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A tale of two bellies: systematics of the oval frogs (Anura: Microhylidae: *Elachistocleis*)

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Oval frogs (*Elachistocleis*) have a broad geographic distribution covering nearly all of South America and parts of Central America. They also have a large inter- and intraspecific variation of the few morphological characters commonly used as diagnostic traits among species of the genus. Based on molecular data, we provide the most complete phylogeny of *Elachistocleis* to date, and explore its genetic diversity using distance-based and tree-based methods for putative species delimitation. Our results show that at least two of the most relevant traditional characters used in the taxonomy of this group (belly pattern and dorsal median white line) carry less phylogenetic information than previously thought. Based on our results, we propose some synonymizations and some candidate new species. This study is a first major step in disentangling the current systematics of *Elachistocleis*. Yet, a comprehensive review of morphological data is needed before any new species descriptions can be properly made.

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INTRODUCTION

The developments in taxonomy and of analytical methods in systematics have continued to challenge old interpretations of Neotropical biological diversity and to provide new estimates of diversity across animal and plant groups (e.g. Fouquet *et al.*, 2007; Clare *et al.*, 2011; Raven *et al.*, 2020).

There are approximately 8470 species of amphibians in the world (Frost, 2022). The Neotropical region is home to over a third of this diversity (Raven *et al.*, 2020) and many estimates suggest that a large number of species are still to be discovered and described in the region (e.g. Fouquet *et al.*, 2014; Lyra *et al.*, 2017; Vacher *et al.*, 2020). A recent estimate for the potential of discovery of new taxa revealed that Amphibia and the Neotropics are, respectively, among the top zoological groups and bioregions of the world for such discoveries (Moura & Jetz, 2020).

Three subfamilies of narrow-mouthed frogs, Microhylidae, occur in the Neotropics: Adelastinae, restricted to the Guiana Shield region; Gastrophryninae, distributed from temperate North America, through Central and northern South America, to temperate regions of Argentina, Brazil and Uruguay; and Otophryninae, restricted to the Amazon. These three groups are relatively poorly studied given their secretive life-history traits: terrestrial, semi-fossorial or fossorial frogs that are 'explosive breeders' with cryptic, dull-coloured adults and fast-developing larvae. In these three microhylid clades, species diversity appears to be underestimated, with multiple species descriptions in recent years and additional new species remaining to be described and named (Peloso *et al.*, 2014; de Sá *et al.*, 2019; Fouquet *et al.*, 2021a, b).

Gastrophryninae is the most species-rich microhylid clade in the Americas, with 83 formally recognized species in 11 genera. Of these, *Elachistocleis* Parker, 1927 is one of the most diverse and taxonomically challenging groups in the Neotropics. *Elachistocleis* is found from Costa Rica south to Uruguay and northern Argentina, and is the second-most species-rich genus of gastrophrynines, with 22 nominal species currently accepted (Frost, 2022). Species-level taxonomy of *Elachistocleis* is in flux, with half of its species named during the last decade (2010–21). Dubois *et al.* (2021) revised the taxonomy of amphibians based on a phylogeny first published by Jetz & Pyron (2018) and designated *Elachistocleis* as a junior synonym of *Engystoma* Fitzinger, 1826. Segalla *et al.* (2021) discussed some of the nomenclatural changes made by Dubois *et al.* (2021), including the synonymy of *Elachistocleis* and *Engystoma*. Segalla *et al.* (2021) argue that, with the word '*Repräsentant*', Fitzinger clearly designates *Rana gibbosa* Linnaeus, 1758 as the type species of the genus *Engystoma*, invalidating the subsequent designation by Duméril & Bibron (1841) of *Rana ovalis* Schneider, 1799 as the type species of that genus. We also examined the work of Fitzinger (1826) and reach the same conclusion – hence we support the proposal to retain *Elachistocleis* as a valid generic name.

Several factors make the taxonomy of *Elachistocleis* confusing and challenging. Species of this genus have a conserved morphology, with few obvious external characters that can be used as diagnostic traits. These are typically, and historically, restricted to the belly and thigh colour pattern, the proportions of the head, the degree of development of a postcommissural gland, and the presence/absence and relative length of a mid-dorsal white line (Parker, 1927; Lavilla *et al.*, 2003; Caramaschi, 2010; Pereyra *et al.*, 2013). Recently, a phenetic classification into two groups was proposed, based on the ventral colour pattern: one group with immaculate bellies and another group with maculate bellies (Piva *et al.*, 2017). Furthermore, other authors have noticed substantial variation in the characters used as diagnostic and some degree of intraspecific variation, including ontogenetic variation in belly colour patterns (Lavilla *et al.*, 2003; Marinho *et al.*, 2018; Bueno-Villafañe *et al.*, 2020). Although several authors have suggested that its conserved morphology might be hiding several unnamed species under species complexes in *Elachistocleis* (e.g. Caramaschi, 2010; de Sá *et al.*, 2012; Piva *et al.*, 2017; Ferraro *et al.*, 2018; Barrio-Amorós *et al.*, 2019; Vaz-Silva *et al.*, 2020; Jowers *et al.*, 2021), the use of genetic data and phylogenetic analyses focusing on *Elachistocleis* are scarce. Only the descriptions of *E. araios* (Sánchez-Nivicela *et al.*, 2020) and *E. nigrogularis* (Jowers *et al.*, 2021) included molecular data and presented phylogenetic hypotheses – all other species were diagnosed based solely on morphology, most often only external morphology. To date, only 12 out of 22 recognized species have been included in phylogenetic studies (Sánchez-Nivicela *et al.*, 2020; Jowers *et al.*, 2021; Acosta-Galvis *et al.*, 2022). As an additional hurdle, sampling density of included species is limited, as most are represented by a small number of samples from few localities that do not cover the known geographic distribution of the nominal taxa.

The advance of theoretical and computational areas in genomic studies in the last two decades has resulted in numerous algorithms to help delimit lineages and species (e.g. Rannala & Yang, 2003; Pons *et al.*, 2006; Fontaneto *et al.*, 2007; Yang & Rannala, 2010; Jones *et al.*, 2011, 2014, 2017; Puillandre *et al.*, 2012, 2021; Fujisawa & Barraclough, 2013; Zhang *et al.*, 2013; Yang, 2015; Kapli *et al.*, 2017; see review by: Rannala & Yang, 2020). Although such analyses are commonly named 'species delimitation methods' (hereinafter SDM), the groups resulting from those analyses might represent either 'true species' or structured populations within species (see: Sukumaran & Knowles, 2017; Sukumaran *et al.*, 2021), especially when in allopatry (Rannala & Yang, 2020). Some of the authors acknowledge this caveat by applying other terms, instead of species, to the groups delimited by those analyses, such as MOTU (molecular operational taxonomic unit; Jones *et al.*, 2011) or by remarking that the output of the analysis should be viewed not as a final species delimitation, but as a preliminary delimitation, an initial hypothesis to be further assessed by taxonomists with additional data from other sources (Puillandre *et al.*, 2012; 2021; Sukumaran & Knowles, 2017; Rannala & Yang, 2020). To account for the drawbacks and advantages of different SDMs, a recommended and frequently applied protocol to approach DNA-based SDMs is to use more than one of these methods and to discuss and interpret the results in light of biological knowledge and additional sources of evidence, such as phenotypes (e.g. Esselstyn *et al.*, 2012; Carstens *et al.*, 2013; Schwarzfeld & Sperling, 2015; Luo *et al.*, 2018; Chambers & Hillis, 2020; Parslow *et al.*, 2021).

