Five new pseudocryptic land planarian species of *Cratera* (Platyhelminthes: Tricladida) unveiled through integrative taxonomy

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Con formato: Portugués (Brasil)

Con formato: Inglés (Estados Unidos)

Abstract

Background. *Cratera* is a genus of land planarians endemic to the Atlantic forest. The species of this genus are distinguished from each other by a series of external and internal characters, nonetheless they represent a challenging taxonomic issue due to the extreme alikeness of the species analysed on the present work. To resolve these difficulties, we have performed morphological analyses and used five nuclear and mitochondrial genes in an integrative taxonomic study.

Methods. To unveil eventual cryptic species, we applied a molecular species delimitation approach based on molecular discovery methods, followed by a validation method. The putative species so delimited were then validated on the basis of the existence of morphological features that differentiated them.

Results. By doing so, we have discovered and described four new species, namely *Cratera piguaiassu, C. piguaiatui, C. piguaiaboja,* and *C. imbiri*. A fifth new species, *C. paraitinga* was

not highly supported by molecular evidence but was described because its morphological attributes were unique. As a result, we find again the existence of high similarity among terrestrial planarians as has already been described in other genera. The high number of young species poorly differentiated might be explained by the recent history of the area.

Introduction

Land planarians (Platyhelminthes: Tricladida: Geoplanidae) are mostly soil inhabitants of forested areas. There are over 900 known species (Sluys, 2016), 332 of them belonging to Geoplaninae (http://planarias.each.usp.br; accessed in 18. March 2020), an exclusively Neotropical subfamily. Anatomy and histology of the copulatory apparatus are central for the identification and systematics of these organisms (e.g. E. M. Froehlich, 1955; Negrete & Brusa, 2016). Nonetheless, when traditional, morphology-based taxonomic approaches had been complemented with molecular methodologies in different studies, some nominal species were found to be polyphyletic (Sluys et al., 2016; Carbayo et al., 2018; Almeida, Marques & Carbayo, 2019). Reanalyses of the morphological evidence in those cases revealed that morphological variation assumed to represent within species polymorphisms, turned out to signal distinct species. From another perspective, reinterpretation of intra-specific morphological variation revealed pseudocryptic species (see references above; Sáez & Lozano, 2005).

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The systematics of Geoplaninae above the species level has also benefited from the molecular approach. Molecular phylogenetic analyses of this group revealed a number of polyphyletic genera~~,~~ ; one of them, *Geoplana* Stimpson, 1857 was subsequently split into several genera (Carbayo et al., 2013). The genus *Cratera* Carbayo et al., 2013 emerged from *Geoplana* as a monophyletic group with 9 species to which were gradually added another 9 species with similar features. The most conspicuous diagnostic feature of *Cratera* is an ejaculatory duct with

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its distal section dilatated (Marcus, 1951; Lago-Barcia & Carbayo, 2018). However, this ~~feature~~ trait is not found in all members of the genus, probably as a result of a secondary loss (Lago-Barcia & Carbayo, 2018).

In the course of extensive land planarian samplings across the Atlantic forest we have found many individuals that can be attributed to the *Cratera* genus, most of them presenting very similar or even identical features in their external aspect or their internal anatomy. Given the precedents of the existence of cryptic, or pseudocryptic species in other land planarian genera, we set an integrative taxonomic analysis to unveil eventual cryptic species. We adopted the General Lineage Species Concept, that defines species as independently evolving metapopulation lineages (de Queiroz, 1998). To implement this concept, we have used an integrative approach to species delimitations. We applied molecular species delimitation methods to delineate a Primary Species Hypothesis (PSH) based on discovery methods, and thereafter applied a validation method to formulate the Secondary Species Hypothesis (SSH; Puillandre et al., 2012a). We then searched whether the putative species presented morphological features giving support to their validity or not. By doing so we unveiled four species for which molecular and morphological data are coherent with each other. Molecular data of a putative fifth species did not fully support its distinctness, but morphological data did; in this case we gave priority to the latter ~~line~~ source of evidence to propose a new species.

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Materials & Methods

Specimens sampling and morphological study

Intensive samplings were performed in four localities (75 hours sampling in Campos do Jordão; 200 hours sampling in the remaining localities) (Fig 1). Field experiments were approved by COTEC - Instituto Florestal do Estado de São Paulo (Proc. SMA 12.640/2011), Museu de

Comentario [#4]: How many sampling campaigns were done in each locality? When? How many people was involved in fieldwork? Please provide more details of the localities:
- geographic location.
- are they protected natural areas?
- conservation degree.
Were specimens collected in anthropized areas?

Con formato: Portugués (Brasil)

Zoologia (EBBAut.020/2013) and Instituto Chico Mendes de Conservação da Biodiversidade (Proc. 32779-1; 11748-4). Animals were collected from the soil litter during the day and at night. The worms were photographed and, subsequently, killed in boiling water, after which a small tissue sample was taken and preserved it in 100% ethanol for DNA extraction. Vouchers of frozen tissues are kept in FC's laboratory. The remaining part of the body was fixed in 10% formalin and, subsequently, transferred to 80% ethanol. Parts of the body were embedded in paraffin Histosec®, sectioned at 2-7 µm, mounted on glass slides, and subsequently stained with Mallory method as modified by Cason (1950). Slides were examined with a compound microscope. Reconstruction drawings were done with a camera lucida attached to the microscope. Photomicrographs were taken with the help of a digital camera attached to the microscope. Enhancement of the contrast of the microphotographs and a whitish background of the photomicrographs were done with GIMP (GNU Image Manipulation Program 2.8.16; The GIMP team www.gimp.org, 1995-2016). Drawings and photomicrographs of sagittal and horizontal views are orientated with anterior end to the left. The width of the creeping sole was measured on transverse sections of the pre-pharyngeal region. Type material was deposited in the Museu de Zoologia da Universidade de São Paulo (MZUSP).

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LSID for this publication is: urn:lsid:zoobank.org:pub:F6B30CB7-6114-434F-9B2A-A2F4CE625A20. The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central and CLOCKSS.

Molecular data acquisition

Extractions of genomic DNA were performed using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA) following Álvarez-Presas et al. (2011). Two mitochondrial and four nuclear markers were selected. The mitochondrial markers are a cytochrome oxidase I gene fragment (hereafter referred to as COI), and a mitochondrial fragment which includes the end of the nad4 gene, all the sequence of trnF and the beginning of the cox1 gene. This latter marker, hereafter referred to as Nd4toCox1, is tested for the first time in this work. The four nuclear genes correspond to the 18S rDNA type II (18S), a fragment of the 28S rDNA (28S), a partial coding region of the elongation factor 1-alpha gene (hereafter referred to as EF), and an anonymous nuclear marker (hereafter referred as Tnuc813) developed from NGS data (as exposed in Leria et al., ~~2019~~2020) and tested here for the first time. Primers used to amplify and sequence the genes are available in Table S1. For some individuals (indicated in Table 1), it was not possible to obtain a long COI sequence (~900 bp) of good quality (fragment amplified by the BarS / COIR primers). To overcome this situation, a shorter fragment (COIF / COIR primers), of ~300 bp, was amplified. The polymerase chain reaction (PCR) amplification (25 µL) was performed on a Techne® TC-5000TM (Bibby Scientific Ltd, Staffordshire, UK) and on an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany) personal thermocyclers using initial denaturation step of 5 min at 92–95 °C, followed by 30–35 cycles of 30- to 50-s denaturation at 94–95 °C, 30- to 45-s annealing at 44–54 °C and 50-s – 1-min extension at 72 °C,

with a final extension step of 3–4 min at 72 °C. The PCR results were verified using electrophoresis of the amplification products on 1% agarose gels stained with GelRed (Biotium, Hayward, CA, USA), and visualized under UV transillumination. Amplification products were purified with a vacuum manifold (Multiscreen®HTS Vacuum Manifold; Millipore Corporation, Billerica, MA, USA). Purification products were sent to Macrogen (Amsterdam, Europe), were both strands where sequenced by Sanger sequencing. Chromatograms were revised and contigs constructed in Geneious v 8.1.7. software (Biomatters; available from http://www.geneious.com).

For all the coding genes (COI, Nd4toCox1, EF and Tnuc813), sequences were aligned based on the amino acid sequences using Clustal W (included in the BioEdit software 7.0.9.0 (Hall, 1999)). The genetic code 9 (Echinoderm and flatworms' mitochondrial) was used for translating the mitochondrial genes. Ribosomal RNA gene sequences were aligned using the online version of the software Mafft v7 (Katoh, Rozewicki & Yamada, 2017) applying the G-INS-i iterative refinement method. Misaligned or ambiguously aligned regions were removed using Gblocks v0.91b program (Talavera & Castresana, 2007) allowing 50 as a maximum number of contiguous non-conserved positions and setting the minimum length of a block to 4, and half gap positions allowed. Three different datasets were used for several analyses: (1) *COI* dataset including COI sequences used for the ABGD and mPTP molecular species delimitation approaches; (2) *BPP* datasets 18S, 28S, COI, Nd4toCox1, Tnuc813, and EF independent alignments (completing some sequences with missing data (Ns); see Table 1) used for the BPP molecular species delimitation analysis; and (3) *concatenated* dataset, included the information of the six genes (18S, 28S, EF, Tnuc813, COI and Nd4toCox1) and was used to infer a general phylogeny.

