fig. 12B, D). One possibility is that this specimen corresponds to a different species, which could be part of the cryptic species complex formed by the *Choeradoplana* species herein discussed. It could also be *Ch. langi*, although this is unlikely due to the distance of Paraguay with the populations from southern Brazil and the great barriers to dispersal above mentioned. It is worth mentioning that the specimen from Paraguay was collected by the same person (A. Borelli) who carried out samplings at the end of the 19th century both in Argentina and Paraguay. Therefore, another possibility is that he accidentally mixed this specimen with other planarians, namely, specimens of *Ch. malaria* found in Argentina. Until there is a greater knowledge of the diversity of land planarians in Paraguay, this issue will remain unresolved since, apparently, this specimen has not been preserved.

Choeradoplana minima vs. *Choeradoplana malaria* sp. nov.

The colour pattern of the dorsal surface of *Choeradoplana minima* (Fig. 12G, Table 2) consists of a median band with pale yellow pigment, two wide lateral bands formed by tiny specks of dark-brown pigment that thicken toward their outer margins forming an irregular stripe on each side of the body, and two marginal bands, whose yellow pigment is darker than that of the median band, being wider than the latter (Lemos et al., 2014). Although this general pattern resembles that of *Ch. malaria* sp. nov., the new species distinguishes itself by the presence of a median stripe along the median band, which is absent in *Ch. minima*. However, the main difference between both species lies in the internal anatomy, essentially in the copulatory apparatus. In this sense, *Ch. minima* can be easily distinguished from *Ch. malaria* sp. nov. and other species of *Choeradoplana* by possessing an inverted penis. This type of penis seems to be unique in Geoplaninae (Lemos et al., 2014). Additionally, *Ch. minima* shows, like most species of *Choeradoplana* (except for *Ch. malaria* sp. nov. and *Ch. gladismariae*), only the ventral cutaneous musculature layer insunk in the parenchyma, while the dorsal cutaneous musculature is not (Lemos et al., 2014).

Choeradoplana gladismariae vs. *Choeradoplana malaria* sp. nov.

Choeradoplana gladismariae, only known from its type locality (Serra da Cantareira, state of São Paulo, Brazil), shows a yellowish median band with a thin dark brown median stripe and two yellowish para-median bands with a marmoreal appearance by the splashing of minute specks of brown pigment (Carbayo & Froehlich, 2012). These specks accumulate on the external margins of each para-median band forming an irregularly contoured dark stripe (Fig. 12H, Table 2). The marginal bands, whose pigment is the same as the median band, show a similar width to the latter. At first sight, the dorsal pigment pattern of *Ch. gladismariae* seems to be the same as *Ch. malaria* sp. nov. However, some details allow us to differentiate both species externally. For instance, the longitudinal mid-stripe of the new species is about twice as wide as in *Ch. gladismariae*. Additionally, each marginal band of *Ch. malaria* sp. nov. is half the width of the median band, while in *Ch. gladismariae* the marginal bands are roughly as wide as the median band. Ventrally, the creeping sole of *Ch. malaria* sp. nov. occupies about 100% of the body width *vs.* the creeping sole of *Ch. gladismariae* occupying ∼80% of body width. These differences, which could be considered subtle, are reinforced by others related to the internal anatomy, namely: (1) the thickness of the cutaneous musculature of *Ch. malaria* sp. nov. represents 13–14% of body height *vs.* 21–22% in *Ch. gladismariae*; (2) the ovovitelline ducts arise from the mid-dorsal side of the ovaries in *Ch. malaria* sp. nov. *vs.* arising from the external side of the ovaries in *Ch. gladismariae*; (3) the female atrium shows a similar length to the male atrium or even a little longer in *Ch. malaria* sp. nov. *vs.* length of the female atrium corresponding to 2/3 of the length of the male atrium in *Ch. gladismariae*; (4) epithelium of the female atrium columnar with stratified appearance in *Ch. malaria* sp. nov. *vs.* columnar in *Ch. gladismariae*; and (5) the paired portions of the prostatic vesicle open separately into the unpaired portion of the vesicle in *Ch. malaria* sp. nov. *vs.* the paired portions unite, forming a short tube before entering the unpaired portion in *Ch. gladismariae* (Carbayo & Froehlich, 2012). All these anatomical differences between the two species, although conclusive, are still minimal, so they should undoubtedly be considered cryptic species.

Phylogenetic analyses reinforce the identity of Ch. malaria *sp. nov.*

It is known that Cox-1 by itself may be insufficient to resolve species interrelationships or at deeper levels, leading to low support values on inner nodes, but contains enough information to identify and delimit species (Carbayo et al., 2018; Lago-Barcia et al., 2021; Lemos et al., 2014). In this sense, the phylogenetic reconstructions based on the Cox-1 sequences include the new species herein proposed into the genus *Choeradoplana*, although with low statistical support; while the species of *Choeradoplana* included in our analyses are recovered with high support values, except for *Ch. bocaina* as was also observed by Carbayo et al. (2018).

The molecular analyses show a sister-taxon relationship between *Ch. malaria* and *Ch. gladismariae*, species with which it has the lowest genetic distance among the *Choeradoplana* species; whereas *Ch. minima*, which belongs to a different clade (though with low support), shows the highest genetic distance with the new species.

The robust clade formed by *Ch. malaria* and *Ch. gladismariae* is reflected in the great similarity both in their external appearance and many traits of the internal anatomy, as stated above. Certainly, without any molecular support, their uniqueness as different species could have been masked as intraspecific variation. A similar situation was observed between *Ch. iheringi* and *Ch. abaiba*, species distributed in southern Brazil. Both species, whose genetic distance has shown to be even smaller compared with *Ch. malaria*/*Ch. gladismariae* in our analysis, are also cryptic and only discriminated by molecular analyses (Carbayo et al., 2018). An interesting argument by Carbayo et al. (2018) is that the similarity between these species, not only morphological but also at a molecular level, reflects a process of ongoing speciation by isolation, so, although the mitochondrial genome already shows signs of differentiation, other genes, such as nuclear ones, do not. Contrarily to the case of *Ch. iheringi*/*Ch. abaiba* (with populations separated by about 200 km), the great geographic distance (more than 2000 km) between the populations of *Ch. malaria* sp. nov. and *Ch. gladismariae* could explain that both species diversified longer ago and therefore, although there are enough morphological similarities to consider them cryptic species, phylogenetically they are differentiable with a mitochondrial marker.

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ORCID

Lisandro Negrete http://orcid.org/0000-0003-3398-6606
Agustina Zivano http://orcid.org/0009-0003-5563-6356
Marina Lenguas Francavilla http://orcid.org/0000-0002-8296-6065

Cristina Damborenea http://orcid.org/0000-0002-6411-1282
Francisco Brusa http://orcid.org/0000-0003-2206-1560

Data availability statement

The new DNA sequences included in this study will be available on GenBank. All other datasets generated and/or analysed during the current study are available from the corresponding author (LN) upon reasonable request.

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