We here present the most comprehensive phylogenetic hypothesis of *Elachistocleis* to date and estimate the putative species richness of the genus, including a dense sampling of individuals covering most of its known geographic distribution. We applied three different types of molecular SDM and examined the results of these analyses in the light of the known distribution, phylogenetic position of type specimens and the two morphological characters most frequently employed in the taxonomy of *Elachistocleis*, namely the ventral colour pattern and the absence/presence of a mid-dorsal white line.

MATERIAL AND METHODS

Sampling

We obtained DNA sequences from 19 of the 22 currently recognized nominal species: *Elachistocleis araios* Sánchez-Nivicela *et al.*, 2020, *E. bicolor* (Guérin-Méneville, 1838), *E. bumbameuboi* Caramaschi, 2010, *E. carvalhoi* Caramaschi, 2010, *E. cesarii* (Miranda-Ribeiro, 1920), *E. haroi* Pereyra *et al.*, 2013, *E. helianneae* Caramaschi, 2010, *E. magna* Toledo, 2010, *E. matogrosso* Caramaschi, 2010, *E. muiraquitan* Nunes-de-Almeida & Toledo, 2012, *E. nigrogularis* Jowers *et al.*, 2021, *E. panamensis* (Dunn *et al.*, 1948), *E. pearsei* (Ruthven, 1914), *E. piauiensis* Caramaschi & Jim, 1983, *E. sikuani* Acosta-Galvis *et al.*, 2022, *E. skotogaster* Lavilla *et al.*, 2003, *E. surinamensis* (Daudin, 1802), *E. surumu* Caramaschi, 2010 and *E. tinigua* Acosta-Galvis *et al.*, 2022. We could not sample *E. corumbaensis* Piva *et al.*, 2017 and *E. erythrogaster* Kwet & Di-Bernardo, 1998 (see Results and Discussion below). We also considered *E. ovalis* (Schneider, 1799) as *nomen dubium* and *species inquirenda* (see Discussion). Whenever possible, we included specimens of taxonomic importance, especially holotypes, paratypes and specimens from relevant localities, such as topotypes or samples obtained from the vicinities of type localities. Sequences used in this study were generated *de novo* from museum samples or downloaded from GenBank (Benson *et al.*, 2013). In all, we included sequences from 434 specimens of *Elachistocleis* from 254 localities throughout its geographic distribution.

We examined 148 voucher specimens for two morphological characters: ventral pattern and mid-dorsal white line. Both characters vary greatly in microhylids (e.g. Peloso *et al.*, 2014: figs 10, 12) and they vary in *Elachistocleis* as well. We summarized this variation into two binary characters: ventral pattern maculate or immaculate, and mid-dorsal white line present or absent. The distinction between immaculate and maculate ventral patterns was used previously to create two groups in *Elachistocleis* (e.g. Piva *et al.*, 2017), but the terminology was inconsistent (see Discussion). We considered as maculate-bellied any specimen that presented any stains or blotches (either being dark stains over a clear background or the reverse), therefore encompassing what has been named in the literature as vermiculated, spotted, mottled, blotched and so on. On the other hand, any specimen lacking such marks was considered immaculate-bellied (see Discussion). Historic taxonomic literature of *Elachistocleis* suggested both the presence/absence and the extension of the mid-dorsal white line as diagnostic traits between species (e.g. Caramaschi, 2010). However, some degree of variation in the extension of these lines was already reported in *Elachistocleis* (e.g. Marinho *et al.*, 2018) and in other Gastrophryninae genera (e.g. Peloso *et al.*, 2014). In order to simplify our analysis, we considered only two alternative states: absent, when there was no trace of a white line; or present, when there was a white line, regardless of its extension. Specimens were examined either directly or from photographs. In addition, we measured 109 voucher specimens for three morphometric characters: snout-to-vent length (SVL),

head length (HL) and head width (HW), and the ratio HL/HW; these characters have also been used in the literature to diagnose some species of *Elachistocleis* (e.g. Caramaschi, 2010; Toledo, 2010).

Institutional acronyms of analysed specimens are: AMNH (American Museum of Natural History, New York, USA), AAG-UFU (Museu de Biodiversidade do Cerrado, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil), CAS (California Academy of Sciences, San Francisco, California, USA), CHFURG (Coleção Herpetológica da Fundação Universidade Federal Coleção Herpetológica da Fundação Universidade Federal do Rio Grande, Rio Grande, Rio Grande do Sul, Brazil), CFBH (Amphibian collection 'Célio F. B. Haddad', Departamento de Biodiversidade, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil), CNP.A (Herpetological Collection of Instituto de Diversidad y Evolución Austral, IDEAus-CONICET, Puerto Madryn, Chubut, Argentina), IIBP-H (Herpetology Collection of the Instituto de Investigación Biológica del Paraguay, Asunción, Paraguay), KU (University of Kansas Biodiversity Institute, Lawrence, Kansas, USA), KUDA (Digital Archive of the Division of Herpetology of the University of Kansas), LGE (Laboratorio de Genética Evolutiva, CONICET – Universidad Nacional de Misiones, Posadas, Misiones, Argentina), MACN (Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Buenos Aires, Argentina), MCP (Museu de Ciências e Tecnologia da PUC-RS, Porto Alegre, Rio Grande do Sul, Brazil), MNRJ (Museu Nacional, Rio de Janeiro, Brazil), MPEG (Coleção de Herpetologia do Museu Paraense Emilio Goeldi, Pará, Brazil), MLP (Museo de La Plata, La Plata, Buenos Aires, Argentina), MTR (Coleção Miguel Trefaut Rodrigues, São Paulo, Brazil), MZUSP (Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil), QCAZ (Museo de Zoología de la Pontificia Universidad Católica del Ecuador, Quito, Ecuador), UFBA (Museu de Zoologia da Universidade Federal da Bahia, Salvador, Bahia, Brazil), URCA-H (Herpetological Collection of Universidade Regional do Cariri, Crato, Ceará, Brazil), USNM (Smithsonian Institution, National Museum of Natural History, Washington D.C., USA), UWIZM (The University of the West Indies Zoology Museum, St. Augustine, Trinidad, Trinidad and Tobago) and ZUFG (Zoological Collection of the Universidade Federal de Goiás, Goiânia, Goiás, Brazil).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

DNA was extracted from frozen, ethanol-preserved samples of muscle or liver. We amplified and sequenced up to four gene fragments: two mitochondrial [the 16S ribosomal RNA subunit (16S), up to 557 bp; and the cytochrome *c* oxidase I (*COI*), up to 658 bp], and two nuclear genes [cellular myelocytomatosis proto-oncogene (*CMYC*), up to 429 bp; and seven in absentia homolog 1 (*SIAH1*), up to 397 bp]. Polymerase chain reaction (PCR) amplification and sequencing methods are those described in Lyra *et al.* (2017) and Peloso *et al.* (2014). Primers used to amplify and sequence the fragments are given in the Supporting Information, Table S1.