For the individual gene alignments DNA sequence evolution model that better fits the data was estimated by using jModelTest v2.1.4 (Darriba et al., 2012), applying the Akaike information criterion (AIC). For the concatenated dataset PartitionFinder2 version 2.1.1 (Lanfear et al., 2017) was run on the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010) to identify an appropriate partition scheme and their corresponding DNA evolutionary models. The data were divided by gene, with unlinked branch lengths, the 'raxml' models for selection and the AICc model selection criteria with the 'greedy' search algorithm. The phylogenetic trees for the concatenated dataset were inferred using the Bayesian Inference (BI) method using MrBayes software v3.2.6. (Ronquist et al., 2012) implemented in CIPRES and using BEAGLE (Ayres et al., 2012), setting the evolutionary model and appropriate partitions according to the PartitionFinder results with the unlinked parameters. Two runs of four chains were applied producing 5 million generations and, for each of them, 5,000 trees were stored. It was checked that the probability values (logarithm) of the cold chain reached the stationarity state and the convergence of the two runs, verifying that the average standard deviation of the split frequencies was lower than 0.01. A default burn-in of 25% was used and a consensus tree was obtained from the remaining trees. The maximum likelihood (ML) method was used to infer phylogenies with the software IQtree v1.6.10 (Nguyen et al., 2015). The IQtree searches were carried out using the default configuration in CIPRES, with a starting random tree (option -t RANDOM), and assessing branch support using 1000 ultrafast bootstrap approximation replicates (Minh, Nguyen & Von Haeseler, 2013). The best fit models for each partition were the selected by PartitionFinder and each partition was allowed to have its own set of branch lengths (option -sp).

Molecular species delimitation

For the molecular species delimitation analyses, two discovery methods (ABGD and mPTP) and one validation method (BPP) were applied. Using the COI dataset, the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al., 2012b) was applied through the website http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. The default values of Pmin = 0.001 and Pmax = 0.10, steps = 10 and number of intervals = 20 were used. Using the relative gap width value (X) = 1.0 and correcting the distance matrix under the K80 Kimura model with a MinSlope = 1.5.

The multi-rate Poisson Tree Process (mPTP), which is another single locus analysis, was also used. This model incorporates different levels of intraspecific genetic diversity derived from differences in the evolutionary history or in the sampling of each species, accommodating different coalescence rates within the lineages (Kapli et al., 2017). mPTP analysis was performed in a ML tree reconstructed by IQtree in CIPRES with the *COI* dataset (Suppl Fig. 1). For this analysis the command line version of the mPTP v 0.2.4. software was used without considering the outgroup. Four independent runs of 5,000,000 Monte Carlo Markov chains (MCMC) were carried out sampling every 10,000 generations.

The use of these discovery methods leads to the Primary Species Hypothesis (PSH), used as starting point for the validation step.

For the validation step, a Bayesian multilocus method of delimiting species (Yang & Rannala, 2010~~,~~ ; Yang & Rannala, 2014) implemented in the BPP v3.3 software (Yang, 2015) was applied. Different hypotheses of species delimitation and estimation of the posterior probability (PP) of each model were tested using reversible jump MCMC (rjMCMC). The previous species assignment resulting from the ABGD discovery analysis was used as a starting

hypothesis for the BPP analysis, because it was the analysis that gave the largest number of PSHs. Some species were excluded from this validation analysis since only one individual was available for each one (*Cratera arucuia* Lago-Barcia & Carbayo, 2018 and *Cratera picuia* Lago-Barcia & Carbayo, 2018) or because it only had COI gene sequenced (*Cratera ochra* Rossi et al., 2016) . As they were not the target species for our study, their removal from the analysis was not relevant. A guide tree generated by 100 million generations (stored every 5000) in * BEAST2 v2.5.2 (Bouckaert et al., 2014) was built in CIPRES with the six single gene datasets (*BPP* datasets), applying the evolutionary model for each gene resulting from the previous jmodeltest analysis (18S=GTR+I; 28S=HKY+I+G; Cox1=GTR+G; EF=GTR+I+G; Nd4toCox1=HKY+I+G and Tnuc=GTR+I).

The molecular clock was set as log normal relaxed for all markers (unlinked) and the Birth and Death model was used for speciation. In the BPP analysis, both the size of the ancestral population (theta, θ) and the time of origin for each species (tau, τ) were parameterized with four different models (named M1-M4): M1 for large ancestral population size and deep divergence G (1, 10) (for θ and τ); M2 for small ancestral population size and shallow divergence G (2, 1000) (for θ and τ); M3 for large ancestral population size and shallow divergence (G (1 10) for θ and G (2 1000) for τ); and M4 for small ancestral population size and deep divergence (G (2 1000) for θ and G (1 10) for τ). Under the algorithm 0 it was run the rjMCMC analysis in 100,000 generations (with a sampling interval of 2) excluding 10% as burn-in. To test the robustness of the results, these executions were replicated using different starting seeds. These analyses were done without outgroup. Results of BPP lead to the Secondary Species Hypothesis (SSH).

Abbreviations used in figures

(cg) cyanophil gland cells

(co) common glandular ovovitelline duct

(e) eye

(ed) dilatated portion of ejaculatory duct

(ej) ejaculatory duct

(ep) esophagus

(fa) female atrium

(fc) female genital canal

(fo) fold

(g) gonopore

(gl) glands

(i) intestine

(lc) longitudinal cutaneous muscle fibers

(m) muscle fiber

(ma) male genital atrium

(mc) common muscle coat

(mo) mouth

(o) ovary

(ov) ovovitelline duct

(ph) pharyngeal pouch

(pb) penis bulb

(pp) penis papilla

(px) pharynx

Comentario [#6]: Is correct? Do you mean dilated?

Comentario [#7]: In some figures, the female canal is indicated with “fd”. Please, check.

(sb) subintestinal transverse muscle fibers

(sd) sperm duct

(sg) shell glands

(sp) supraintestinal transverse muscle fibers

(t) testis

(vi) vitellaria

(vn) ventral nerve plate

Results

Molecular datasets

The *COI* dataset consists of 40 sequences with a length of 822 bp, and the *concatenated* dataset (28 *Cratera* sequences plus 3 outgroups from the genus *Obama*) has a length of 5671 bp. The individual gene datasets for the *BEAST analysis are constituted by 26 sequences with a length of 1349 bp (18S), 1544 bp (28S), 825 bp (COI), 612 bp (EF), 730 bp (Nd4toCox1) and 611 bp (Tnuc813).

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Phylogenetic analysis

The partitions obtained with PartitionFinder and applied to the phylogenetic analysis of the *concatenated* dataset are Cox1_codon2, EF_codon2, 18S, Tnuc813-1_codon3, Tnuc813-3_codon2, Tnuc813-3_codon3, Tnuc813-5_codon1, Tnuc813-5_codon2, Tnuc813-5_codon3 = K81UF + G, 28S = TIM + G, Cox1_codon1, EF_codon1 = TRN + G, Cox1_codon3 = GTR + G, EF_codon3, Tnuc813-1_codon2, Tnuc813-3_codon1 = TVM + G, Nd4toCox1-1_codon1, Nd4toCox1-1_codon2, Nd4toCox1-3_codon1, Nd4toCox1-3_codon3, Tnuc813- 1_codon1 = GTR + G, Tnuc813-2, Tnuc813-4 = GTR + G, Nd4toCox1-1_codon3, Nd4toCox1-2,

Nd4toCox1-3_codon2 = HKY + G. For the concatenated analyses, the topology obtained is the same in both methods (ML and BI) as shown in Fig. 2. There is only a small difference in the relationships between the specimens within two of the new species described in the present paper (*Cratera piguaiatui* sp. nov. and *C. ratera piguaiassu* sp. nov.), but without statistical support for any of the two methods. All known species are monophyletic. Three of ~~T~~the new species described here (*C. piguaiatui* sp. nov., *C. ratera imbiri* sp. nov. and *C. ratera paraitinga* sp. nov.) are sister species, forming a large monophyletic group, which in turn is sister to the species *C. ratera cuarassu* Carbayo & Almeida, 2015. *Cratera pseudovaginuloides* (Riester, 1938) is a sister lineage of ~~the new species described here,~~ *C. ratera piguaiaboja* sp. nov., both constituting the sister group of a large clade formed by the species *C. piguaiassu* sp. nov. and *C. ratera crioula* (Froehlich, 1955) on the one hand and the group constituted ~~by the three species~~ *C. picuia*, *C. arucuia* and *C. ratera tamoia* (Froehlich, 1955). The vast majority of the relationships present high support values at the nodes for both methodologies (PP and bootstrap values) (Fig. 2).

Molecular species delimitation

On the concatenated tree in Fig. 2 is displayed a summary of the species delimitation results. The ABGD method delimits, within the genus *Cratera*, ~~genus~~ 13 Molecular Operational Taxonomic Units (MOTUs) with a p-value of P 0.001 - 0.0046. ~~-~~The mPTP method delimits 11 candidate species for the ingroup with average support of 0.90. In both delimitations, mPTP and ABGD, *C. ochra* is delimited as a single candidate species. However, it is not present in the concatenated tree in Fig. 2 because there are only COI sequences in GenBank for this species (Table 1 and Suppl Fig. 1). For this same reason, ~~the~~ this species ~~is~~ was not included in the BPP

290 analyses.

291 ABGD predicts a higher number of candidate species than mPTP. In consequence,
292 ABGD candidate set of species is adopted as a reference to designate the PSHs in order to test as
293 many scenarios as possible in the validation with the BPP method. The assignment is as follows.
294 All known species are delimited by both ABGD and mPTP methods as candidate species. Hence,
295 each is assigned a PHSs (PSH-4 is *C. cuarassu*, PSH-5 is *C. crioula*, PSH-8 is *C. tamoia* and
296 PSH-10 corresponds to *C. pseudovaginuloides*). Regarding the rest of *Cratera* individuals
297 included in this work, the two discovery methods coincide in assigning individuals F2789,
298 F2809, F5178 F2040, F2031, F2054 to one candidate species (PSH-1) and individuals F2828 and
299 F2829 to another candidate species (PSH-9). Finally, in two cases a clade that mPTP considers
300 as a single MOTU is divided in two candidate species by ABGD these are designated as
301 candidate species PSH-2, PSH-3, PSH-6 and PSH-7. The species *C. picuia* and *C. arucuia* are
302 not assigned any PSH because they are singletons (constituted by a single individual) and so are
303 not included in the validation step. So, finally 10 PSHs are set to be validated in BPP.

