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ORIGINAL ARTICLE

Tracking the diversity of the flatworm genus *Imbira* (Platyhelminthes) in the Atlantic Forest

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Abstract

The genus *Imbira* Carbayo et al., 2013 encompasses two species, *Imbira guaiana* (Leal-Zanchet & Carbayo, 2001) and *Imbira marcusi* Carbayo et al., 2013, which occur in south Brazil, in areas originally covered by the Atlantic Forest. In the present study, we examine the genetic diversity within the genus, investigate the occurrence of molecular autapomorphies for its species and describe a new species for the genus based on an integrative approach. The Bayesian and maximum likelihood analyses based on DNA barcoding recovered the monophyly of the genus *Imbira*, but indicate that specimens representing *I. marcusi* correspond to five distinct lineages. These analyses, as well as sequence divergence data, revealed that the new species herein described is closely related to *I. guaiana* and that the specific status of specimens of *I. marcusi* available in GenBank should be reviewed. In addition, sequence analysis revealed 32 molecular autapomorphies for all independent evolutionary units within the genus. The new species described herein seems to be endemic to its type locality, a private area without legal protection.

Keywords Geoplaninae · Taxonomy · Phylogeny · COI

Introduction

Land planarians (Tricladida: Continenticola), soil animals of cryptic behaviour, depend on the humidity of their microhabitat because they do not have water-conserving adaptations (Kawaguti 1932; Froehlich 1955a; Winsor et al. 1998). These flatworms have restricted locomotion capacity over long distances, so that there are many endemic species (Sluys 1995). The highest species richness of land planarians worldwide has been documented in the southern hemisphere (Winsor et al. 1998), in areas which were originally covered by the Brazilian Atlantic Rain Forest (Sluys 1998, 1999; Carbayo et al. 2002; Fick et al. 2006; Leal-Zanchet et al. 2011). Land flatworms are predators of other soil invertebrates (Froehlich 1955b; Boll et al. 2015; Boll and Leal-Zanchet 2015, 2016). Many land triclad species select relatively undisturbed areas, such as native forests and plantations of a native tree species in southern Brazil, rather than plantations of exotic trees (Carbayo et al. 2002; Fonseca et al. 2009; Oliveira et al. 2014). The coexistence of several species in the same habitat is possible due to differences in prey items, which reduces interspecific competition (Boll and Leal-Zanchet 2016).

Land flatworms belong to the family Geoplanidae, which is subdivided into four subfamilies, viz. Bipaliinae, Microplaninae, Rhynchodeminae and Geoplaninae (Sluys et al. 2009). The subfamily Geoplaninae has a Neotropical distribution and comprises about 270 species in 23 genera (Sluys et al. 2009; Carbayo et al. 2013; Lemos et al. 2014; Negrete et al. 2014; Rossi et al. 2014; Álvarez-Presas et al. 2015; Carbayo and Almeida 2015; Rossi et al. 2015; Carbayo et al. 2016a, b).

Usually, taxonomic descriptions of land flatworms detail external features, such as colour pattern and eye arrangement, and anatomical features mainly related to the pharyngeal anatomy and anatomy of the reproductive apparatus. However, in some genera, such features are very homogeneous among species, making species delimitation a difficult task. Therefore, more recently integrative approaches employing molecular and morphological data have been used for species identification in the subfamily Geoplaninae (Lemos et al. 2014; Álvarez-Presas et al. 2015; Rossi et al. 2015; Carbayo et al. 2016a, b).

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The genus *Imbira* Carbayo et al., 2013 is one of the six new genera proposed in a recent appraisal of the phylogeny of Geoplaninae, based on molecular analyses (Carbayo et al. 2013). It comprises two species, which were formerly included in the genera *Notogynaphallia* Ogren & Kawakatsu, 1990 and *Geoplana* Stimpson, 1857. *Imbira marcusi* Carbayo et al., 2013 occurs in areas with dense ombrophilous forest in Southeast Brazil (Marcus 1951; Álvarez-Presas et al. 2011), and *Imbira guaiana* (Leal-Zanchet & Carbayo, 2001) in areas with ombrophilous forest mixed with *Araucaria angustifolia* in southern Brazil (Leal-Zanchet and Carbayo 2001), both phytophysiognomies belonging to the Atlantic Rain Forest.

Imbira marcusi (*Geoplana goetschi* sensu Marcus) was firstly studied by Marcus (1951) and considered conspecific with *Geoplana goetschi* Riester, 1938, later being transferred to the genus *Notogynaphallia* by Ogren & Kawakatsu (1990). However, they are different species, as pointed out by Álvarez-Presas et al. (2011) and therefore *Geoplana goetschi* sensu Marcus was named *Imbira marcusi* by Carbayo et al. (2013). It is a relatively abundant species with a wide distribution in the South Atlantic Forest, with records from at least four areas in Southeast Brazil (Álvarez-Presas et al. 2011). Besides one other land flatworm species, *I. marcusi* was tested as a model organism for phylogeographic studies. In that study, the population of *I. marcusi* showed a high level of genetic variability (Álvarez-Presas et al. 2011). In contrast, *I. guaiana* is a species known only from its type locality, despite extensive studies on land flatworms having been performed in neighbouring areas (Leal-Zanchet and Carbayo 2001; Carbayo et al. 2002; Baptista et al. 2006; Fick et al. 2006; Leal-Zanchet and Baptista 2009; Leal-Zanchet et al. 2011).

By sampling land flatworms in an area of the Atlantic Forest, relatively close to the type locality of *I. guaiana*, we encountered specimens that match the diagnostic features of the genus *Imbira* and that are herein described as representing a new species. Further, we examine the genetic diversity within the genus *Imbira* and investigate possible molecular apomorphies for its species.

Materials and methods

Specimens were collected during the night by direct sampling in leaf litter. The specimens collected, locality data, and Genbank accession numbers are detailed in the taxonomic account presented below, as well as in the Supplementary Material, Appendix 1.

Live specimens were analysed for colour pattern and body shape and dimensions. Before fixation, the posterior tip of three specimens was cut off and fixed in 100% ethyl alcohol for molecular analysis. After that, specimens were killed with boiling water, fixed in 10% neutral formalin and, subsequently, stored in 70% ethyl alcohol. Methods described by Rossi et al. (2015) were used for histological processing of the material and analysis of external and internal characters. The material was sectioned at intervals of 6 μm.

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

Molecular analyses

DNA isolation, PCR amplification and sequencing of amplicons' DNA were performed from specimens preserved in 100% ethanol using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The following primers were used to amplify approximately 800 bp fragment of the cytochrome *c* oxidase subunit I (COI), as well as for sequencing: BarT (5′-ATG ACD GCS CAT GGT TTA ATA ATG AT-3′) (Álvarez-Presas et al. 2011) and COIR (5′-CCW GTY ARM CCH CCW AYA GTA AA-3′) (Lázaro et al. 2009). Each PCR was conducted in a 25-μl reaction volume containing 20–50 ng of genomic DNA, 0.2 μM of each primer, 200 μM dNTPs, 1× buffer, 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase (Invitrogen, USA) and ultrapure H_2O. The following PCR conditions were used: 95 °C for 3 min (denaturation), 38 cycles of 50 s at 94 °C (denaturation), 60 s at 50 °C (annealing) and 50 s at 72 °C (extension), then 72 °C for 5 min. PCR results were verified through electrophoresis of the amplicons on 1% agarose gels stained with GelRed (Biotium, Hayward, CA, USA), and visualized under UV transillumination. PCR products were 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.