The molecular matrix was completed using sequences gathered from GenBank (www.ncbi.nlm.nih.gov/genbank/) (Sayers *et al.*, 2020). The complete dataset, including GenBank accession numbers and specimen data, is given in the Supporting Information, Table S2.

Not all terminals had sequences for every gene: 16S was available for 384 individuals of *Elachistocleis* and 57 of the outgroup; *COI* for 345 individuals of *Elachistocleis* and 37 of the outgroup; *CMYC* for 89 individuals of *Elachistocleis* and 16 of the outgroup; *SIAH1* for 91 individuals of *Elachistocleis* and 22 of the outgroup.

Sequences were verified, quality trimmed, assembled and aligned in GENEIOUS R7 (http://www.geneious.com/). Alignments were made separately for each gene using the MAFFT v.7.017 (Katoh, 2002) plugin implemented in GENEIOUS, with automatically chosen algorithms for each gene. Sequences were then concatenated in SEQUENCEMATRIX v.1.8 (Vaidya *et al.*, 2011).

PHYLOGENETIC ANALYSES

Phylogenetic analyses were performed using maximum likelihood (ML) in IQ-TREE v.2.0.5 (Nguyen *et al.*, 2015; Chernomor *et al.*, 2016) on XSEDE available from CIPRES Science Gateway (Miller *et al.*, 2010). Best partitioning and model selection were made by ModelFinder (Kalyaanamoorthy *et al.*, 2017) implemented in IQ-TREE. Branch supports were assessed with SH-like approximate likelihood ratio test (SH-aLRT) (Guindon *et al.*, 2010) and ultrafast bootstrap (UFBoot) (Minh *et al.*, 2013; Hoang *et al.*, 2018), both with 10 000 replicates. Clades with SH-aLRT > = 80% and UFBoot > = 95% are considered highly supported (Minh *et al.*, 2020).

The complete dataset included the four genes and all sampled species for a total of 496 terminals and 2067 characters, including the outgroup, which was composed of sequences from 62 specimens of 33 other microhylid species (see Supporting Information, Table S2). We rooted the tree with *Otophryne* Boulenger, 1900, from the subfamily Otophryninae, which are widely corroborated as the sister of Gastrophryninae (Peloso *et al.*, 2015; Hime *et al.*, 2021). The percentage of missing data, gaps and ambiguities, which are treated alike by IQ-TREE, was over 50% for 178 terminals and was 45.6% in the whole matrix.

Species delimitation

Molecular species delimitation methods (SDM) can be distance-based, which are grounded on the 'barcode gap' concept, such as the automatic barcode gap discovery (ABGD) (Puillandre *et al.*, 2012) and the 'assemble species by automatic partitioning' (ASAP) (Puillandre *et al.*, 2021), or tree-based methods, which take the phylogeny into account, such as the general mixed Yule coalescent (GMYC) (Pons *et al.*, 2006; Fontaneto *et al.*, 2007; Fujisawa & Barraclough, 2013) and the Poisson tree processes (PTP; Zhang *et al.*, 2013) and the multi-rate Poisson tree processes (mPTP; Kapli *et al.*, 2017). We performed three different types of SDM to investigate the underlying diversity of *Elachistocleis*: one distance-based (ASAP) and two tree-based (GMYC and mPTP). All SDM analyses were performed using a reduced matrix, which included only *Elachistocleis* sequences – all outgroup taxa removed. ASAP was performed separately for each mitochondrial gene, whereas GMYC and mPTP were performed using a tree made with the two concatenated mitochondrial genes.

ASAP used a pairwise genetic distance matrix as input to calculate the barcode gap. Although the K2P model for distance calculation is the most commonly used in barcode literature, there are some critiques to its use (Srivathsan & Meier, 2012; Barley & Thomson, 2016; Zinger & Philippe, 2016) with some studies finding that uncorrected distances (*p*-distances) performed equally or better than more complex models (Collins *et al.*, 2012; Srivathsan & Meier, 2012). We used MEGA v.5.2 (Tamura *et al.*, 2011) to generate genetic distances matrices with uncorrected distances (*p*-distances) with pairwise deletion, for each mitochondrial gene separate (16S and *COI*). Matrices were uploaded into ASAP graphical web-interface (https://bioinfo.mnhn.fr/abi/public/asap/) with default options. We selected the best-scoring partitions schemes (each partition is defined for a different number of candidate species) following Puillandre *et al.* (2021).

For the tree-based SDMs, we produced an ML tree in IQ-TREE from a reduced matrix. This matrix holds only *Elachistocleis* samples, using *E. araios* as the root, and with only the mitochondrial genes, 16S and *COI*, for a total of 434 terminals and 1210 characters. Overall gaps/ambiguity percentage was 20.7%, and 91 sequences contained more than 50% gaps/ambiguity. Tree search used the same settings described above. The resulting ML tree was used in the mPTP analysis. The same tree was transformed into an ultrametric timetree to be used in GMYC. For transformation, we used RelTime (Tamura *et al.*, 2012) as implemented in the time tree tool in MEGA X (Kumar *et al.*, 2018). We ran the single-threshold version of GMYC, which has been shown to outperform the multiple-threshold version (Fujisawa & Barraclough, 2013; Talavera *et al.*, 2013; Michonneau, 2015) using the SPLITS (Ezard *et al.*, 2009) package for R (R Core Team, 2020). We ran mPTP using the command line version downloaded from http://github.com/Pas-Kapli/mptp. For each analysis, we first used the '--minbr' function to calculate the minimum branch length of the tree to be taken into account in the delimitation run. We executed two independent Markov chain Monte Carlo (MCMC) runs with ten million steps, sampling at each 1000 steps and with a 1000 burn-in.

Finally, as our final species delimitation scheme, we considered not only the congruence between all SDMs, but also between them and the phylogeny (assuming species as monophyletic), and the morphology and geographic location of voucher specimens – especially from holotypes, paratypes, topotypes or specimens from vicinities of type localities.

RESULTS

Species delimitation methods (SDM)

SDM analyses resulted in a total of five species partition sets: two from ASAP with 16S, which delimited two sets with equally best scores, one from ASAP with *COI* (ASAP-*COI*), one from mPTP and one from GMYC (Fig. 1; Supporting Information, Figs S1, S2). These results suggest between four and 27 putative species in *Elachistocleis*. Both extremes, the most conservative and the most divisive schemes, were the two equally best-scored results from ASAP based on 16S (ASAP-16S-lumper and ASAP-16S-splitter) (Fig. 1; Supporting Information, Fig. S1). The other analyses resulted in intermediate numbers: mPTP delimited 19 putative species; ASAP-*COI* delimited 22 putative species; and GMYC delimited 26 putative species. Although we illustrate the partition sets side-by-side for the sake of comparison (Fig. 1), we highlight that they should not be considered equal, as ASAP analyses were based on a single gene (16S or *COI*) and do not consider the phylogeny, whereas GMYC and mPTP were based on two genes (16S and *COI*).