304 The species tree resulting from the *BEAST analysis (Fig. 3), used as the input tree to
305 implement the BPP method, differs slightly from the tree inferred using the concatenated dataset.
306 In the ML and BI phylogenies inferred from the concatenated dataset (Fig. 2), the clade formed
307 by PSH-9 and PSH-10 is sister to the clade constituted by PSH-5, PSH-6, PSH-7 and PSH-8.
308 However, in the *BEAST tree PSH-9 + PSH-10 is sister group of the rest of species. The small
309 differences could be due to the fact that *C. picuia* and *C. arucuia* were not included in the
310 *BEAST analysis, but this does not affect the subsequent species assignment.

311 The different values of θ (ancestral population size) and τ (time of divergence) that are
312 used in the 4 BPP analyses (M1, M2, M3 and M4) do not have a significant effect on the results

of the BPP analyses (Fig. 3), except for the node that separates PSH-2 and PSH-3, the PP value of which is higher than 0.95 in the M2 and M4 models (small ancestral population size) and a little lower than 0.95 in the M1 and M3 models (large ancestral population size). Of the 10 PSHs that are analysed as starting hypothesis for BPP, only 9 are validated since PSH-6 and PSH-7 would form a single SSH (SSH-6 in Fig. 3). In conclusion, the BPP results determine the presence of 9 SSHs, validating five new species of *Cratera* (SSH-1, SSH-2; SSH-3, SSH-6, and SSH-9) that will be described in the following section.

Taxonomic account

Order Tricladida Lang, 1884

Suborder Continenticola Carranza et al., 1998

Family Geoplanidae Stimpson, 1857

Genus *Cratera* Carbayo et al., 2013

***Cratera piguaiassu* sp. nov., Araujo, Carbayo, Riutort & Álvarez-Presas**

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Con formato: Español (alfab. internacional)

Synonymy.

Cratera sp. 1: Carbayo et al. (2013).

Etymology. The name *piguaiassu* is a free composition of the Tupi (indigenous Brazilian language) words pyguaia (meaning hole, cave) and assu (meaning large) (Tibiriçá, 1984). It refers to the large distal dilatation of the ejaculatory duct.

Comentario [#8]: Add to References.

Type locality. Parque Nacional da Serra da Bocaina, São José do Barreiro, State of São Paulo,

Con formato: Portugués (Brasil)

Brazil.

Material studied. All specimens collected in the Parque Nacional da Serra da Bocaina, São José do Barreiro, State of São Paulo, Brazil (-22.75, -44.62) by Carbayo et al. Holotype F2825 (MZUSP PL 2150): 8 September 2008. Horizontal sections of pre-pharyngeal region-1 on 5 slides; transverse sections of pre-pharyngeal region-2 on 5 slides; sagittal sections of pharynx and copulatory apparatus on 34 slides. Paratypes: F2025 (MZUSP PL 2146): 5 February 2008. Horizontal sections of pre-pharyngeal region-1 on 15 slides; transverse sections of pre-pharyngeal region-2 on 7 slides. Sagittal sections of copulatory apparatus on 15 slides. F2807 (MZUSP PL 1050): 7 September 2008. Transverse sections of cephalic extremity on 7 slides; horizontal sections of ovaries on 5 slides; transverse sections of pre-pharyngeal region on 6 slides; sagittal sections of pharynx on 12 slides; sagittal sections of copulatory apparatus on 11 slides. F2821 (MZUSP PL 1052): 8 September 2008. Sagittal sections of a piece of the body behind cephalic extremity on 71 slides; sagittal sections of pharynx and copulatory apparatus on 71 slides. F2834 (MZUSP PL 2151): 8 September 2008. Preserved in 80% ethanol.

Diagnosis

Species of *Cratera* 50-62 mm long (34-38 mm preserved); dorsum chestnut brown to black brown, excepting the yellow orange or grayish body margins. A thin grayish median stripe may be present. Eyes dorsal. Pharynx bell-shaped. Penis papilla horizontal; distal portion of ejaculatory duct very dilated, ~~to~~ occupying most of the length of the penis papilla. Common glandular ovovitelline duct absent.

Description

When crawling, the longest specimen (paratype F2807), up to 62 mm in length and 3.5 mm in width; preserved, 38 and 7 mm, respectively. Body elongate, slightly lanceolated, with

maximum width at the level of the pharynx. Anterior to it, body narrows gradually towards the rounded tip; posterior to the level of the pharynx, body becomes narrower abruptly near posterior, pointed tip. Dorsum slightly convex, ventral side flattened. Creeping sole as wide as 92-94% of body width at the pre-pharyngeal region. In holotype, ~~Mouth~~ mouth and gonopore at a distance from anterior extremity equal to 70% and 85%~~of body length; gonopore at 85 % in holotype~~. In paratype F2807, 74% and 85%, respectively.

Color of the dorsum varies from chestnut brown (Fig. 4A) to black brown (Fig. 4B). A submarginal yellow orange or grayish stripe is present in anterior tenth of the body; posteriorly, this stripe becomes marginal. The stripe, measuring 8% of body width, may be inconspicuous (Fig. 4B). A thin grayish median stripe, 4% of body width, may also occur (Fig. 4A). Ventral surface varies from deep orange (Fig. 4A, inset) to grayish (Fig. 4B, inset).

Each eye is formed by a single pigmented cup 30 µm in diameter. Clear halos around eyes were not observed. Eyes contour anterior extremity of the body in a single row along the first 5 millimeters (equal to 13% of body length, paratype F2807); then they spread onto the back in a lateral band which maximum width is 1/3th on each side of the body ~~width~~, reaching the posterior tip.

Sensory pits, 15 µm deep, in a uniserial ventro-lateral row, from very anterior tip up to 29% of body length posterior. Dorsal and ventral epidermis in pre-pharyngeal region pierced by necks of two types of cell glands producing erythrophil and cyanophil granules, respectively. Besides, rhabditogen cells discharge their content through dorsal epithelium. Glandular margin constituted by two types of cell glands, one abundant type producing xanthophil granules and a less abundant type secreting erythrophil granules.

Cutaneous musculature constituted of a subepithelial circular layer, followed by two

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Comentario [#10]: it seems light brown

diagonal layers with decussate fibers, and then a strongly developed longitudinal layer with fibers arranged in bundles (Fig. 4C). Longitudinal layer is 40 µm thick dorsally and 50 µm ventrally (paratype F2807); dorsally, fibers gathered into well-delimited and more compact bundles than ventrally (Fig. 4C). Thickness of cutaneous muscle ranges from 8.9% (F2807) to 13.4% (F2025).

Three parenchymal muscle layers throughout the body: a dorsal layer of diagonal decussate fibers (20 µm thick), a transverse supraintestinal layer (150 µm), and a transverse subintestinal one (90 µm). Central nervous system as a ventral nerve plate. ~~Cerebral ganglia were not discerned.~~

Mouth located shortly behind middle of pharyngeal pouch. Pharynx bell-shaped, with dorsal insertion posterior to the ventral at the equivalent of 40% of pharyngeal length (Fig. 4D). Esophagus length, 33% of pharyngeal length. Outer pharyngeal epithelium underlain by a one-fiber-thick layer of longitudinal muscle, followed by a circular one (7 µm thick); inner epithelium underlain by a layer (70-150 µm) of circular fibers with interspersed longitudinal fibers, followed by a thinner longitudinal muscle layer (10 µm). Pharyngeal pouch at 1.6-2.6 mm from prostatic vesicle.

Testes dorsal, located under the supraintestinal transverse muscle layer, partially placed between the intestinal diverticula (Fig. 4C). Sperm ducts run immediately above the subintestinal muscle layer, dorso-medially to the ovovitelline ducts (Fig. 4C). Shortly behind the prostatic vesicle, sperm ducts curve medially and anteriorly, to communicate separately with the respective short lateral diverticulum of the vesicle (Fig. 5A). Prostatic ~~Vesicle~~ vesicle extrabulbar, tubular, with anterior portion slightly dilated. In lateral view, vesicle with the shape of an inverted U, the distal portion of which, anterior to ~~anterior region of~~ penis bulb,

communicates with ejaculatory duct. Prostatic vesicle lined with a ciliated, tall ~~and tortuous~~ epithelium, which is traversed by cells producing fine erythrophil granules. This epithelium is surrounded by a circular muscle layer. Proximal portion of the ~~Ejaculatory~~ ejaculatory duct straight, running slightly dorsally. At the basis of the penis papilla, duct widens to give rise to a very large cavity inside the penis papilla (Figs. 5A-C). At the tip of penis papilla this cavity narrows to open into male atrium. Ejaculatory duct lined with a cuboidal, ciliated epithelium and surrounded by a 25-µm thick circular muscle layer. Under this muscle layer, there is an additional, one-fiber-thick layer of longitudinal muscle.

Penis papilla a long, roughly conical, protrusible organ extending along the length of the male atrium or even beyond (Fig. 5A-C). Lining epithelium of the penial cavity cuboidal, not ciliated, pierced by necks of cell producing minute erythrophil granules (<0.2 µm), and underlain by a layer (25 µm) of circular muscle, followed by a simple layer of longitudinal muscle. The penis papilla is clothed with a cuboidal, non-ciliated epithelium, which is traversed by necks of cells producing erythrophil granules and is underlain by a 20-µm-thick layer of circular muscle with some interspersed longitudinal fibers.

Male atrium ample and smooth, not narrowed distally. It is lined with a cuboidal, non-ciliated epithelium, which is pierced by necks of cells producing granules with weak xanthophil affinity. Male atrial epithelium underlain by a muscle layer (40 µm) of circular fibers.

Ovaries roughly ovoid, with maximum length 400 µm anteroposteriorly. They are located immediately above the ventral nerve plate, at a distance from anterior tip equivalent to 21% of body length. Ovovitelline ducts arise from external side of the ovaries, subsequently run backwards above ventral nerve plate. The ducts ascend laterally to the female atrium, posteriorly and medially inclined, to unite dorsally to the atrium (Fig. 5A). Half of the ascending portion of

Comentario [#11]: The tortuous appearance could be an effect of the sectioning. What seems tortuous is the course of the vesicle.