Sequence and phylogenetic analysis

We visually inspected the chromatogram quality in Chromas Pro 1.5 software (http://www.technelysium.com.au). Sequences were also checked using the BLASTn online tool for comparison with sequences deposited in the GenBank database (NCBI). Sense and antisense sequences for each specimen were aligned by MAFFT (Katoh and Toh 2008) using the E-INS-i algorithm, manually inspected and, when necessary, corrected using BioEdit 5.0.9 (Hall 1999) to obtain a consensus sequence of 550 bp length of high quality for the new species. The amino acid translation was examined to

ensure that no gaps or stop codons were present in the alignment.

We performed phylogenetic analyses based on maximum likelihood (ML) using RAxML (Stamatakis 2006), which was run on the CIPRES Science Gateway cluster (Miller et al. 2010), and Bayesian inference (BI) using MrBayes (Ronquist et al. 2012). We used jModelTest 2.1.1 software (Darriba et al. 2012) to assess the best-fitting model of nucleotide substitution based on the Akaike information criterion (AIC) (Akaike 1974). Based on likelihood scores, the best-fit model for the two genes in this study was the General Time Reversible (GTR; Tavaré 1986) model with the proportion of invariant sites (I) estimated, and gamma distributed rate variation (G). ML analyses were performed using RAxML (Stamatakis 2006). Each RAxML run employed the GTR model and estimated the gamma rate distribution. Moreover, we evaluated nodal support using 1000 bootstrap pseudoreplicates of the data (Felsenstein 1985). We performed BI with default priors; three heated and one cold Markov chains were run from two random starting points. We ran the Markov chain Monte Carlo search with 10,000,000 generations (repeated 3 times), sampled at every 1000 generations; the first 25% trees were discarded as "burn-in." At that point, the chain reaches a stationary state; this ensures that the average split frequency between the runs is less than 1%. We considered to be significantly supported those nodes with bootstrap values > 70 (ML) and Bayesian posterior probabilities > 0.95 (BI). We calculated pairwise nucleotide distances between all sequences according to Kimura's 2-parameter model and 1000 bootstraps (Kimura 1980) using MEGA version 6 (Tamura et al. 2011). Additionally, we also analysed the dataset in PAUP*4.0b10 (Swofford 2002) to detect the molecular autapomorphies of species and to calculate the consistency index of each character using heuristic parsimony analysis, with 100 random stepwise additions of taxa (tree-bisection-reconnection branch swapping).

The Molecular Operational Taxonomic Unit

Barcode sequences have frequently been used to infer Molecular Operational Taxonomic Units (MOTUs), in which clusters of sequences that differ from each other are proposed as independent taxonomic units (Floyd et al. 2002). In the present study, we used the automatic barcode gap discovery (ABGD) tool (Puillandre et al. 2012) and the Generalized Mixed Yule Coalescent (GMYC) method for delimiting species (Fujisawa and Barraclough 2013). The ABGD was run based on all COI sequences (Supplementary Material, Appendix S1), using the online version of the program (http://wwwabi.snv.jussieu.fr/public/abgd/), with default value of relative gap width ($X = 1.0$) and Kimura-2-parameter (K2P) model (Kimura 1980) as the model of nucleotide evolution.

The single-threshold GMYC method was implemented in the R package "splits" (SPecies LImits by Threshold Statistics) (Monaghan et al. 2009), using the unique threshold method to detect the transition point between intra- and interspecific relationships, considering the ultrametric tree based on the COI gene generated by BEAST v.2.2.1 (Bouckaert et al. 2014). The nucleotide substitution models were estimated in jModelTest with the following conditions: GTR (−lnL = 4.062,659) (R(a) 1.12636, R(b) 9.06216, R(c) 6.22129, R(d) 1.13706, R(e) 4.60467, R(f) 1.0) + gamma distribution (0.8140) + invariable sites (0.4160).

The parameters estimated in the jModelTest were used in the BEAUti program assuming a relaxed molecular clock with a lognormal distribution and Yule model. Three independent runs were carried out in BEAST 2 with 4×10^7 generations each. Later, the effective sample size (ESS > 200) of each run was verified in Tracer v1.5 (Rambaut and Drummond 2009). The TreeAnnotator software was used to summarize the information from a sample of trees (30% burn-in percentage, 0.5 posterior probability limit and mean height node) onto a maximum clade credibility tree.

Abbreviations used in the figures

The following abbreviations are used in the figures: cmc, common muscle coat; cov, common glandular ovovitelline duct; db, dorsal band; de, dorsal epidermis; di, dorsal insertion; dm, dorsal cutaneous musculature; e, eyes; ej, ejaculatory duct; ep, epithelium; fa, female atrium; fc, female canal; gm, glandular margin; go, gonopore; i, intestine; im, internal musculature; l, lateral stripes; lm, longitudinal musculature; lu, pharyngeal lumen; m, muscle fibres; mo, mouth; ma, male atrium; mm, mesenchymal musculature; n, nerve cord; o, ovary; oe, oesophagus; om, outer musculature; ov, ovovitelline duct; pp, pharyngeal pouch; pv, prostatic vesicle; sg, shell glands; sp, sensory pit; sv, spermiducal vesicle; t, testes; v, vitellaria; ve, ventral epidermis; vi, ventral insertion; vm, ventral cutaneous musculature.

Results

Molecular results

Maximum likelihood and Bayesian analyses based on the set of specimens (see Fig. 1 and Supplementary Material, Appendix S1) showed the same topology and recovered *Imbira* as a monophyletic group (Bayesian posterior probability—BPP = 0.99; bootstrap = 92%). We detected a total of 95-bp (17.3%) variable nucleotide sites in the COI gene (out of a total of 550-bp nucleotide sites included in the analyses of the specimens of *Imbira* shown in Fig. 1). Considering the dataset, we did not identify putative NUMTs (nuclear DNA