Elachistocleis araios and *E. panamensis*, both represented by a single terminal, were delimited equally by all analyses. Besides those two, the ASAP-16S-lumper grouped all the other terminals into two groups, corresponding to the two major clades in *Elachistocleis*, herein treated as species groups (Fig. 1). The other four delimitation sets were congruent; 18 clusters were congruently delimited by at least two analyses, whereas seven clusters were congruently delimited by four analyses. The ASAP-16S-splitter delimited several groups that were not monophyletic and several singletons. When it comes to only the tree-based analyses, which were both run using the same matrix and topology, 11 putative species were

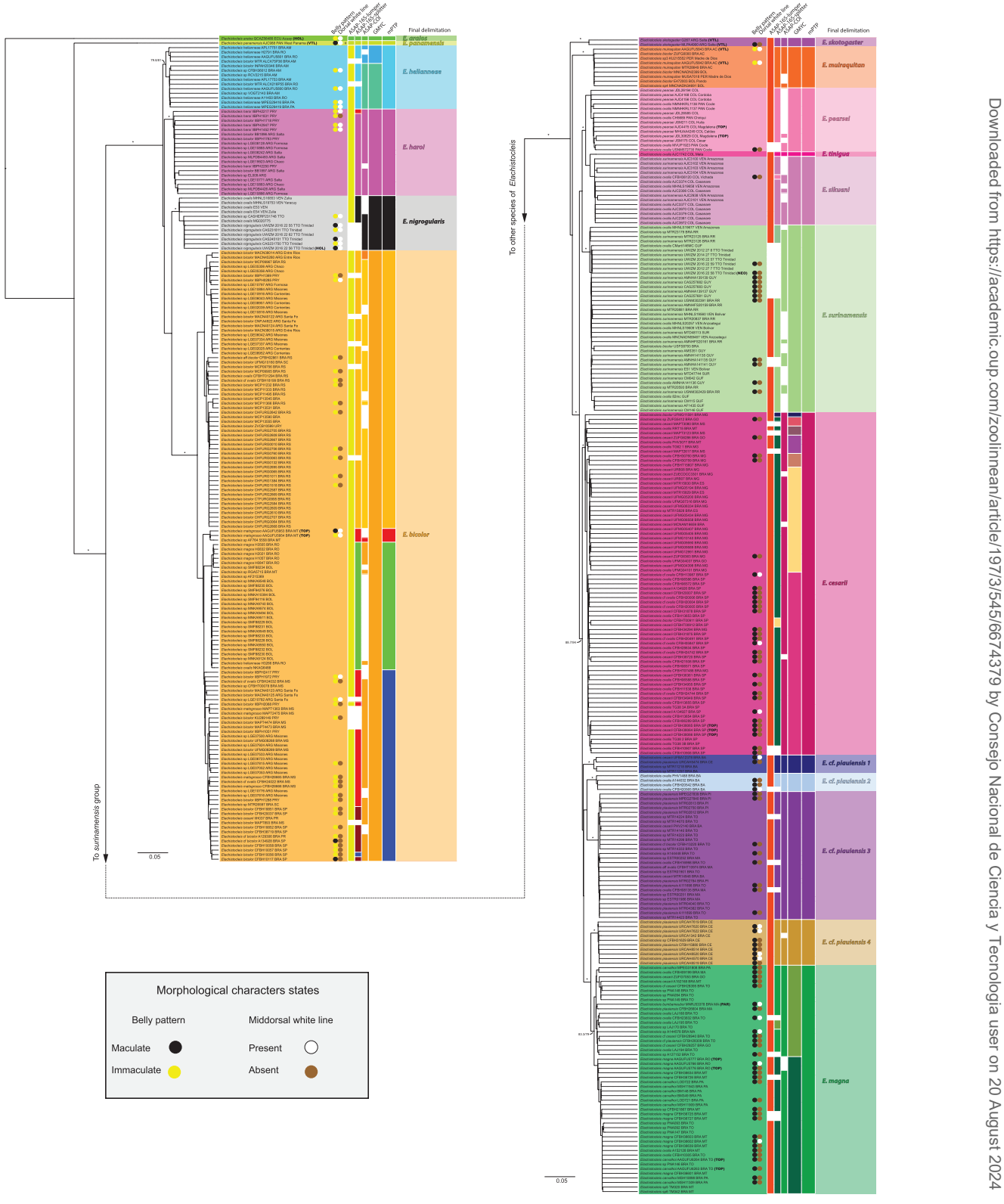

Figure 1. Species delimitation in *Elachistocleis*. The tree shown is from the maximum likelihood (ML) analysis with only 16S + *COI* from only *Elachistocleis* samples (tree rooted in *E. araios*). Numbers on branches are SH-aLRT/UFBoot support values. Asterisks represent support above 80/95. The terminals of the tree are named as: original labelling of

congruently delimited, including *E. araios* and *E. panamensis*. Regarding the incongruent groups, GMYC was usually a better splitter than mPTP, with a single exception (Fig. 1).

Original labelling of sequenced samples (i.e. as initially identified by collectors and incorporated into collection databases) did not correspond well with their phylogenetic placement. We found terminals with different *nomina* grouped in the same subclade, as well as terminals with the same *nomen* in different subclades in the phylogenetic tree (Fig. 1). This might be due to two reasons: (1) flawed morphological diagnosis of currently recognized species; and (2) misidentification of specimens, which in turn can be exacerbated when species diagnoses are dubious or when differences between species are subtle.

Nonetheless, for the integrative delimitation scheme, we were able to confidently assign samples to 14 nominal species: *E. araios*, *E. bicolor*, *E. cesarii*, *E. haroi*, *E. helianneae*, *E. muiraquitan*, *E. nigrogularis*, *E. magna*, *E. panamensis*, *E. pearsei*, *E. sikuani*, *E. skotogaster*, *E. surinamensis* and *E. tinigua*, plus four candidate new species (Figs 2, 3). These candidate new species, composed mainly of samples from north-eastern Brazil (Fig. 3), were herein labelled as *Elachistocleis* cf. *piauiensis* 1–4, but we could not confidently assign any of them to *E. piauiensis s.s.* because we did not have samples from the type locality of *E. piauiensis* (Picos, State of Piauí). Although we had samples originally labelled as *E. 'ovalis'*, we followed Caramaschi (2010), who considered *Rana ovalis* Schneider as a *nomen dubium* and *species inquirenda* and, as such, we did not assign this name to any delimited species.