Comentario [#12]: Only circular? Thickness?

Comentario [#13]: I do not see it very straight. Judging from the images of the sagittal sections (5b, c), I would say that it is rather zigzagging in its convex course.

Comentario [#14]: Mature?

Comentario [#15]: Vitellaria? Are well developed?

these ducts receives secretion of shell glands (Fig. 5A). Ovovitelline ducts open the female genital canal. Common glandular ovovitelline duct absent. ~~This~~ The female canal is a long canalicular projection of the posterior portion of the female atrium. This canal is directed forwards (Fig. 5B, D). Female atrium, as long as male atrium, and funnel-shaped. Lateral walls of female atrium partially occlude its lumen. Its lining epithelium is columnar, and the free surface of which is erythrophil and displays small recesses that appear as small gaps (Fig. 5D). In the anterior portion of female atrium, the epithelium is 40 µm high; posteriorly it becomes taller and its nuclei arranged at different heights within the cells, so giving to the epithelium a multilayered aspect. Necks of cells producing erythrophil granules pierce this epithelium, which is clothed with two layers of muscles; clothing the anterior half of the atrium there is a 5-µm-thick circular muscle layer followed by a 3-µm-thick longitudinal one. Arrangement of these layers is inverted in posterior half of female atrium, i. e., a layer (5 µm) of longitudinal is followed by a layer (20 µm) of circular fibers.

Comentario [#16]: In these figures, you should change fd to fc.

Remarks

Regarding the external aspect, *C. tamoia* is the only species of the genus resembling *C. piguaiassu* in that both display a uniform dark dorsum with clear margins. However, although color patterns are alike, *C. piguaiassu* differs in being darker. Moreover, the phylogenetic analyses show that these two species are highly differentiated at the genetic level and do not even represent sister groups. Regarding the internal organs, *C. piguaiassu* is, together with *C. cuarassu*, the unique among the representatives of *Cratera* in the very large dilatation of the ejaculatory duct, which. ~~This dilatation~~ occupies most of the penis papilla. ~~This situation is also present in *C. cuarassu*, but~~However, the penis papilla of *C. cuarassu*~~the latter~~ is vertically oriented (vs. horizontally in *C. piguaiassu*). The molecular delimitation methods all clearly point

to *C. piguaiassu* being a species differentiated from the rest of species molecularly analysed in the present study. On the other hand, although the primary hypothesis found this lineage could be divided in two species (ABGD analysis), neither the molecular validation nor the morphological analysis found evidence of this possibility.

***Cratera piguaiaboja* sp. nov., Araujo, Carbayo, Riutort & Álvarez-Presas**

urn:lsid:zoobank.org:act:46D76DD7-B129-461D-8803-3E918AA4601C

Etymology. The name *piguaiaboja* is a free composition of the Tupi (indigenous Brazilian language) words pyguaia (meaning hole, cave) and bojá (meaning intermediate, middle) (Tibiriçá, 1984). It refers to the middle size of the distal dilatation of the ejaculatory duct.

Type locality. Parque Nacional da Serra da Bocaina, São José do Barreiro, State of São Paulo, Brazil.

Material studied. All specimens collected in Parque Nacional da Serra da Bocaina, São José do Barreiro, State of São Paulo, Brazil (-22.75, -44.62), in September 2008 by Carbayo et al. Holotype F2829 (MZUSP PL 0459): transverse sections of cephalic extremity on 4 slides; horizontal sections of a portion of the body behind cephalic extremity on 4 slides; transverse sections of pre-pharyngeal portion on 7 slides; sagittal sections of pharynx on 6 slides; sagittal sections of copulatory apparatus on 4 slides ~~(plus one slide lost)~~. F2828 (MZUSP PL 0458): horizontal sections of a portion behind anterior extremity on 6 slides; transverse sections of the pre-pharyngeal portion on 22 slides; sagittal sections of pharynx on 13 slides; sagittal sections of copulatory apparatus on 14 slides.

Diagnosis

Species of *Cratera* 34 mm long preserved; dorsum olive grey with large black dots, with these being more concentrated in paramedian bands; eyes marginal; pharynx bell-shaped; distal portion of ejaculatory duct dilatated to occupy half of the penis papilla; male atrium separated from female by a narrowing; penis papilla shorter than male atrium; prostatic vesicle with inverted-U shape in lateral view; penis papilla postero-dorsally oriented; numerous cyanophil cell necks piercing roof of male atrium; female atrium half the length of the male atrium; common glandular ovovitelline duct absent.

Description

Fixed, holotype measures 34 mm in length and 4 mm in width. Body elongate, with nearly parallel margins along most of body length. Anterior extremity rounded; posterior pointed. Dorsal side convex; ventral side flattened. Creeping sole, 95% of body width at the pre-pharyngeal region. Mouth at a distance from anterior extremity equal to 71% of body length; gonopore at 87% (holotype).

Dorsum spotted black on olive grey ground color (Fig. 6A-D). Large black spots join each other. Spots distributed all over dorsum and especially concentrated in a median band (33% of body width). This band may be divided by a thin midline, measuring 6% of body width (Fig. 6C). Ventral surface olive gray (Fig. 6A), gray at anterior extremity.

Each eye is formed by a single pigmented cup 40 µm in diameter. No ~~Clear~~ clear halos around eyes ~~were not found~~. Eyes contour anterior extremity in a single row, posteriorly they are arranged marginally until posterior tip of the body.

Comentario [#17]: In a single row?

Sensory pits were not found despite intensive search on the sections, which are partially damaged. Dorsal and ventral epidermis in pre-pharyngeal region pierced by necks of two types of cell glands producing ~~granules,~~ erythrophil and cyanophil granules, respectively. Besides,

rhabditogen cells discharge their content through dorsal epithelium and, scarcely, ventral epithelium as well. Glandular margin not well defined (Fig. 7A), constituted by three types of cell glands, one abundant type producing xanthophil granules and a less abundant type secreting erythrophil granules, and rhabditogen cells.

Cutaneous musculature constituted of a subepithelial circular layer, followed by two diagonals with decussate fibers, and ~~then~~ an inner strongly developed longitudinal layer with fibers arranged in bundles (Fig. 7A). Longitudinal layer 55 µm thick dorsally and 90 µm ventrally. Dorsal ~~Fibers~~ fibers of this layer are gathered into well-delimited and more compact bundles than ventrally. Thickness of cutaneous muscle, 12.5% of body height (holotype).

Three parenchymal muscle layers throughout the body: a dorsal layer of diagonal decussate fibers (20 µm thick, holotype), a transverse supraintestinal layer (60 µm), and a transverse subintestinal one (70 µm). Central nervous system as a ventral nerve plate. Cerebral ganglia starting at 0.5 mm from anterior extremity (1.5% of body length, holotype).

Mouth located at the end of second third of pharyngeal pouch (Fig. 7B). Pharynx bell-shaped, with dorsal insertion posterior to the ventral at the equivalent of 36% of pharyngeal length. Esophagus length, 21% of pharyngeal length. Outer pharyngeal epithelium underlain by a one-fiber-thick longitudinal muscle layer followed by a circular one (4 µm thick); inner epithelium underlain by a circular muscle layer (30-60 µm thick), followed by a thinner longitudinal muscle one (5 µm). Pharyngeal pouch of holotype at 2.5 mm (equivalent to 7% of body length) from prostatic vesicle.

Testes dorsal, located under the supraintestinal transverse muscle layer, partially placed between the intestinal diverticula (Fig. 7A). Posteriormost testes lateral to ventral insertion of the pharynx. Sperm ducts run immediately above the subintestinal muscle layer, dorso-medially to

the ovovitelline ducts (Fig. 7A). Below prostatic vesicle, sperm ducts curve medially and anteriorly, to communicate separately with the respective short lateral diverticulum of the vesicle (Fig. 7C, D). Prostatic vesicle extrabulbar and tubular. In lateral view, this vesicle is seen as an inverted U. Prostatic vesicle approaches anterior region of penis bulb and communicates with ejaculatory duct. ~~Ventral portion of penis bulb posterior to dorsal portion.~~

Prostatic vesicle lined with a ciliated, columnar epithelium, traversed by necks of two types of cell producing erythrophil and cyanophil granules, respectively, the cyanophil glands being much more abundant (Fig. 8A). Epithelium of prostatic vesicle surrounded by a 130-µm-thick circular muscle layer. Proximal portion of ejaculatory duct sinuous; distal portion straight, traversing center of penis papilla; midway of penis papilla, ejaculatory duct widens to give rise to a relatively large, funnel-shaped cavity, which opens at the tip of the penis papilla (Fig. 7C-D, 8A). Ejaculatory duct lined with a cuboidal, ciliated epithelium; in its dilatated portion, pierced by necks of cells producing xanthophil granules. Epithelium of ejaculatory duct surrounded by a 20-µm thick circular muscle layer.

Penis papilla conical cylindrical, 1.3-1.5 times longer than its diameter and as long as the male atrium or shorter (Fig. 7C-D, 8A). Penis papilla clothed with non-ciliated epithelium. Columnar epithelium lines basal half of papilla; cuboidal epithelium lines distal half. Epithelium of papilla pierced by necks of three types of cells producing erythrophil, xanthophil and cyanophil granules, respectively (but the latter appears erythrophil in paratype); necks of cyanophil glands only piercing epithelium of basal half of penis papilla. Epithelium of penis papilla underlain by a 10-µm-thick layer of circular muscle fibers followed by a longitudinal layer, 10 µm thick. Parenchyma of penis papilla richly traversed by circular and longitudinal muscle fibers.

Comentario [#18]: Irrelevant.

Comentario [#19]: In figure 8A the prostatic vesicle needs to be marked. Also, there is an arrow that seems incomplete.

Comentario [#20]: intermingled?

Male atrium ample and smooth, lined with a columnar, non-ciliated epithelium. Epithelium clothing roof of male atrium pierced by ~~very~~ numerous necks of cells producing cyanophil granules. Ventral portion of male atrium pierced by less numerous necks of glands producing erythrophil granules. Atrial epithelium underlain by a layer (12 µm thick) of circular muscle fibers, followed under dorsal epithelium by a 5-µm-thick longitudinal muscle layer.