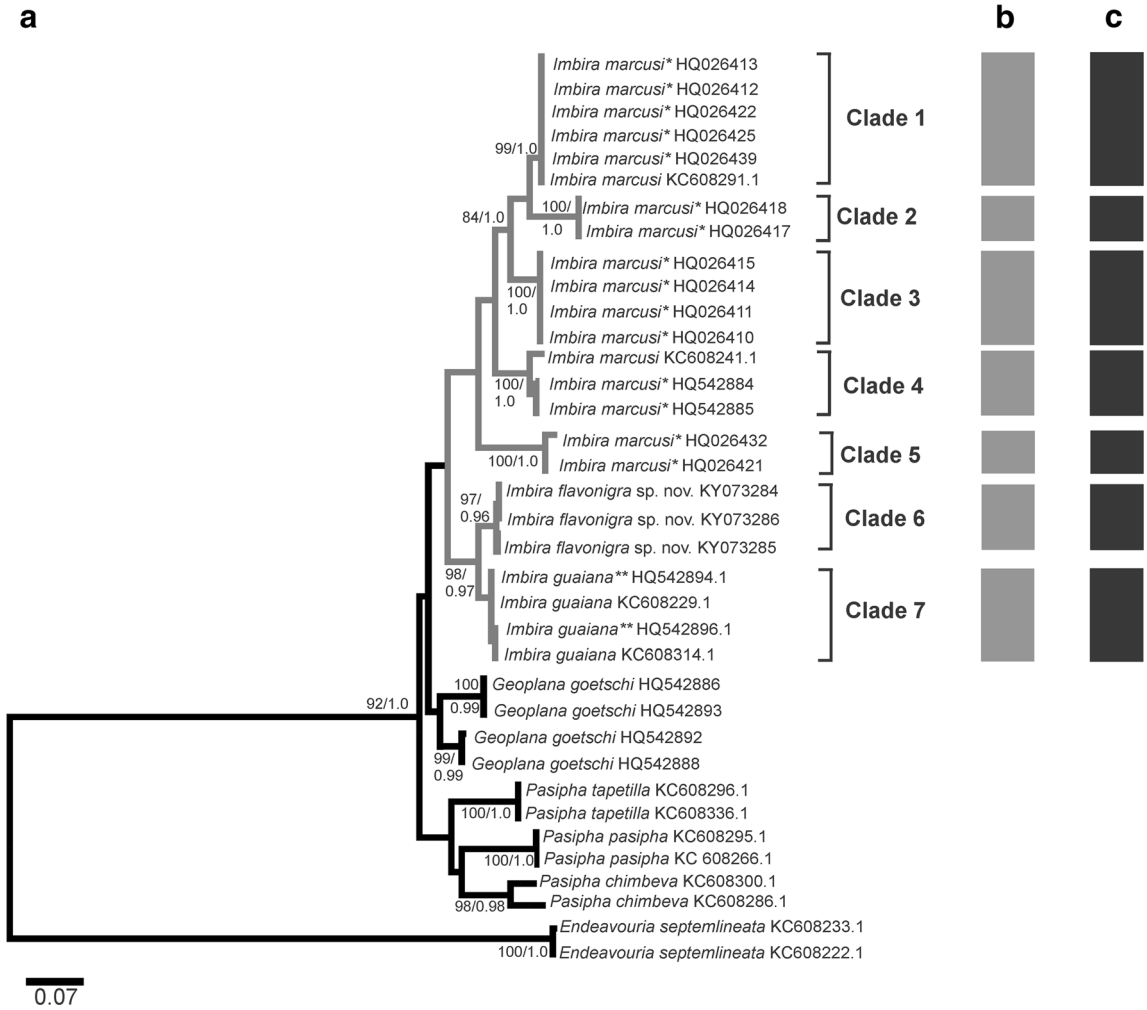

Fig. 1 **a** Maximum likelihood phylogenetic tree inferred from the 550 bp of cytochrome c oxidase subunit I gene. **b** ABGD analysis and **c** GMYC analysis. Values indicate support for each node according to the bootstrap support values > 80 and maximum posterior probabilities > 0.95, respectively. Scale bar: number of changes per site. *Specimens recorded as *Geoplana goetschi* in GenBank; **Specimens listed as *Notogynaphallia guaiana* in GenBank

sequences originating from mitochondrial DNA sequences), insertions, deletions or stop codons, which indicates that all amplified sequences are consistent with functional mitochondrial genes.

The members of the in-group formed seven well-supported clades (bootstrap > 92%; BPP > 1.0) (Fig. 1), including two currently recognized species (*Imbira marcusi* and *Imbira guaiana*) and also a new species herein described as *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov., which is closely related to *I. guaiana*. *Imbira flavonigra* and *I. guaiana* occur in southern Brazil (state of Rio Grande do Sul) with their type localities about 70 km apart from each other. The type locality of *I. guaiana* is situated at 950 a.s.l., at the border of the Araucaria Plateau, whereas that of *I. flavonigra* is approximately at sea level. The remaining specimens, identified as *Imbira marcusi* (*Geoplana goetschi* sensu Marcus, 1951), correspond to five distinct lineages, which occur in at least four localities in southeastern Brazil (Fig. 2). One of these areas, the Serra da Bocaina National Park (SBNP) in São Paulo, houses two lineages (clades 1 and 3), occurring in sympatry with an intraspecific divergence of 3.5%. Likewise, in the Boracéia Biological Station (BBS, Salesópolis, São Paulo), two independent groups (clades 2 and 4) live in sympatry with a divergence of 7.4%. However, the specimens forming clade 4 represent an allopatric group separated by a distance of 65 km, occurring both in BBS, at 850 m a.s.l., and in São Sebastião (SB/SMSP, Serra do Mar State Park, São Paulo), at sea level, showing an intraspecific divergence smaller than 1.4% (Appendix S2). In the state of São Paulo, the southernmost records concerned the specimens of clade 5, found in the Intervales State Park (ISP, Ribeirão Grande, São Paulo). Measures of intra-clade variation of the COI gene indicated that the genetic divergence of these specimens was 1.4 ± 0.7%, while all comparisons with the other clades of the state of São Paulo were higher than the mean intra-clade divergence (> 8%; see Supplementary Material, Appendix S2).