Remarkably, samples of a paratype of *E. bumbameuboi* and topotypes of *E. carvalhoi* (Aragominas, State of Tocantins, Brazil) were recovered nested within samples of *E. magna*. Similarly, samples of topotypes of *E. matogrosso* (Cuiabá, State of Mato Grosso, Brazil) were recovered within *E. bicolor*. One sample from Corumbá (state of Mato Grosso do Sul, Brazil), very close (less than 20 km) to the type locality of *E. corumbaensis* (Corumbá, Parque Municipal de Piraputangas), was also found nested in *E. bicolor*, but we were not able to access the voucher to examine its morphology; its whereabouts is unclear. We make taxonomic comments and suggest actions in the Discussion below.

Phylogeny of Gastrophryninae and *Elachistocleis*

The phylogenetic analysis based on the complete matrix did not recover *Elachistocleis* as monophyletic, with *E. araios* as sister of a clade composed of *Gastrophryne* Fitzinger, 1843, *Hypopachus* Keferstein, 1867 and all other species of *Elachistocleis*. However, this arrangement received low support (Fig. 2; Supporting Information, Fig. S3). Apart from *E. araios*, all other species of *Elachistocleis* consistently form a clade (which we refer as *Elachistocleis s.s.*) with *E. panamensis* being the sister-taxon of all remaining congeners (except, of course, *E. araios*). The other species were grouped in two well-supported clades, which we label herein as the *E. bicolor* species group (composed of *E. bicolor*, *E. haroi*, *E. helianneae* and *E. nigrogularis*) and the *E. surinamensis* species group (composed of *E. cesarii*, *E. magna*, *E. muiraquitan*, *E. pearsei*, *E. piauiensis*, *E. sikuani*, *E. skotogaster*, *E. surinamensis*, *E. tinigua* and three candidate new species). Regarding *Elachistocleis* samples, the topologies in the trees with the complete and with the reduced matrices were similar, the main difference being that *E. muiraquitan* and *E. skotogaster* were recovered as sister-groups in the tree with the complete matrix, albeit with low support (Fig. 2).

The analysis did not recover the previously suggested phenetic groups, supposedly diagnosed by having immaculate-bellied or maculate-bellied species, as monophyletic (Fig. 1). Moreover, our data suggest and support an overlooked, wide, within-species variation of belly patterns and of the absence/presence of a mid-dorsal line (Fig. 1).

DISCUSSION

Species diversity in *Elachistocleis*: species complexes or complex species?

Many studies use SDMs with genetic data to identify underestimated diversity (as cryptic species) in traditionally phenotypic-based taxonomic groups (e.g. Jaramillo *et al.*, 2020; Silva *et al.*, 2020). Complementarily, other studies have shown that SDM can also aid identification in situations where species number is overestimated by phenotypic-based

sample voucher + voucher code + country code ISO 3166-1 alpha-3 + state/departament/province code (when available; for Brazilian states we used the state code). Coloured circles on the right side of the samples' names represent the two states of the characters belly pattern (black: maculate; yellow: immaculate) and mid-dorsal white line (brown: absent; white: present). Columns on the right side of the tree indicate results from the species delimitation methods analyses, named on top. On each column, bars with identical colours indicate conspecific samples according to the respective method. Blank spaces are missing samples. Background coloured squares and names on the right of the columns indicate our final species delimitation. HOL, holotype; NEO, neotype; PAR, paratype; TOP, topotype; VTL, vicinity of type locality.

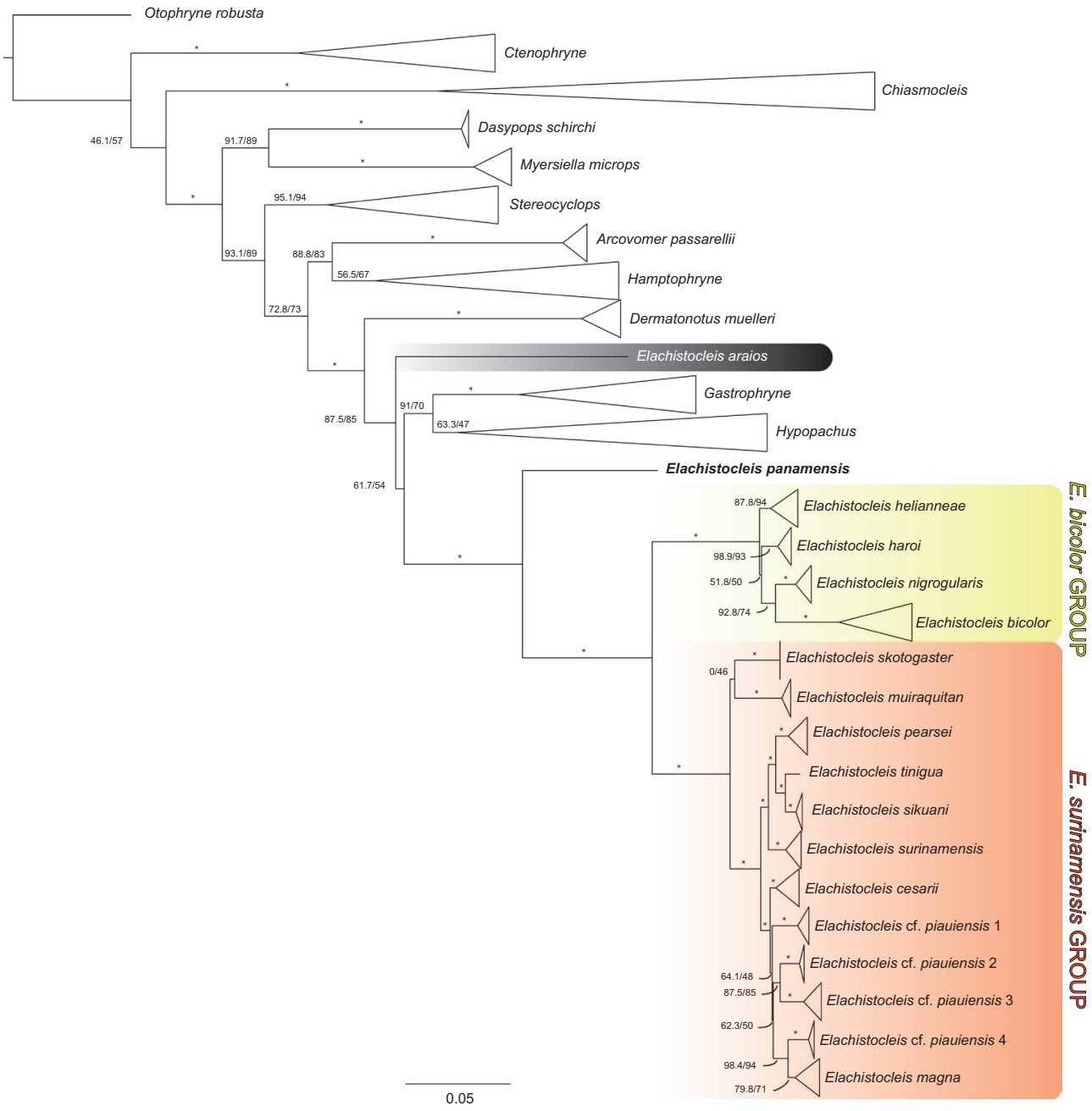

Figure 2. Phylogeny of Gastrophryninae, showing the positioning of *Elachistocleis*. Maximum likelihood (ML) tree from IQ-TREE with the complete dataset (see Material and methods). Numbers on branches are SH-aLRT/UFBoot support values. Asterisks represent support above 80/95.