Ovaries were not found in histological sections and were probably destroyed in the portion from which a tissue sample was extracted. Ovovitelline ducts ascend laterally to proximal portion of the female atrium, posteriorly and medially inclined. Subsequently, the ducts unite dorsally to the posterior portion of atrium. ~~Half~~ The distal half of ascending portion of these ducts receives shell glands. Ovovitelline ducts open into female genital canal, i.e., a common glandular ovovitelline duct is absent. Female genital canal projects forwards from the postero-dorsal portion of the female atrium. Female atrium ample, 55-57% the length of male atrium and lined with 30-70 µm high, non-ciliated epithelium, with stratified aspect (Fig. 8A-C). Surface of epithelium sinuous; subapical portion of epithelial cells cyanophil (Fig. 8B) or erythrophil (Fig. 8C). Necks of these two types of cells~~, producing erythrophil and cyanophil granules, respectively,~~ pierce ~~this~~ the atrial epithelium, which is underlain by a 25-µm thick and dense layer of circular muscle, followed by a 3 µm thick longitudinal muscle layer.

Remarks

C. piguaiaboja is distinctive among all species of the genus in the spotted color pattern of the dorsal side. Regarding its copulatory apparatus, there are five species with a similar aspect of the copulatory apparatus and size of the dilatation of the ejaculatory duct, namely, *C. aureomaculata* Rossi & Leal-Zanchet, 2017, *C.* ~~–~~ *ratera nigrimarginata* Rossi & Leal-Zanchet, 2017, *C. pseudovaginuloides*, *C. tamoia*, and *C.* ~~–~~ *ratera viridimaculata* Negrete & Brusa, 2016.

Comentario [#21]: In the holotype? In both specimens (F2828 and F2829)?
I think you should add something about this, in view of denoting that the specimens studied were sexually mature.

However, (a) all of these five species have a common glandular ovovitelline duct (vs. absent in *C. piguaiaboja*; but see redescription of *C. pseudovaginuloides* in Riester, 1938); (b) the male and female atria are not separated by a constriction in *C. pseudovaginuloides* (vs. separated in *C. piguaiaboja*); (c) the penis papilla is longer than the male atrium in *C. nigrimarginata* and *C. pseudovaginuloides* (vs. shorter in *C. piguaiaboja*); (d) the prostatic vesicle is horizontal in *C. nigrimarginata* (vs. inverted-U shaped in *C. piguaiassu*); (e) the female atrium is funnel-shaped, and its posterior section oriented upwards in *C. viridimaculata* (vs. horizontal, and not funnel-shaped in *C. piguaiaboja*); (f) the prostatic vesicle runs postero-dorsally in *C. aureomaculata* and *C. tamoia* (vs. inverted U-shaped in *C. piguaiaboja*); (g) the penis papilla exhibits a postero-ventral orientation in *C. tamoia* (vs. postero-dorsal in *C. piguaiaboja*); and (h) there is no accumulation of necks of cyanophil cells piercing the roof of the male atrium in *C. nigrimarginata*, *C. pseudovaginuloides*, and *C. tamoia* (vs. present in *C. piguaiaboja*).

The phylogenetic tree as well as the molecular species delimitation analyses also show that this species is distinct and well-delimited from *C. pseudovaginuloides* and *C. tamoia*, the two species included in these analyses that show similarities with *C. piguaiaboja*.

***Cratera piguaiatui* sp. nov., Araujo, Carbayo, Riutort & Álvarez-Presas**

urn:lsid:zoobank.org:act:FB96BE96-86AD-41FB-961F-C0337C56A596

Synonymy. *Cratera* sp. 4: Carbayo et al. (2013).

Etymology. The name *piguaiatui* is a free composition of the Tupi (indigenous Brazilian language) words pyguaia (meaning hole, cave) and tui (meaning tiny, insignificant) (Tibiriçá, 1984). It refers to the small distal dilatation of the ejaculatory duct.

Type locality. Parque Nacional da Serra da Bocaina, São José do Barreiro, State of São Paulo, Brazil.

Distribution. Parque Nacional da Serra da Bocaina, São José do Barreiro, State of São Paulo; Parque Nacional Itatiaia, Resende, State of Rio de Janeiro, Brazil.

Material studied. Holotype F2809 (MZUSP PL 1051): Parque Nacional da Serra da Bocaina, São José do Barreiro, State of São Paulo (-22.75, -44.62). F. Carbayo et al., 7 September 2008; transverse sections of cephalic extremity on 4 slides; sagittal sections of a portion immediately behind cephalic extremity on 19 slides; horizontal sections of a immediately behind on 12 slides; transverse sections of pre-pharyngeal on 16 slides; sagittal sections of pharynx on 17 slides; sagittal sections of copulatory apparatus on 23 slides. Paratypes: F2031 (MZUSP PL 1014): Ibidem, 7 September 2008; sagittal sections of copulatory apparatus on 6 slides. F2054 (MZUSP PL 2148): Ibidem, 9 September 2008; sagittal sections of pharynx and copulatory apparatus on 13 slides (including 3 slides lost). F2040 (MZUSP PL 2147): Ibidem, 9 February 2007, preserved in 80% ethanol. F2798 (MZUSP PL 2149): Ibidem, 7 September 2008; preserved in 80% ethanol. F5178 (MZUSP PL 2154): Parque Nacional de Itatiaia, Resende, State of Rio de Janeiro (-22.43328, -44.61539), Brazil, F. Carbayo et al., 5 April 2012, horizontal sections of a body portion behind cephalic extremity on 7 slides; transverse sections of pre-pharyngeal on 15 slides; sagittal sections of pharynx on 17 slides; sagittal sections of copulatory apparatus on 30 slides.

Comentario [#22]: horizontal sections of a portion immediately before the pre-pharyngeal?

Diagnosis

Species of *Cratera* 45-70 mm long preserved; dorsum with a melon yellow median stripe, bordered on either side by a jet-black stripe external to which a marginal traffic white stripe; body margins jet black. Anterior 1/5th of the body colored with a gradient of carmine red; eyes

Comentario [#23]: Beautiful!!!

marginal; pharynx cylindrical; ~~distal dilatation of ejaculatory duct relatively small; pharyngeal pouch 0.6 mm anterior to the prostatic vesicle (equal to 1% of body length);~~ penis papilla shorter than male atrium; distal dilatation of ejaculatory duct relatively small; female atrium 2.5 times longer than the male atrium; common glandular ovovitelline duct long.

Description

Preserved 45-58 mm in length and 7 mm in width. Body slightly lanceolate, with maximum width at the level of the pharynx. Anterior to it, body becomes thinner gradually to the rounded tip; posterior to the level of the pharynx, body becomes thinner abruptly close to posterior pointed tip. Dorsum slightly convex, ventral side flattened. Creeping sole, 95% of body width at pre-pharyngeal region. Mouth at a distance from anterior extremity equal to 73% of body length; the gonopore at 82% (holotype).

Dorsum with a melon yellow median stripe, 28% of body width, which is bordered on either side by a jet-black stripe, 22% of the body width (Fig. 9A-B). External to jet black stripes, a traffic white marginal stripe, 10% of body width; body margin (3% of the body width) jet black. Anterior 1/5th of the body colored with a gradient of carmine red, dorsally and ventrally; otherwise, ventral side grey white (Fig. 9B).

Each eye is formed by a single pigmented cup with 35 µm in diameter. No clear halos around eyes. Eyes contour the anterior extremity in a single row and extend marginally until posterior tip.

Sensory pits, 20 µm deep, as a uniserial ventro-lateral row, from anterior extremity through a body length at least equal to 9% of body length (holotype). ~~Besides r~~Rhabditogen cells, and necks of two types of cell glands, producing xanthophil and erythrophil granules, respectively, pierce pre-pharyngeal dorsal epithelium; ~~the latter also pierce~~ ventral epithelium is

only pierced by erythrophil granules. Conspicuous glandular margin constituted of abundant glands producing erythrophil granules (Fig. 10A).

Cutaneous musculature composed of three layers: a subepithelial circular layer, followed by two diagonals with decussate fibers, and a subjacent ~~then a strongly developed~~ longitudinal one, 30 µm thick dorsally and 40 µm thick ventrally (holotype). Fibers of the longitudinal layer gathered in bundles, which are dorsally better delimited than ventrally. Relative thickness of cutaneous muscle, 9.1% (holotype).

Three parenchymal muscle layers throughout the body, all constituted by fibers relatively densely packed: a dorsal layer of diagonal decussate fibers (15 µm thick, holotype), a transverse supraintestinal layer (25 µm), and a transverse subintestinal one (25 µm).

Central nervous system as a ventral nerve plate. Clearly evident cerebral ganglia were not found.

Mouth in middle of pharyngeal pouch (Fig. 10B). Pharynx cylindrical, with dorsal insertion posterior to the ventral at the equivalent of 20% of pharyngeal length (paratype F2054). Esophagus length, ca. 10% of pharyngeal length (paratype F2054). Outer pharyngeal epithelium underlain by a one-fiber-thick layer (4 µm) of longitudinal muscle fibers followed by a layer of circular fibers (7 µm); inner pharyngeal epithelium underlain by a layer (80 µm) of circular fibers, followed by a layer (10 µm) of longitudinal fibers (holotype). Pharyngeal pouch at 600 µm from prostatic vesicle in holotype (or 1% of body length).

Testes dorsal, located under supraintestinal transverse muscle layer, partially placed ~~between~~ among the intestinal diverticula. The testes extend from level of ovaries to nearly root of pharynx. Distal course of sperm ducts running posteriorly and medially to communicate with the respective lateral diverticulum of the prostatic vesicle (Fig. 11A). Paired portion of prostatic

Comentario [#24]: This is how I understood it.

Comentario [#25]: Mature?