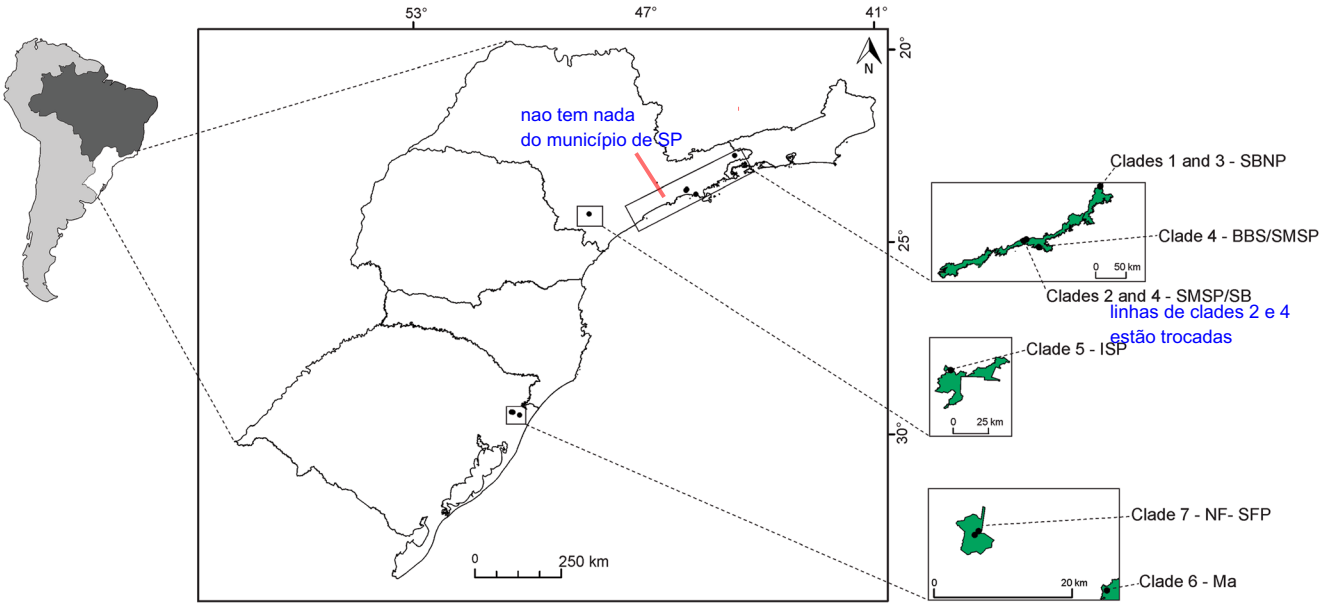

Fig. 2 Distributional range of the genus *Imbira*, showing the sampling sites (black dots) of the seven lineages studied herein (clades 1–7) and outlines of their sampling areas (green areas). *Ma*: private area in Maquiné, Rio Grande do Sul, Brazil; *FN-SFP*: National Forest of São Francisco de Paula (São Francisco de Paula, Rio Grande do Sul, Brazil); *SBNP*: Serra da Bocaina National Park (São José do Barreiro, São Paulo, Brazil); *SB*: São Sebastião (São Paulo, Brazil); *BBS*: Boracéia Biological Station (Salesópolis, São Paulo, Brazil); *ISP*: Intervales State Park (Ribeirão Grande, São Paulo, Brazil)

The pairwise distance among the well-supported monophyletic groups defined as Molecular Operational Taxonomic Units (MOTUs) presents the mean divergence of 7.2%, with a range of 2.5–11.1%, while the measures of intraspecific variation ranged from 0 to 1.4% (Supplementary Material, Appendix S2). The smallest distance was found between the specimens of *I. flavonigra* and *I. guaiana* (2.5 ± 0.3%), being, however, much higher than the highest intraspecific distance found within the genus.

Under ABGD and GMYC approaches, the numbers of OTUs (putative species) were congruent and supported the clades obtained in the phylogenetic reconstruction (Fig. 1). The ABGD showed a multimodal pairwise genetic distance (K2P) distribution with a barcode gap in the range of 1.4–2.4% distance (Supplementary Material, Appendix Figure S1). The maximum likelihood for the GMYC model was significantly higher ($\log L_{GMYC} = 177.4113$) than the null model ($\log L_{NULL} = 175.6191$; $p > 0.01$). In accordance with the ABGD approach, the selected single-threshold GMYC model also suggested the same three primary species hypotheses for the seven evolutionary units.

The seven clades recovered in the phylogenetical analyses share 64 (18.8%) molecular synapomorphies as revealed by the maximum-parsimony analysis of a segment of the COI gene of 340 bp. Molecular transformations were optimized on a strict consensus tree (consistency index [CI] 0.9206). Moreover, 32 molecular autapomorphies were inferred for the species of *Imbira*, distributed as follows: 1 (*I. guaiana*), 3 (*I. flavonigra*), 3 (*I. marcusi* MOTU 1), 4 (*I. marcusi* MOTU 4), 5 (*I. marcusi* MOTU 3), 6 (*I. marcusi* MOTU 2) and 10 (*I. marcusi* MOTU 5) (Supplementary Material, Appendix S3).

Taxonomic description

Family Geoplanidae Stimpson, 1857
Subfamily Geoplaninae Stimpson, 1857
Imbira Carbayo et al., 2013
Imbira flavonigra Amaral & Leal-Zanchet, sp. nov.

Etymology: The specific name is a composite of the Latin adjectives *flavus* (yellow) and *niger* (black), referring to the dorsal pigmentation.

Type material

Holotype: *MZUSP PL. 1689*: *coll*. S. V. Amaral, 04 April 2014, area of dense ombrophilous forest (29° 30′ 5.2″ S, 50° 13′ 15.8″ W), alt. of 409 m above sea level, Maquiné, Rio Grande do Sul (RS), Brazil—anterior tip: transverse sections on 21 slides; anterior region at the level of the ovaries: sagittal sections on 26 slides; posterior region to ovaries in two parts: sagittal sections on 37 slides; pre-pharyngeal region: transverse sections on 10 slides; pharynx: sagittal sections on 19 slides; copulatory apparatus: sagittal sections on 51 slides.

Paratypes: both collected by S.V.A. at the same locality as the holotype. *MZU PL.00215*: 03 April 2014—copulatory apparatus (in two parts): horizontal sections on 14 slides. *MZU PL.00216*: 04 April 2014—preserved in ethanol.

Diagnosis: *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov. is characterized by the dorsal surface with a yellow median band, bordered by two lateral black bands, a short oesophagus and a bell-shaped pharynx, as well as a prostatic vesicle long, forked, tubular-shaped and laterally sinuous. Molecular diagnosis: this species includes all populations that cluster with specimens KY073284 to KY073286 with significant support in phylogenetic analyses and show the following molecular autapomorphies in the mitochondrial cytochrome oxidase I gene (COI): C(483), C(543), C(675).

Type locality: Maquiné, Rio Grande do Sul, Brazil.

Distribution: only known from its type locality.

Description

External features

Body slender, flattened, with parallel margins. Anterior tip rounded and posterior tip pointed (Fig. 3a–c). When creeping, maximum length reaches 135 mm. After fixation, maximum length was 81 mm (Table 1). Mouth and gonopore located in the posterior third of the body (Table 1).

In live animals, ground colour of the dorsal surface greyish with a yellow median band, bordered by two black lateral bands (Fig. 3a–c). After fixation, ground colour of the dorsal surface becomes pale greyish, while the lateral bands become dark greyish and the median band pale yellow. Dorsal band with maximum width of about 1/6th of body width. Ventral surface pale yellow in live and preserved specimens.