taxonomy (e.g. Walther *et al.*, 2016; Correa *et al.*, 2017; Barrasso & Basso, 2019; Cassini *et al.*, 2020; Abraham *et al.*, 2021). Historically, the taxonomy of *Elachistocleis* has emphasized the importance of colour patterns – ventral pattern, mid-dorsal white line and posterior surface of the thighs – with insufficient coverage of character variation in and among populations. Nevertheless, variation in colour patterns is widely spread among amphibians (Hoffman & Blouin, 2000), including Neotropical microhylids (Peloso *et al.*, 2014). There are various cases in anuran taxonomy in which colouration characters used to diagnose new species were subsequently found to be a subset of more inclusive variation (e.g. Moore, 1942; Volpe, 1955; Correa *et al.*, 2017; Barrasso & Basso, 2019; Novaes-e-Fagundes & Solé, 2021). Our results suggest

Table 1. Average uncorrected p-distances (in percentages) between 16S (below the mid-diagonal) and COI (above the mid-diagonal) sequences of *Elachistocleis* species (as delimited in this study). Values in the mid-diagonal are the distances within groups (16S before the slash, COI after the slash). All distances were calculated with pairwise deletion in MEGA v.5.2. NC: not calculated

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	***E. araios***	NC	16.8	14.9	15.6	16.3	16.0	16.4	16.3	16.4	16.8	16.6	16.3	15.8	16.4	16.1	16.0	15.6	16.5
2	***E. panamensis***	8.3	NC	14.4	14.3	13.5	15.6	15.1	14.2	14.8	14.0	13.8	15.3	14.0	14.8	15.0	14.5	16.2	14.8
3	***E. helianneae***	11.9	8.2	0.3/0.9	4.0	5.6	8.4	12.3	13.8	12.7	11.9	12.4	12.9	12.6	12.9	13.1	13.2	13.1	13.1
4	***E. haroi***	11.3	7.8	1.0	0.3/0.6	6.0	9.2	12.6	13.8	12.1	12.3	12.9	12.8	12.3	12.8	13.1	13.2	13.2	12.3
5	***E. nigrogularis***	11.6	8.1	1.7	1.6	0.4/0.1	8.3	13.2	14.4	13.5	13.4	13.2	13.0	13.6	14.4	13.6	14.3	13.7	13.3
6	***E. bicolor***	11.6	7.4	2.3	2.0	2.4	1.3/3.4	12.9	14.2	13.4	12.5	12.6	13.1	13.2	13.3	13.1	14.4	13.1	13.1
7	***E. skotogaster***	8.9	7.1	9.0	8.7	9.0	8.3	0.0/0.0	7.3	7.0	6.7	6.7	6.5	6.8	8.4	7.7	6.6	7.5	7.5
8	***E. muiraquitan***	9.7	7.0	7.7	7.6	7.8	7.3	2.3	0.1/0.5	7.8	6.7	6.9	7.7	6.7	7.7	8.6	8.5	8.0	8.1
9	***E. pearsei***	10.3	7.0	7.4	7.4	8.0	7.3	3.7	3.0	0.4/0.7	3.9	4.0	4.4	4.6	6.3	5.7	6.3	5.9	5.9
10	***E. tinigua***	10.8	6.9	7.4	7.1	7.8	7.1	3.9	2.7	1.7	NC	2.1	4.3	4.4	6.0	5.0	5.5	5.3	5.6
11	***E. sikuani***	10.0	7.1	7.3	7.5	7.8	7.4	3.6	3.2	1.7	1.1	0.3/0.2	3.9	3.9	5.6	4.5	5.6	5.1	5.2
12	***E. surinamensis***	10.6	8.3	7.7	8.1	8.6	8.0	3.3	2.3	1.9	2.2	2.1	0.4/0.3	3.6	5.5	4.5	5.3	5.7	5.2
13	***E. cesarii***	10.3	7.0	7.1	7.2	7.4	6.9	2.6	2.0	2.3	2.5	2.4	2.1	0.5/1.0	3.7	4.0	3.9	4.6	4.9
14	***E. piauiensis 1***	10.1	6.9	6.5	6.7	7.2	6.5	3.0	2.8	3.0	2.8	2.7	2.7	1.7	0.1/1.2	5.2	5.5	5.7	5.8
15	***E. piauiensis 2***	10.6	7.0	8.0	7.7	7.9	7.7	3.1	2.6	3.0	2.7	2.6	2.6	2.0	2.4	0.0/0.3	4.8	4.7	5.1
16	***E. piauiensis 3***	9.8	6.7	7.5	7.6	8.2	7.5	3.1	2.6	2.8	2.6	2.5	2.5	1.9	2.1	1.4	0.2/0.7	5.8	6.1
17	***E. piauiensis 4***	10.2	7.5	7.4	7.5	8.1	7.4	3.1	2.6	2.7	2.9	2.8	2.4	1.4	1.9	2.1	2.0	0.0/0.4	3.4
18	***E. magna***	10.3	7.3	7.4	7.4	8.0	7.3	3.0	2.2	2.7	2.9	2.8	2.4	1.4	1.9	2.0	2.0	1.1	0.9/1.4

that rampant overlooking of character variation in *Elachistocleis* has led to much taxonomic confusion in this genus.

It is interesting to note that the mean genetic distances between *Elachistocleis araios* and the other species (16S, 8% or more; *COI*, 15% or more), between *E. panamensis* and the other species (16S, 7% or more; *COI*, 14% or more) and between species from the *E. bicolor* group and the *E. surinamensis* group (16S, 7% or more; *COI*, 12% or more) can be considered high. In contrast, the mean genetic distances between species in each of these two major clades are comparatively much lower (16S, 2% or less in the *E. bicolor* group; 16S, 4% or less in the *E. surinamensis* group; *COI*, 9% or less in both groups) (Table 1). These interspecific distances between species in the same species group are under, or close to, the limits of interspecific uncorrected genetic distance proposed for both 16S and *COI* as tentative thresholds for flagging candidate species of amphibians: Vences *et al.* (2005) proposed 5% and 10%, respectively; Fouquet *et al.* (2007) 3% for 16S; Crawford *et al.* (2010) 2% and 8%, respectively; Lyra *et al.* (2017) 3% and 6%, respectively.