Comentario [#26]: the pharyngeal pouch could be marked in figure 11A.

vesicle represents ca. half of the total length of this organ. This vesicle, extrabulbar, approximately pear-shaped in lateral view, with posterior portion running posteriorly and upwards until anterior region of penis bulb. Vesicle lined with a ciliated epithelium, which is pierced by necks of cells producing fine erythrophil granules. A 20 µm thick layer of circular muscle fibers surrounds vesicle. Inside penis papilla vesicle communicates with a horizontal, initially sinuous ejaculatory duct which is lined with a cuboidal, ciliated epithelium. This epithelium surrounded by a 5 µm thick layer of circular muscle fibers. Near tip of penis papilla, lumen of the ejaculatory duct doubles its width to form a small cavity (Fig. 11A-C).

Comentario [#27]: Columnar? Cuboidal?

Penis papilla short, conical and blunt, horizontally placed~~, and roughly bulb-shaped~~; shorter than male atrium (Fig. 11A-B). Male atrium slightly folded in its proximal part. Penis papilla and male atrium clothed with a cuboidal non ciliated epithelium, this pierced by necks of two types of cells, producing erythrophil and xanthophil granules, respectively. Epithelium of penis papilla and that of male atrium underlain by a 7-µm-thick layer of circular muscle fibers, followed by a 7-µm-thick layer of longitudinal muscle fibers.

Ovaries elongate, roughly ovoid, with 250 µm anteroposteriorly. They are located immediately above the ventral nerve plate, at a distance from anterior tip equivalent to 30% of body length (holotype). Ovovitelline ducts arise from dorso-external side of ovaries and run backwards above the ventral nerve plate. They ascend laterally to female atrium to unite with the common glandular ovovitelline duct dorsally to ~~female~~ the atrium (Fig. 11A). Distal ascending portion of these ducts receive shell glands. Common glandular ovovitelline duct long (1.2 mm, ie., 1/3th of the length of female atrium in holotype) communicating with female genital canal, the latter being a projection of the posterior portion of female atrium directed forwards and dorsally. Female atrium funnel-shaped, with a length 2.5 times that of male atrium. Lateral wall

of female atrium with folds narrowing its lumen. Female atrium lined with a non-ciliated, 25 µm tall epithelium along anterior 4/5th of its length. Posterior 1/5th lined with a 50-µm-high epithelium that might display a multilayered one; quality of the sections precluded confirmation. Necks of cells producing erythrophil granules pierce female epithelium, this underlain by a layer of circular muscle fibers (7 µm thick, holotype) followed by a layer of longitudinal fibers (7 µm thick).

Remarks

Among all species of the genus, only *C. ratera taxiarcha* (Marcus, 1951) resembles *C. piguaiatui* in the three-color striped pattern of the dorsum composed of white, yellow, and black colors. However, in *C. taxiarcha*, the median stripe is white (vs. yellowish in *C. piguaiatui*). Regarding the copulatory apparatus, all species in the genus possess a female atrium as long as the male atrium, with minor variations whereas in *C. piguaiatui* it is 2.5 times longer. The molecular delimitation methods all clearly point to *C. piguaiatui* being a species differentiated from the rest of species molecularly analysed in the present study.

***Cratera imbiri* sp. nov., Araujo, Carbayo, Riutort & Álvarez-Presas**

urn:lsid:zoobank.org:act:CC5B22EB-9E7C-490F-A6FF-03757BA03C26

Etymology. The name *imbiri* refers to Vila de São Matheus do Imbiri, former name of Campos do Jordão, type locality of the species.

Type locality. Parque Estadual Campos do Jordão, Campos do Jordão, State of São Paulo, Brazil.

Distribution. Type locality only.

Material studied. Holotype F5512 (MZUSP PL 2155): Parque Estadual Campos do Jordão, Campos do Jordão, State of São Paulo, Brazil (-22.68878, -45.48068). F. Carbayo et al., 15 November 2012. Horizontal sections of a behind cephalic extremity on 7 slides; transverse sections of pre-pharyngeal on 9 slides; sagittal sections of pharynx on 13 slides; sagittal sections of copulatory apparatus on 9 slides.

Diagnosis

Species of *Cratera* 26 mm long preserved; dorsal median stripe sulfur yellow bordered on either side by a khaki grey band; body margins cream; in anterior 1/4th of the body, this pattern covered with a color gradient of coral red; eyes marginal; pharynx cylindrical, with dorsal insertion posteriorly shifted at the equivalent of 20% the length of pharynx; pharyngeal pouch 600 μm anterior to prostatic vesicle; paired portion of the prostatic vesicle with 1/3th of total length of this organ; epithelium of penis papilla underlain by a layer of circular muscle fibers only; female atrium 2.5 times longer than male atrium; female atrium narrows gradually towards its posterior section; common glandular ovovitelline duct long.

Description

When creeping, body 38 mm long and 2.5 mm wide. Preserved 26 mm and 4 mm, respectively. Body margins parallel along most of its length. Extremities of the body rounded. The dorsum slightly convex, ventral side flattened. Creeping sole, 94% of body width at the pre-pharyngeal region. Mouth at a distance from anterior extremity equal to 70% of body length; gonopore, 78%.

Dorsal color with a sulfur yellow median stripe, 14% of the body width, this bordered on either side by a khaki grey band, 34% of the body width. Body margins (9% of body width) cream (Fig. 12A). In anterior 1/4th of body, this pattern covered with a gradient color of coral

red. Ventral side coral red along anterior 1/4th, and cream colored behind (Fig. 12B).

Eyes of one pigmented cup with 25 µm in diameter. No clear halos around eyes ~~were seen~~. Eyes contour the anterior extremity in a single row and extend marginally until posterior extremity. Anterior extremity of body with 3mm long not available (used for DNA extraction). Sensory pits, 20 µm deep, as a uniserial ventro-lateral row in along a body portion initiating 3 mm behind anterior tip of the body and extending backwards 3.7 mm. Necks of cell glands producing erythrophil granules pierce dorsal and ventral epithelium in pre-pharyngeal region. Besides, rhabditogen cells discharge their content through dorsal epithelium. Glandular margin constituted of abundant glands producing erythrophil granules.

Cutaneous musculature composed of a subepithelial circular layer, followed by two diagonals with decussate fibers, and ~~then a strongly developed~~a subjacent longitudinal one, 35 µm thick dorsally and 30 µm thick ventrally. Thickness of cutaneous muscle, 11.3% of body height in the pre-pharyngeal region.

Three parenchymal muscle layers are present throughout the body: a dorsal layer of diagonal decussate fibers (10 µm thick), a transverse supraintestinal layer (20 µm), and a transverse subintestinal one (20 µm).

Central nervous system as a ventral nerve plate. Cerebral ganglia not discerned.

Mouth located at the end of the anterior half of pharyngeal pouch (Fig. 12C). Pharynx cylindrical, with dorsal insertion posterior to the ventral at the equivalent of 7% of pharyngeal length. Esophagus length, 20% of pharyngeal length. Outer pharyngeal epithelium underlain by a one-fiber-thick layer of longitudinal muscle fibers followed by a layer of circular fibers (5 µm thick); inner pharyngeal epithelium underlain by a well-developed layer of circular muscle fibers (60-100 µm), followed by a thinner layer of longitudinal fibers (8 µm). Pharyngeal pouch 80 µm

Comentario [#28]: In Fig. 12, "px" indicates the pharyngeal pouch. Is a detail, but please fix it.

anterior to prostatic vesicle.

Testes dorsal, located under the supraintestinal transverse muscle layer, partially placed ~~between~~ among the intestinal diverticula. The testes extend from 200 µm behind the level of the ovaries to 1 mm anterior to the root of pharynx. Sperm ducts very narrowed at the point of communication with the respective branch of prostatic vesicle. Paired portion of this vesicle with ca. 1/3rd of the total length of the organ. Prostatic vesicle extrabulbar, running postero-dorsally until anterior region of penis bulb. Vesicle lined with a ciliated epithelium, this pierced by necks of cells producing fine erythrophil granules. A 50-µm-thick circular muscle surround the vesicle. Inside the penis papilla, vesicle communicates with ~~the horizontal, sinuous~~ ejaculatory duct, sinuous in its proximal course. This duct is dilatated distally at the tip of the penis papilla the equivalent of 2/5th of length of penis papilla. Ejaculatory duct lined with a cuboidal, ciliated epithelium, its cilia being as long as cell height, i.e., 10 µm. This epithelium is surrounded by a 5-µm-thick layer of circular muscle fibers.

Penis papilla short, horizontally placed, cylindrical, with rounded tip; it is shorter than male atrium (Fig. 13A-C). Male atrium as long as 1.2 its height, with smooth folds. A large, transverse, annular fold strongly narrows communication with female atrium (Fig. 13A, C). Penis papilla and male atrium clothed with a cuboidal-to-columnar, non-ciliated epithelium; the subapical portion of its cells is xanthophil. Papillar epithelium pierced by necks two types of cells producing granules, one erythrophil, another weakly basophil. Additionally, cells with gross necks (6 µm in diameter) and erythrophil amorphous appearance are located immediately under the epithelium. Epithelium of penis papilla and that of male atrium underlain by a 6-µm-thick layer of circular muscle fibers.

Ovaries ellipsoid, with 100 µm anteroposteriorly (Fig. 13D), located immediately above

Comentario [#29]: Columnar? Cuboidal?

Comentario [#30]: Judging from the scale, the ovaries appear longer. Or the scale is wrong. Please, check.

the ventral nerve plate, at a distance from anterior tip equivalent to 21% of body length. Ovovitelline ducts arise from dorso-external side of ovaries, subsequently run backwards above the ventral nerve plate. They ascend laterally to the female atrium to unite the common glandular ovovitelline duct dorsally to mid female atrium (Fig. 13A). The distal half ascending portion of the ducts receives shell glands. 0.9-mm-long common glandular ovovitelline duct (47% of the length of female atrium) communicates with the female genital canal, this a projection of the postero-dorsal portion of the female atrium directed forwards and dorsally. Female atrium with 3.2x the length of male atrium. Posterior third of female atrium with lateral folds narrowing its lumen. Female atrium lined with a non-ciliated, 20 µm tall epithelium along anterior 3/4 of its length; tissue of posterior portion is damaged. Gland cells producing erythrophil granules discharge their secretion into female atrium. It seems to be underlain by two muscle layers, but sections are suboptimal in quality.