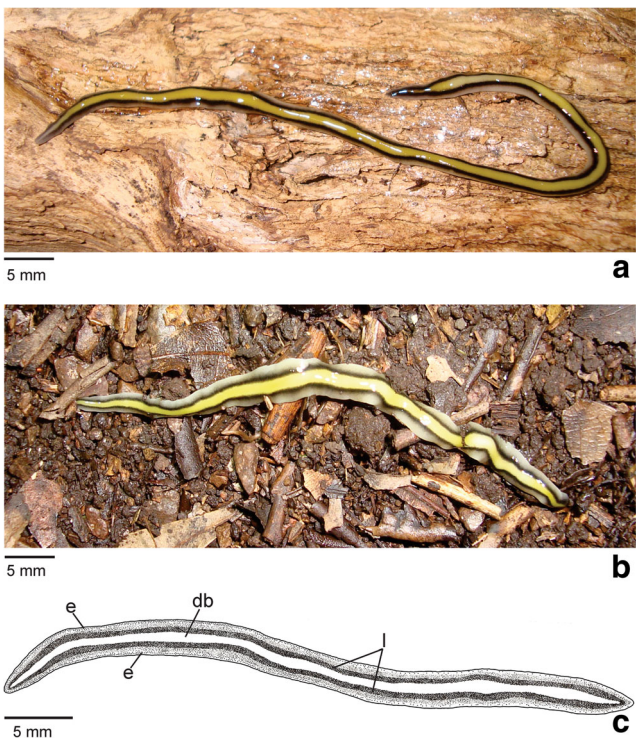

Fig. 3 *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov., holotype: **a**, **b** photograph of the live specimen, in dorsal view, and **c** colour pattern of a fixed specimen in dorsal view. Anterior tip to the left

The eyes are monolobated and arranged exclusively on the body margins. They are initially uniserial, with pigment cups of about 10 μm in diameter, surrounding the anterior tip. After the second millimetre, the eye cups become larger (about 20 μm in diameter). The eyes extend to the posterior millimetre of body, albeit that they become less numerous towards the posterior tip (Fig. 3c). Clear halos around eyes are absent.

Sensory organs, epidermis and body musculatures

Sensory pits (Fig. 4a) occur as simple invaginations ventromarginally in an irregular, single row in the anterior 1/10th of the body; their depth is about 25 to 30 μm.

Two types of glands discharge through the entire epidermis of the pre-pharyngeal region, without forming a glandular margin: rhabditogen cells with xanthophil secretion and cells with amorphous, cyanophil secretion (Fig. 4b–e). Creeping sole occupies 93% of body width (Table 1, Fig. 4e). On the anterior tip, glands are similar to those of the pre-pharyngeal region.

Cutaneous musculature with the usual three layers: circular, oblique and longitudinal muscle layers; longitudinal layer with thick bundles (Fig. 4a–e). Musculature thicker medially, being between two and three times higher than the epidermis (25–35 μm high), becoming progressively thinner towards body margins; ventral and dorsal musculatures with similar thickness at the sagittal plane (about 80 μm thick). Cutaneous musculature thinner in the pre-pharyngeal region than in the anterior region of the body, gradually diminishing in thickness towards anterior tip. Musculature becoming progressively thinner towards body margins. Mc:h ratio of 14%.

Mesenchymal musculature (Fig. 4a–e) well developed, mainly composed of three layers: (1) dorsal subcutaneous, mainly located close to the cutaneous musculature, with decussate oblique fibres (about 2–5 fibres thick); (2) supraintestinal transverse (about 3–5 fibres thick); (3) subintestinal transverse (about 4–8 fibres thick). In addition, there are scattered transverse subneural fibres, ventral subcutaneous oblique fibres and numerous dorso-ventral fibres, as well as bundles of longitudinal muscles around the intestine (about 3–4 fibres per bundle). In the anterior region of the body (Fig. 4a), the mesenchymal musculature is less developed than in the pre-pharyngeal region.

Pharynx

Pharynx bell-shaped, about 1/12th of body length. Mouth anterior to the dorsal insertion, but located in the median third of the pharyngeal pouch (Fig. 5). Oesophagus short with a length

Table 1 Measurements, in millimetres, of type specimens of *Imbira flavonigra* Amaral & Leal-Zanchet, sp. n. 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. –: not measured; *: after fixation. The numbers given in parentheses represent the position relative to body length

	Holotype MZUSP PL.1689	Paratype MZU PL.00215	Paratype MZU PL.00216
Maximum length in extension	135	135	85
Maximum width in extension	2	3	1
Length at rest	71	75	20
Width at rest	5	5	4
Length*	81	75	37
Width*	3	5	1.5
DM*	67 (83)	60 (80)	33 (89)
DG*	77 (95)	70 (93)	–
DMG*	10	10	–
DPVP*	0.8	–	–
Creeping sole %	93	–	–
Ovaries	16 (20)	–	–
Anteriormost testes	20 (25)	–	–
Posteriormost testes	63 (78)	–	–
Prostatic vesicle	3	2.8	–
Male atrium	2.2	1.7	–
Female atrium	1.3	1.3	–

corresponding to 1/3rd of the pharynx length; oesophagus: pharynx ratio 17% in the holotype.

Pharynx and pharyngeal lumen lined with ciliated, cuboidal epithelium with insunk nuclei. Outer pharyngeal musculature (about 20–50 μm thick) comprised of thin subepithelial layer of longitudinal muscles, followed by a thicker layer of circular fibres. Inner pharyngeal musculature (about 60–160 μm thick) comprises a thick subepithelial layer of circular fibres, interposed with various longitudinal fibres and scarce oblique fibres. Outer and inner musculatures gradually become thinner towards pharyngeal tip. Pharyngeal glands of four types: erythrophil glands of two types (with finely granular and coarse granular secretion), xanthophil glands with finely granular secretion and cyanophil glands with amorphous secretion. Oesophagus lined with ciliated, cuboidal to columnar epithelium, with some insunk nuclei, and covered with a thick muscle layer comprised of circular fibres interposed with various longitudinal fibres (about 40–70 μm thick).

Reproductive organs

Testes well developed in the holotype, arranged in two irregular rows on either side of the body, located beneath the dorsal transverse mesenchymal muscles (Fig. 4c). The follicles extend from the anterior fourth of the body, slightly posteriorly to the ovaries, to near the root of the pharynx (Table 1). Sperm ducts dorsal to ovovitelline ducts, subdivided into two or three ductules in the pre-pharyngeal region. They form spermiducal vesicles laterally to the pharynx. The sperm ducts open terminally into the proximal portion of the prostatic vesicle (Figs. 6a, b, 7a and 8a). Extrabulbar prostatic vesicle composed of two long, tubular-shaped and laterally sinuous portions with an enlarged proximal end (Figs. 6a and 8a). The forked portions of the prostatic vesicle unite to an intrabulbar common segment that opens into the proximal part of the male atrium as an ejaculatory duct (Figs. 6a, b and 7a, b). Male atrium long and with highly folded wall.

Lining epithelium of sperm ducts cuboidal and ciliated; muscularis (6–12 μm) mainly constituted of intermingled circular and longitudinal fibres. Prostatic vesicle and ejaculatory duct lined with ciliated, columnar epithelium, receiving openings from erythrophil glands (with finely granular secretion) and cyanophil glands (amorphous secretion). Muscle coat of the extrabulbar portions of prostatic vesicle thick (45–55 μm thick), comprising interwoven longitudinal, and circular fibres. This coat becomes progressively thinner in the intrabulbar segment of the prostatic vesicle and in the ejaculatory duct (5–15 μm thick), being mainly constituted of circular fibres.