One of the distance-based analyses' results, the ASAP-16S-lumper, delimited only four putative species in *Elachistocleis*: (1) *E. araios*, (2) *E. panamensis*, (3) one species corresponding to all lineages in the *bicolor* group, and (4) one species corresponding to all lineages in the *E. surinamensis* group (Fig. 1). Under this delimitation scheme, the interspecific genetic distances would fit into the proposed thresholds cited above, and the low distances might suggest that other SDMs would have recovered genetic structure between and within populations, instead of separate species. However, low genetic distances might suggest a scenario of recent and rapid speciation or with low substitution rates (Avise, 2000). It is acknowledged that the genetic distance should always be analysed in context, taking other criteria into consideration (Grant *et al.*, 2006; Funk *et al.*, 2012; Peloso *et al.*, 2014). Several studies have found lower-than-expected genetic distances between species well delimited by another set of characters (e.g. Ron *et al.*, 2006; Vieites *et al.*, 2009; Pereyra *et al.*, 2021). Even for the characters we analysed, morphological variation within the *E. bicolor* and *E. surinamensis* groups is extensive. Thus, a proposal with only four species in *Elachistocleis*, with two of them not readily diagnosable by any means other than reciprocal monophyly, does not seem adequate. Whether this large morphological variation is an effect of historical events or a result of environmental pressure remains to be determined.

Thus, the limits between these different hypotheses (distinct species vs. structured populations) should be debated on the grounds of independent evidence, such as morphology and behaviour. Unfortunately, we could not thoroughly check all voucher specimens sequenced for all traits traditionally suggested as diagnostic characters for *Elachistocleis*. We also did not check some other characters used in the taxonomy of the genus (e.g. morphology of the commissural gland and colour pattern of the thigh). However, this review is paramount. We noticed from the literature that many traits have subjective definitions (e.g. poorly developed commissural gland) or vary significantly (thigh pattern) and we were unable to define comparable states at this moment. Therefore, we focused on two more clearly defined characters, better illustrated and consistently considered relevant in the literature: ventral colour pattern and mid-dorsal white line. Thus, our taxonomic decisions are as conservative as possible, and based on the integrative analyses of the phylogeny, SDM and morphology.

TAXONOMY OF *ELACHISTOCLEIS* – A COMPLEX MATTER

Parker (1927) erected *Elachistocleis* to allocate *Rana ovalis* Schneider, 1799 and *Engystoma ovale bicolor* (Guérin-Méneville, 1838). As usual at the time, the descriptions of both species were brief and did not designate type specimens. Lavilla *et al.* (2003) suggested that these names apply to complexes of species, because species of this genus are morphologically similar, with few obvious external characters that can be used as reliable diagnostic characters (i.e. non-overlapping and fixed). Ultimately, as mentioned throughout our contribution, diagnoses in this genus rely heavily on the ventral colour pattern, head proportions and the presence/absence and extension of body lines, such as a mid-dorsal white line and lines on the hidden surface of hindlimbs (Parker, 1927; Lavilla *et al.*, 2003; Caramaschi, 2010; Pereyra *et al.*, 2013).

Caramaschi (2010) and Piva *et al.* (2017) suggested the ventral colour pattern as a diagnostic character for two internal species groups: a group with immaculate bellies (uniformly clear, free of markings) and another with maculate bellies. We found that the groups proposed by the above-cited authors are not monophyletic. For example, *Elachistocleis muiraquitan* (immaculate-bellied) is not related to the remaining immaculate-bellied species; instead, it was recovered in the *E. surinamensis* species group, a clade composed of species with predominantly maculate bellies. Furthermore, whereas *E. panamensis* has a maculate belly (Dunn *et al.*, 1948; Nelson, 1972), *E. araios* has an immaculate belly (Sánchez-Nivicela *et al.*, 2020). Given their phylogenetic position as successive sister-taxa to the remaining species of the genus (but see our comments on the position of *E. araios*), the description of *E. araios* already rendered the immaculate-bellied species group as paraphyletic. Also, it implies that the optimization of the ventral pattern of the *Elachistocleis*

ancestor is ambiguous, given our topology and that of Sánchez-Nivicela *et al.* (2020). More importantly, we found intraspecific variation of the ventral pattern in some species, such as *E. bicolor* and *E. nigrogularis* (Figs 1, 4). Ontogenetic variation of the ventral pattern has also been recently reported for *E. haroi* (Bueno-Villafañe *et al.*, 2020). Therefore, the available phylogenetic and morphological evidence emphatically rejects the existence of groups diagnosed solely by the colour of their bellies.

It is noteworthy that this discussion seems far from settled. Ventral patterns have been recorded idiosyncratically in the literature. Sánchez-Nivicela *et al.* (2020) cite *E. cesarii* as 'having uniform, immaculate, ventral colouration', although Toledo *et al.* (2010) when resurrecting the species clearly state that it has 'ventral colouration white or yellow with grey marks and reticulations', and their figure 3d depicts a specimen with a maculate belly. Jowers *et al.* (2021) state that *E. cesarii* has a 'uniform' belly. This same was said of *E. erythrogaster* and *E. bicolor*. While *E. cesarii* and *E. erythrogaster* are maculate (the latter comparatively less maculate), *E. bicolor* bellies usually present no markings whatsoever (but see discussion on *E. bicolor* below). Given the historical use of the ventral pattern in the taxonomy of *Elachistocleis*, we attempted to verify as many specimens as possible for this trait. We have not extensively checked our vouchers for other morphological diagnostic traits (male throat colour; presence and shape of the femoral stripe; post-commissural gland). Nevertheless, our preliminary data suggest that none of the above-cited morphological traits is as reliable as suggested in the literature [see also comments in Nunes *et al.* (2010) and Marinho *et al.* (2018)].

Data from other sources are scarce. Tadpoles of few species are described (e.g. Rossa-Feres & Nomura, 2006; Pereyra *et al.*, 2013; Schulze *et al.*, 2015; Ferreira & Weber, 2021), but it is difficult to confidently assign these descriptions to lineages recovered because most lack an association with available DNA sequences. Moreover, there is no comprehensive understanding of variation, although Gómez & Kehr (2012) reported some variability in larval morphology related to chemical cues in the presence of predators. Advertisement calls of several *Elachistocleis* have been described (e.g. Nelson, 1972; Duellman, 1997; Kwet & Di-Bernardo, 1998; Lavilla *et al.*, 2003; Nunes *et al.*, 2010; Toledo, 2010; Marinho *et al.*, 2018; Pansonato *et al.*, 2018; Jowers *et al.*, 2021). Some of these are associated with vouchers present in our phylogeny (see: Marinho *et al.*, 2018; Jowers *et al.*, 2021). These are not adequately distributed in our topology and most are described from a few specimens and localities. Published data have already shown that even slight increases in sample size may affect recorded variation (see: Marinho *et al.*, 2018). Finally, some localities present at least two sympatric species of *Elachistocleis*. For instance, both *E. nigrogularis* and *E. surinamensis* are syntopically found at the type locality of *E. nigrogularis* (Jowers *et al.*, 2021), and both *E. bicolor* and *E. erythrogaster* are found at the type locality of *E. erythrogaster* (Kwet & Di-Bernardo, 1998) (also see Fig. 3). This makes it even more difficult to associate larvae with adults and advertisement calls of unvouchered specimens to named species without molecular data.