Comentario [#31]: According to figure 13D (longitudinal section), the oviduct arises from the posterior aspect of the ovary, or at least not externally.

Remarks

This small species displays a color pattern that cannot be confounded with any of its congeners. Regarding the internal morphology, only *C. piguaiatui* resembles *C. imbiri* in that both species have an uncommonly long female atrium, at least 2.5 times longer than the male one. Indeed, *C. piguaiatui* and *C. imbiri* are very alike each other in the general aspect of the copulatory apparatus. They distinguished from each other in a set of minor anatomical details: (a) the pharyngeal pouch is 600 µm anterior to the prostatic vesicle (vs. practically leveled with the prostatic vesicle in *C. imbiri*); (b) dorsal insertion of the pharynx is posteriorly shifted at the equivalent of 20% the length of pharynx (vs. 7% in *C. imbiri*); (c) paired portion of the prostatic vesicle is 1/3th of total length of this organ in *C. piguaiatui* (vs. half in *C. piguaiatui*); (d) epithelium of penis papilla is underlain by a layer of circular muscle fibers followed by a layer of

longitudinal one in *C. piguaiatui* (vs. only a layer of circular muscle in *C. imbiri*); and (e) the female atrium narrows abruptly towards its posterior section in *C. piguaiatui* (vs. gradually in *C. imbiri*). The molecular based phylogeny shows these two species as very close genetically, nonetheless, the molecular delimitation shows them to be two clearly distinct species which reinforces the minor anatomical differences found to be in fact species specific.

***Cratera paraitinga* sp. nov., Araujo, Carbayo, Riutort & Álvarez-Presas**

urn:lsid:zoobank.org:act:7B3F43A7-2794-42F4-B99F-B8C062F972CF

Etymology. The name *paraitinga* refers to São José do Paraitinga, former name of Salesópolis, type locality of the species.

Type locality. Estação Biológica de Boraceia, Salesópolis, São Paulo State, Brazil.

Distribution. Type locality only.

Material studied. Holotype F5769 (MZUSP PL 2157): Estação Biológica de Boraceia, Salesópolis, São Paulo State, Brazil (-23.65413, -45.88884). F. Carbayo et al., 20 April 2013. Transverse sections of cephalic extremity on 19 slides; horizontal sections of a portion immediately behind on 71 slides; transverse sections of pre-pharyngeal on 22 slides; sagittal sections of the pharynx on 33 slides; sagittal sections of copulatory apparatus on 60 slides. Paratype F5745 (MZUSP PL 2156): Ibidem. Transverse sections of cephalic extremity on 11 slides; horizontal sections of an immediately behind on 23 slides; transverse sections of pre-pharyngeal on 7 slides; sagittal sections of pharynx and copulatory apparatus on 12 slides.

Diagnosis

Species of *Cratera* 76 mm long preserved; dorsal melon yellow median stripe, bordered

on either side by a jet black stripe external to each a marginal traffic white stripe; body margins jet black; eyes marginal; anterior 1/6th of the body colored with a gradient of carmine red; pharynx cylindrical to bell-shaped; pharyngeal pouch leveled with prostatic vesicle; distal dilatation of ejaculatory duct relatively large; penis papilla as long as male atrium; female atrium 2.4 times longer than the male atrium; common glandular ovovitelline duct long.

Description

Preserved holotype is 76 mm in length and 7 mm in width. Paratype (incompletely mature), 27 and mm 4, respectively. The body is slightly lanceolate, with maximum width at the level of the pharynx. Anterior to it, body thinner gradually to the rounded extremity; near posterior extremity thinner more abruptly to the pointed tip. Dorsum convex, ventral side flattened. Creeping sole, 95% of body width at pre-pharyngeal region. Mouth at a distance from anterior extremity equal to 63% of body length; gonopore, 83% (holotype).

Dorsum with an orange luminous median stripe, 40% of the body width, this bordered on either side by a jet-black stripe (14.5%), external which a white band (11%); body margin (4.5%) jet black (Fig. 14A-B). Body sides of anterior 1/5th of the body colored with a gradient of carmine red. Ventrally, body sides of anterior 1/6th orange brown, grey white behind (Fig. 14C).

Each eye is formed by a single pigmented cup with 25 µm in diameter. No clear halos around eyes ~~were seen~~. Eyes contour the anterior extremity in a single row and extend marginally until posterior tip. Sensory pits, 15 µm deep, as a uniserial ventro-lateral row, from anterior extremity through a body length at least equal to 15% of body length (holotype). Necks of two types of cell glands, producing xanthophil and erythrophil granules, respectively, pierce pre-pharyngeal region dorsally and ventrally. Besides, rhabditogen cells discharge their secretion through dorsal epithelium. Conspicuous glandular margin constituted of abundant glands

Comentario [#32]: In the diagnosis, you say “melon yellow median stripe”. Please homogenize.

842 producing xanthophil granules (Fig. 15A).

843 Cutaneous musculature composed of a subepithelial circular layer, followed by two
844 diagonals with decussate fibers, and then a ~~strongly developed~~subjacent longitudinal one, 35-40
845 µm thick (paratype and holotype, respectively) dorsally and 40-45 µm thick ventrally (holotype
846 and paratype, respectively). Thickness of cutaneous muscle ranges from 6.6% (holotype) to
847 10.7% (paratype) to body height in the pre-pharyngeal region.

848 Three parenchymal muscle layers present throughout the body, all constituted by fibers
849 relatively densely packed: a dorsal layer of diagonal decussate fibers (10 µm thick, holotype), a
850 supraintestinal layer of transverse muscle fibers (40 µm), and a transverse subintestinal one (40
851 µm). Dorso-ventral fibers abundant ~~between~~ among intestinal branches.

852 Central nervous system as a ventral nerve plate. Clearly evident cerebral ganglia were not
853 found.

854 Mouth located in the end of the anterior half of pharyngeal pouch (Fig. 15B). Pharynx
855 between cylindrical to bell-shaped, with dorsal insertion posterior to the ventral at the equivalent
856 of 40% of pharyngeal length. Esophagus length, 20% of pharyngeal length (holotype and
857 paratype). Outer pharyngeal epithelium underlain by a one-fiber-thick layer (4 µm) of
858 longitudinal muscle fibers followed by a layer of circular fibers (6 µm); inner pharyngeal
859 epithelium underlain by a layer (50-100 µm) of circular fibers, followed by a layer (20 µm) of
860 longitudinal fibers (holotype). Pharyngeal pouch at 80 µm from prostatic vesicle (holotype), i.e.,
861 0.001% of body length.

862 Testes dorsal, located under the supraintestinal muscle layer, partially placed ~~between~~
863 among the intestinal diverticula (Fig. 15A). These testes extend from shortly behind the level of
864 the ovaries to nearly 3 mm anterior to root of pharynx (holotype). Sperm ducts communicate

with the respective lateral diverticulum of the prostatic vesicle. This vesicle extrabulbar, elongate, with anterior 1/4th of its length branched. Vesicle runs posteriorly and upwards until anterior region of penis bulb. Vesicle is lined with a ciliated epithelium, which is pierced by numerous necks of cells producing fine erythrophil granules. A 15-µm-thick net of muscle fibers surround the vesicle. Inside the penis papilla, vesicle communicates with a straight ejaculatory duct traversing penis papilla at the tip of which dilates to a conspicuous cavity with half of penis papilla length (Fig. 15C, 16A-B). Epithelium of this duct surrounded by a 5-µm-thick layer of circular muscle fibers along most of its length, and of circular and longitudinal fibers in its dilated portion.

Comentario [#33]: Columnar? Cuboidal?

Penis papilla conical and blunt, with dorsal insertion slightly posterior to the ventral one; it is as long as male atrium (Fig. 15C, 16A). Male atrium as long as 1.4x its height, slightly folded. Penis papilla and male atrium clothed with a cuboidal, non-ciliated epithelium, which is pierced by necks of cells producing erythrophil granules. Quality of stain did not provide further details. Epithelium of penis papilla and that of male atrium underlain by a 3-µm-thick layer of circular muscle fibers, followed by a 2-µm-thick layer of longitudinal fibers.

Ovaries elongated, ovoid, with 300 µm anteroposteriorly. They are located immediately above the ventral nerve plate, at a distance from anterior tip equivalent to 25% of body length (holotype). Ovovitelline ducts arise from dorso-lateral side of the ovaries and run backwards above the ventral nerve plate. They ascend laterally to the female atrium to unite with the common glandular ovovitelline duct, dorsally to the posterior third of female atrium (Fig. 15C). Distal ascending portion of the ducts receives shell glands. The long common glandular ovovitelline duct (0.9 mm, ie., 1/3th of the length of female atrium) communicates with female genital canal, the latter being a projection of posterior portion of female atrium that runs