The male atrium is lined with a ciliated columnar epithelium (Figs. 6a, b and 7a, b). Two types of glands empty into the male atrium: glands with a finely granular erythrophil secretion, and glands with an amorphous, cyanophil secretion. Muscularis of male atrium (20–60 μm thick) comprises subepithelial circular fibres, followed by longitudinal fibres.

Vitelline folicles situated between intestinal branches, poorly developed in both specimens analysed. Ovaries, in an initial stage of maturation, oval-elongate with a diameter of about 0.1 mm, being situated dorsally to the ventral nerve

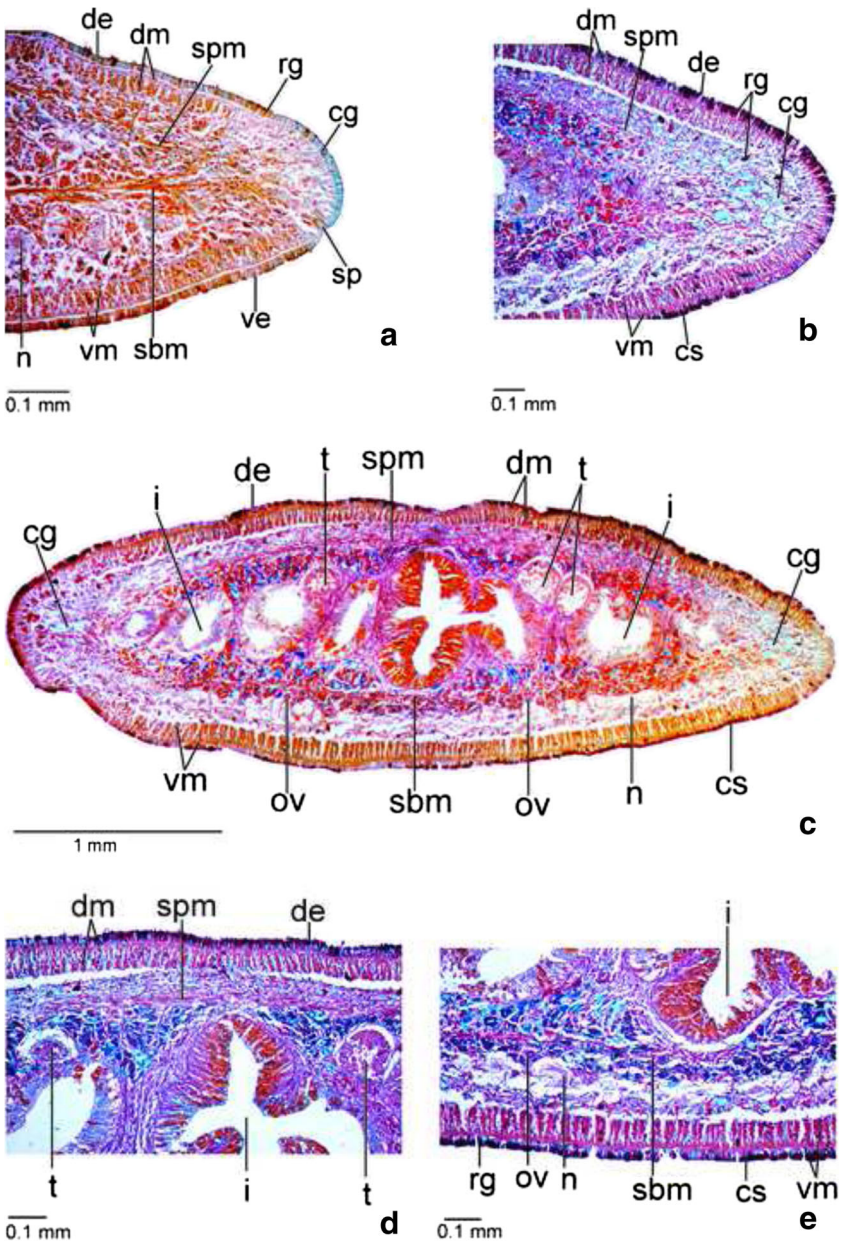

Fig. 4 *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov., holotype, microphotographs of transverse sections: **a** anterior region of the body, **b** pre-pharyngeal region, **c** detail of body margin of the pre-pharyngeal region, **d** detail of the dorsal surface of the pre-pharyngeal region in transverse section and **e** detail of the ventral surface of the pre-pharyngeal region

plate, in the anterior fifth of the body (Table 1). Ovovitelline ducts emerge dorsally from the median third of ovaries and run posteriorly immediately above the nerve plate. Before the gonopore, ovovitelline ducts ascend postero-medially to unite dorsally to the median or posterior third of the female atrium, thus forming a long common glandular ovovitelline duct.

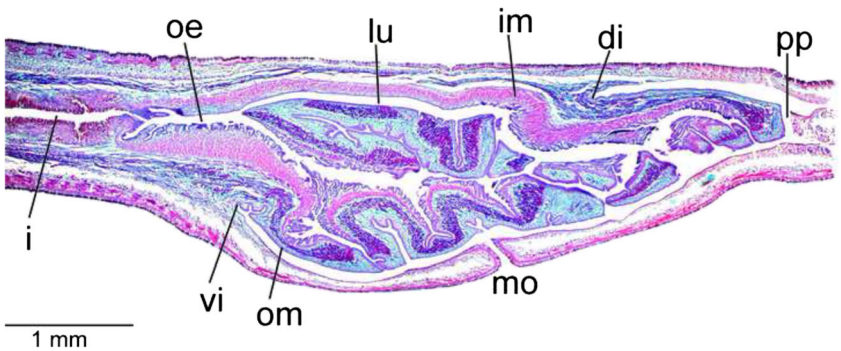

Fig. 5 *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov., holotype: microphotograph of pharynx in sagittal section. Anterior tip to the left