888 forwards and dorsally (Fig. 15C, 16A-C). Female atrium long and funnel-shaped, compressed
889 laterally. Anterior portion of female atrium narrower. Female atrium with 2.4 times the length of
890 male atrium, and lined with non-ciliated epithelium, 40 µm tall and with multilayered aspect in
891 its posteriormost 1/4th; otherwise, 20 µm tall and cuboidal-to-columnar. Necks of cells
892 producing erythrophil granules pierce female epithelium, this underlain by a layer of circular
893 muscle fibers (10 µm thick, holotype) followed by a layer of longitudinal fibers (10 µm thick).
894 **Remarks**
895 Among all species of the genus, *C. taxiarcha* and *C. piguaiatui* resemble *C. paraitinga* in
896 the general color pattern of the dorsum consisting of longitudinal stripes with black, white and
897 yellow color. However, the median stripe in *C. taxiarcha* is white (vs. yellowish in *C.*
898 *paraitinga*). *Cratera piguaiatui*, very similar to *C. paraitinga* in the color pattern, differs from it
899 in the width of the yellowish midstripe (28% of body width in *C. piguaiatui* vs. 40% in *C.*
900 *paraitinga*).
901 Regarding the copulatory apparatus, only *C. piguaiatui* and *C. imbiri* resemble *C.*
902 *paraitinga* in the relatively long female atrium when compared with the male one. However, the
903 two species differ from *C. paraitinga* in minor details: (a) the dilatation of the ejaculatory duct is
904 relatively small in *C. piguaiatui* (vs. relatively large in *C. paraitinga*); (b) the pharyngeal pouch
905 is 0.6 mm anterior to the prostatic vesicle in *C. piguaiatui* (vs. practically leveled in *C.*
906 *paraitinga*); (c) the penis papilla is shorter than the male atrium in *C. piguaiatui* and *C. imbiri*
907 (vs. as long as the male atrium in *C. paraitinga*).
908 The molecular analyses show these three species, *C. piguaiatui*, *C. imbiri* and *C.*
909 *paraitinga*, to constitute a monophyletic group, which will explain their morphological
910 similitudes commented above and could also raise some doubt on their identity as different

911 species. However, the discovery methods of species delimitation, only with the exception of the
912 mPTP method, show the three as independent species. In the case of the validation method (BPP)
913 the significance of the separation of *C. imbiri* and *C. paraitinga* is only highly supported by the
914 models implying a small ancestral size, while the support is slightly lower if we consider
915 ancestral population was large. It could be interpreted from our results that ancestral populations
916 may not have been very large, although, of course, the current situation may be a consequence of
917 the destruction of their habitat or a lack of sampling, since some areas have been explored very
918 intensively and others are still pending sampling. Putting together all the evidence, molecular
919 and morphology reinforce one another and give more weight to the small morphological
920 differences found to be indicators of the different lineages having become different species.
921
922 ## Discussion
923
924 Carbayo et al. (2013) proposed the first phylogenetic framework of Geoplaninae. That
925 phylogeny was inferred from one mitochondrial region (COI) and three nuclear ones (18S, 28S
926 rDNA and EF) of 68 species/morphospecies, eight of them representing *Cratera* lineages (plus
927 one immature representative). At that time, only three species of *Cratera* were known (*C.*
928 *crioula*, *C. pseudovaginuloides* and *C. tamoia*). Later, three of the undescribed species
929 considered in that study were formally described (*C. cuarassu*~~;~~ , *C. picuia* ~~Lago-Barcia &~~
930 ~~Carbayo, 2018~~, and *C. arucuia* ~~Lago-Barcia & Carbayo, 2018~~) (Carbayo & Almeida, 2015;
931 Lago-Barcia & Carbayo, 2018). Two species included in the Carbayo et al (2013) phylogeny that
932 had remained morphologically unstudied, are described in the present work, namely *C.*
933 *piguaiassu* and *C. piguaiatui*.
934 In the present study, three new species (*C. imbiri*, *C. paraitinga*, *C. piguaiaboja*) are also

included ~~for the first time~~. The phylogenetic relationships between all these 11 species have been examined here through comparative analysis of six concatenated DNA regions (two mitochondrial fragments and four nuclear).

Without taking into consideration differences in representativeness, the topology of our phylogeny matches that of Carbayo et al (2013), except for the position of *C. tamoia* and *C. crioula*. In the phylogeny from 2013, *C. tamoia* is sister of the remaining species of an in-group including *C. crioula*, whereas in the present phylogeny, *C. crioula* + *C. piguaiassu* is the sister clade to the remaining members of the in-group, which includes *C. tamoia*. This is a relevant result, due to a difference in taxon sampling. As more species are included in the present study, relationships are resolved that could not be observed in the phylogenies of 2013, with a smaller representation of species of *Cratera*. Lago-Barcia & Carbayo (2018) discussed the evolution of some morphological attributes within *Cratera* by analyzing them against the phylogeny of Carbayo et al. (2013). They considered only the five species whose anatomy was known, namely *C. arucuia*, *C. crioula*, *C. cuarassu*, *C. picuia*, and *C. pseudovaginuloides*. They interpreted that the distal widening of the ejaculatory duct originated in the common ancestor of all *Cratera* members and was secondarily lost in the last common ancestor of *C. tamoia*, *C. crioula*, and *C. arucuia*. For this feature as well as other characters (roof of the male atrium pierced by necks of numerous cyanophil glands; prostatic vesicle dorsally located; ~~90o~~ 90º rotation of the penis papilla) they concluded that "diagnostic character states of the genus can be lost or modified within recently evolved in-groups of *Cratera*, hence puzzling species classification" (Lago-Barcia & Carbayo, 2018).

In the light of this new phylogenetic framework, loss of the widening of distal section of the ejaculatory duct apparently evolved independently in two lineages, thus giving rise to the

condition is *C. crioula*, and that in *C. picuia* and *C. arucuia*. However, this new framework does not invalidate the above-quoted conclusion of Lago-Barcia & Carbayo. Moreover, our data corroborates their conclusion as demonstrated in the following five selected examples. (i) The position of the prostatic vesicle, either internal to the penis bulb or external to it, appears to have independently evolved from an external to an internal position only in *C. tamoia* and *C. picuia*. An equally parsimonious interpretation would be that the internal position of the prostatic vesicle would have evolved in the common ancestor of *C. tamoia* + *C. arucuia* + *C. picuia* and that this condition would have reversed in *C. arucuia*. (ii) In similar vein, a penis papilla longer than the male atrium may have been gained in the common ancestor of *C. piguaiaboja*, *C. pseudovaginuloides*, *C. crioula*, *C. piguaiassu*, *C. picuia*, *C. arucuia* and *C. tamoia*, while this condition would independently secondarily have been lost in *C. piguaiaboja* and *C. tamoia*. (iii) In *C. picuia* and *C. piguaiaboja*, the dorsal surface of the male atrium is traversed by a mass of necks of cells producing cyanophil granules. This situation is best explained as two independent gain events. (iv) The most parsimonious explanation for the very reduced or even absence of the common glandular ovovitelline duct in *C. picuia*, *C. piguaiassu* and *C. piguaiaboja* is independent loss in each of these species, none of which shares a sister-group relationship.

In bucking this trend, the relatively long female atrium, in comparison with the male atrium appears to be homologous in all members of *Cratera*. The female atrium is usually as long as the male one. However, in *C. piguaiatui*, *C. imbiri*, and *C. paraitinga*, the female atrium is >2.4 times longer than the male one. These three species constitute a monophyletic group and most probably this character state evolved in the common ancestor of these species. These three species are similar to each other, not only in this trait, but also in the traditional characteristics used in the classification of Geoplaninae. For this reason, our molecular approach in the species

delimitation proved to be essential in their discovery as independent lineages.

The causes underlying the evolutionary differences between the copulatory organs in land triclads remain unclear. Absence of relevant apomorphies in other related groups, such as the freshwater planarian genus *Girardia* (Dugesiidae), also complicated assignment of species to the genus (Sluys, Hauser & Wirth, 1997). In the case of land planarians, morphological differences may be related to the fact that each species belongs to a lineage that has evolved independently for a long period, as exemplified by the land planarian *Cephaloflexa bergi* (Graff, 1899) (Geoplaninae), a species that originated about 7 Mya (Álvarez-Presas et al., 2014).

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As a result of this argument, it rises that species of *Cratera* ~~land planarians~~ evolved labile features, even those that diagnose the genus, such as the dilatation of the ejaculatory duct. This lability can mislead a natural classification of *Cratera* and its relatives if systematics is solely based on morphology.

An interesting aspect of land planarians is their restricted geographical distribution. Most species are known from only one or a few localities (unpublished results). In the present study only *Cratera piguaiatui* was found in an additional locality apart from the type locality. Even so, the two localities are only 30 km apart from each other. Although sampling artifacts may underlie such presumably restricted distributions (Sluys, 1999) it is also possible that they reflect actual species distribution. Our data support the latter in the case of *Cratera* because we performed an intensive sampling effort in the four studied areas that resulted in the distributional ranges reported in this study.

We hypothesize here that *Cratera* species presenting such labile features, being genetically close among them and with very restricted areas of distribution may be the result of relatively recent speciation events linked to the postglacial history of the area. However,

thorough studies including NGS data and robust population analyses will be necessary to confirm such hypothesis.

Conclusions

Molecular-based phylogenies and species delimitation provide hypotheses on species recognition that are independent from the morphology-based approach. Congruence of both approaches allowed us to recognize evolutionarily independent lineages, i.e, species, and to independently evaluate minor morphological differences among the individuals as a signal of diagnostic attributes of a species. Otherwise, most likely we had ranked *C. piguaiatui*, *C. imbiri* and *C. paraitinga* under one nominal species.

Moreover, the new molecular markers for species delimitation and phylogenetic inference developed for the first time in the present work have shown to be highly resolutive for terrestrial planarians. We have expanded the number of informative molecular markers by adding two new molecules (Tnuc813 and Nd4toCox1) as a result of the use of new generation molecular tools. This result should not be overlooked, since the availability of molecular markers has always been a limitation in the study of the systematics of these animals.

Acknowledgements

We are grateful to the COTEC - Instituto Florestal do Estado de São Paulo (Proc. SMA 12.640/2011), Museu de Zoologia (EBBAut.020/2013) and Instituto Chico Mendes de Conservação da Biodiversidade (Proc. 32779-1; 11748-4) for licensing the field work. We also

thank Celso Barbieri Junior, Cláudia Olivares, Débora Redivo, Erica Panachuk de Souza, Júlio Pedroni, Leonardo Zerbone, Marcos Santos Silva, Marília Jucá and Welton Araújo (EACH, USP) for assistance during the sampling. Thanks are due to Geison Castro da Silveira, Lucas Beltrami, and Ítalo Silva de Oliveira Souza (EACH, USP) for histological processing. Gema Blasco is thanked for the wet lab support.

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