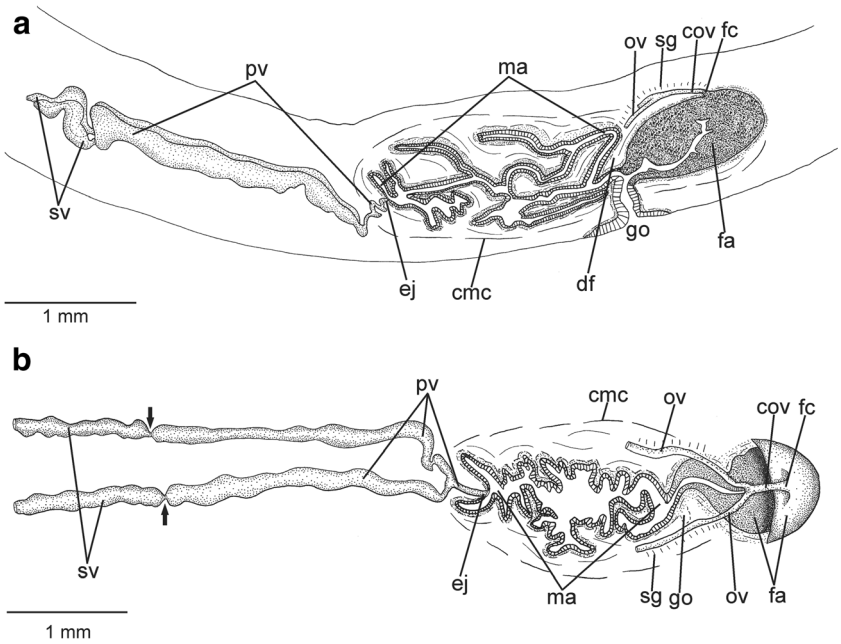

Fig. 6 *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov.: **a** sagittal composite reconstruction of the copulatory apparatus of the holotype and **b** diagrammatic horizontal composite reconstruction of the copulatory apparatus of paratype MZU PL.00215. The arrows indicate the openings of sperm ducts into the prostatic vesicle. Anterior tip to the left

Proximal portion of female atrium with a short and antero-dorsally directed female canal (Fig. 6a, b). Female atrium ovoid, lined with a compact, multilayered epithelium that fills most of the atrium and only leaves a narrow central free space (Figs. 6a, b and 7a, b). Length of female atrium about 60% of male atrial length in the holotype and 75% in paratype MZU PL.00215 (Table 1).

Ovovitelline ducts as well as common ovovitelline duct lined with columnar, ciliated epithelium and covered with intermingled circular and longitudinal muscle fibres (10–20 μm thick). Lining epithelium of ovovitelline ducts and common ovovitelline duct containing cyanophil secretion. Shell glands with coarse granular, erythrophil secretion empty into the common ovovitelline duct and also in the distal, posterior sections of the ovovitelline ducts (Fig. 7a).

Female atrium and canal lined with epithelium with stratified appearance (Fig. 8b). Two types of glands open through the epithelium of the female atrium and canal: abundant glands with coarse granular erythrophil secretion and glands with amorphous cyanophil secretion. Muscularis of female atrium thin (about 30–45 μm), comprising circular muscles mixed with scarce longitudinal fibres.

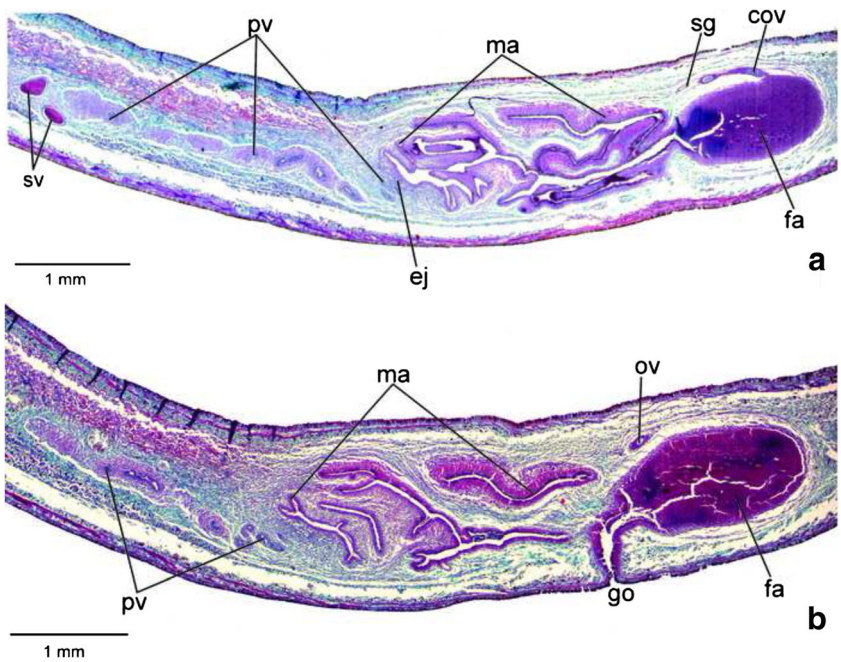

Fig. 7 *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov., microphotographs: **a** copulatory apparatus of the holotype in sagittal section and **b** copulatory apparatus of paratype MZU PL.00215 in horizontal section. Anterior tip to the left

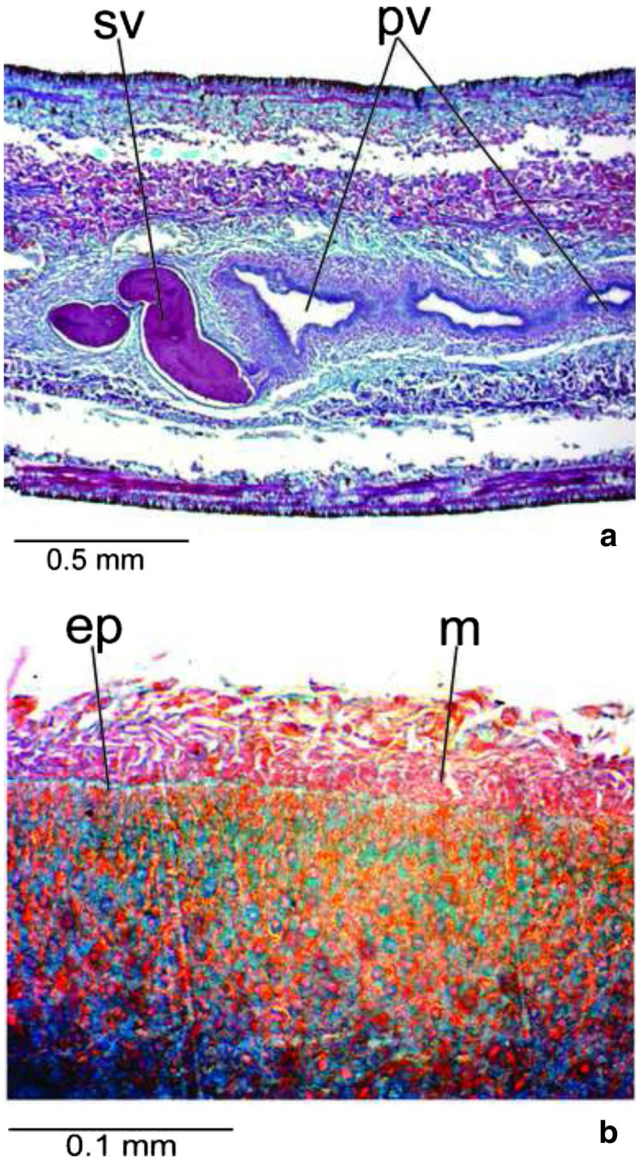

Fig. 8 *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov., holotype, microphotographs: **a** detail of prostatic vesicle in sagittal section and **b** detail of female atrium in sagittal section. Anterior tip to the left

Male and female atria separated by a constriction of the female atrium wall and a dorsal fold proceeding from the male atrium (Fig. 6a, b). Gonopore canal, posteriorly inclined at the sagittal plane, lined with columnar ciliated epithelium, receiving the numerous openings of two types of glands, one producing a finely granular xanthophil and the other an amorphous cyanophil secretion. Muscularis of gonopore canal (15–25 μm) comprised of subepithelial circular fibres and subjacent longitudinal fibres.

Common muscle coat, consisting of longitudinal, oblique and circular fibres, poorly developed, slightly thicker around male atrium (30–35 μm thick) than around female atrium (15–25 μm thick). Male and female atria with continuous muscle coat.

Comparative discussion

Corroborating the phylogenetic analyses, *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov. can be easily differentiated from its congeners by its colour pattern, consisting of a yellowish median band and two black lateral stripes over a greyish background. In contrast, *I. marcusi* shows a dark dorsal surface with two yellowish lateral bands while *I. guaiana* has a homogeneous dorsal surface. In addition, *I. flavonigra* does not have clear halos surrounding eyes, contrasting with *I. marcusi*, which shows small clear halos (Marcus 1951).

Regarding the anatomy of pharynx and oesophagus, *I. flavonigra* differs from its congeners. *Imbira guaiana* has a collar-type pharynx and *I. marcusi* a cylindrical pharynx with dorsal insertion shifted posteriad, thus contrasting with the bell-shaped pharynx of *I. flavonigra*. *Imbira marcusi* and *I. guaiana* have a long oesophagus, with a length that is at least two-thirds of the pharynx length (Marcus 1951; Leal-Zanchet and Carbayo 2001), whereas in *I. flavonigra*, the oesophagus is short, measuring one-third of the pharynx length.

Imbira flavonigra has a prostatic vesicle with two long, tubular-shaped and laterally sinuous portions, receiving the openings of the sperm ducts terminally in their enlarged ends. In contrast, *I. marcusi* has a prostatic vesicle with short and oval-elongate or globose branches, while its sperm ducts open subterminally through the ventral wall of the vesicle (Marcus 1951). Further, in *Imbira flavonigra*, the common glandular oviduct opens into the posterior third of the female atrium, whereas in *I. marcusi*, the common glandular oviduct opens into the median third of the female atrium (Marcus 1951).

The copulatory apparatus of *I. flavonigra* resembles that of *I. guaiana*, but the prostatic vesicle has straight extrabulbar portions with a similar width in all their length, whereas in *I. flavonigra*, these portions are laterally sinuous and show enlarged proximal parts. In addition, the relative length of male atrium and female atrium is greater in *I. flavonigra* than in *I. guaiana* and the common glandular ovovitelline duct shorter in *I. flavonigra* than in *I. guaiana* (Leal-Zanchet and Carbayo 2001).

Notes on ecology and distribution Specimens of *Imbira flavonigra* Amaral & Leal-Zanchet, sp. nov. were collected at a site of dense ombrophilous forest of a private area without legal protection in the northeastern region of the state of Rio Grande do Sul, southern Brazil. Inventories of flatworms in areas covered by the same type of vegetation or in other vegetation types did not encounter the species in other areas of the state or in neighbouring regions, including areas adjacent to the type locality (Carbayo et al. 2002; Baptista et al. 2006; Fick et al. 2006; Leal-Zanchet et al. 2011). Hence, the species seems to be endemic to its type locality, the same area where an endemic fish species was collected and recently described (Pereira et al. 2015). Thus, the creation of a Private Reserve of

Natural Heritage (Reserva Particular do Patrimônio Natural-RPPN) in this area may be necessary to protect its local biodiversity, which seems to be unique and poorly known.

General discussion

The monophyly of the genus *Imbira* was corroborated in the present analysis, as pointed out earlier by a phylogenetic analysis based on molecular characters of Carbayo et al. (2013). Further, we inferred 32 molecular autapomorphies for species of this genus. Its previously known diversity of only two species (Álvarez-Presas et al. 2011; Carbayo et al. 2013) is herein enlarged with the recognition of seven independent evolutionary units, each characterized by autapomorphies. In addition, other two clades, including the new species herein described, occur in areas of its southern part. The mean interspecific distance (7.2%) for species of *Imbira* is slightly lower than that obtained for species of three other genera of the subfamily Geoplaninae (9.3–11.7%) (Rossi et al. 2015; Amaral et al. 2018). In the present work, the closest related species, *I. guaiana* and *I. flavonigra*, showed the smallest interspecific distance within the genus, which is much higher than the highest intraspecific distance.

Regarding pairwise intraspecific divergence, recent studies showed that the greatest intraspecific genetic distances in the subfamily Geoplaninae generally is less than 1% in species of *Cratera* (0 to 0.3%), *Pasipha* (0 to 0.5%) and *Choeradoplana* (0.1 to 0.9%), while it is less than 3% in species of *Obama* (0.3 to 2.8%) (Rossi et al. 2015; Amaral et al. 2018; Lemos et al. 2014). Within *Choeradoplana*, *C. iheringi* forms an exception, with is genetic distance ranging from 0 to 13% (mean 4.5%); it has been suggested already that the taxonomy of this species needs to be reviewed (Lemos et al. 2014). A similar situation may be that of *Imbira marcusi* (*Geoplana goetschi* sensu Marcus, 1951), which shows genetic divergences ranging between 3.5 and 9.3%. The evolutionary independence of two clades named as *I. marcusi* was already suggested in a previous study when specimens corresponding to MOTUs 1 and 4 were used as an out-group (Amaral et al. 2018). Álvarez-Presas et al. (2011) recovered two groups in their maximum likelihood tree, with nucleotide diversity estimated to be 1.7% for the *I. marcusi* specimens occurring in Serra da Bocaina National Park. The average divergence we found for *I. marcusi* ($1.8 \pm 0.4\%$) was similar to that recorded by these authors. However, in our pairwise distance matrix, we found distances greater than 3% between the specimens of clades 1 and 3, while distances within the clades ranged between 0 and 0.1% (Supplementary Material, Appendix S2). Furthermore, the fact that species delimiters pointed to the independence of both groups and that we recorded three and five autapomorphies for clades 1 and 3, respectively, also indicates that this presumed single species is in need of taxonomic revision.

Thus, high genetic divergence within *I. marcusi* and its wide geographical distribution, in addition to the results of ABGD and GMYC analyses, support the notion that various evolutionarily independent species reside under this taxonomic epithet. Although overestimation of taxonomic diversity under the GMYC approach has been observed (Puillandre et al. 2012; Talavera et al. 2013), the present results are supported also by the partition output from ABGD analysis. Therefore, the specimens corresponding to each of the five clades identified as *I. marcusi* should be anatomically analysed in order to test the hypothesis that these represent five evolutionarily independent species as suggested by the results of the present study. In addition, a re-description of *Imbira marcusi*, based on the type specimens, as well as on additional specimens from the type locality, should be undertaken, since many characters concerning its musculature and glands are not known.

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