A fine line

A white or light yellow mid-dorsal line is present in many individuals of *Elachistocleis*, and other genera of Gastrophryninae, such as *Chiasmocleis* Méhelÿ, 1904 (e.g. Peloso *et al.*, 2014), *Ctenophryne* Mocquard, 1904 (e.g. Duellman, 1978), *Hamptophryne* A.L.Carvalho, 1954 (e.g. Parker, 1927; Duellman, 1978), *Hypopachus* [e.g. Cope (1889); see also figure 5 in Greenbaum *et al.* (2011)], *Dasypops* Miranda-Ribeiro, 1924 and *Stereocyclops* Cope, 1870 (PP, personal observation). Many authors have noticed important intraspecific or intrapopulational variation (i.e. being present in some individuals and absent in others) in this character in other genera (e.g. *Chiasmocleis*, Peloso *et al.*, 2014; *Ctenophryne*, Zweifel & Myers, 1989; *Stereocyclops*, GNF, personal observation) and in *Elachistocleis* (e.g. Nelson, 1972; Toledo, 2010; Toledo *et al.*, 2010; Marinho *et al.*, 2018). Notwithstanding, the occurrence of this line and the variation of its extension when present (e.g. from snout to vent or from post-cephalic fold to vent) were used in diagnoses of several *Elachistocleis* species (e.g. Caramaschi & Jim, 1983; Caramaschi, 2010; Nunes-de-Almeida & Toledo, 2012; Piva *et al.*, 2017).

We found a remarkable variation in the occurrence of the mid-dorsal white line in and among species (Fig. 1), similar to the variation reported for *Chiasmocleis* (Peloso *et al.*, 2014). This variation challenges the reliability of the trait as a diagnostic feature and weakens the known diagnoses of several currently recognized species (see below). It was impossible to confidently evaluate the extension of the mid-dorsal line, thus we only scored the presence or the absence of the line. Hence, we recommend caution when using the mid-dorsal line in the systematics of *Elachistocleis*, especially for diagnostic purposes.

Taxonomic comments and actions

Our phylogenetic and species delimitation analyses contrasted with information available in the literature (see discussion above), and their integration with our evaluation of phenotypic data, suggest that the taxonomy of *Elachistocleis* is in urgent need of revision. The evidence

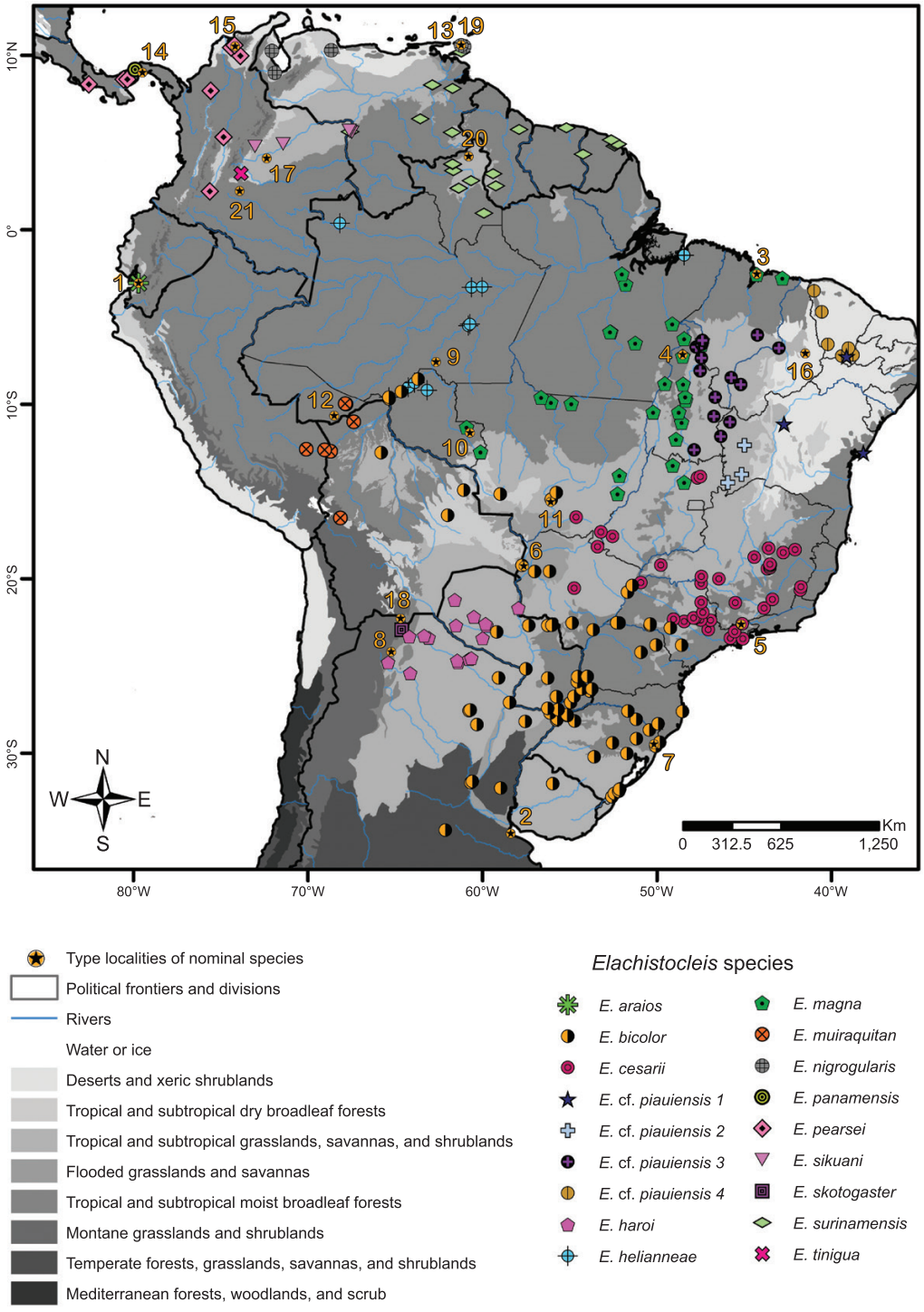

Figure 3. Geographic location of sampled specimens (symbols) and type-localities of the nominal species (numbers). 1, *Elachistocleis araios*; 2, *E. bicolor* (here illustrated as the city of Buenos Aires; see Discussion); 3, *E. bumbameuboi*; 4, *E. carvalhoi*; 5, *E. cesarii*; 6, *E. corumbaensis*; 7, *E. erythrogaster*; 8, *E. haroi*; 9, *E. helianneae*; 10, *E. magna*; 11, *E. matogrosso*; 12, *E. muiraquitan*; 13, *E. nigrogularis*; 14, *E. panamensis*; 15, *E. pearsei*; 16, *E. piauiensis*; 17, *E. sikuani*; 18, *E. skotogaster*; 19, *E. surinamensis*; 20, *E. surumu*; 21, *E. tinigua*.

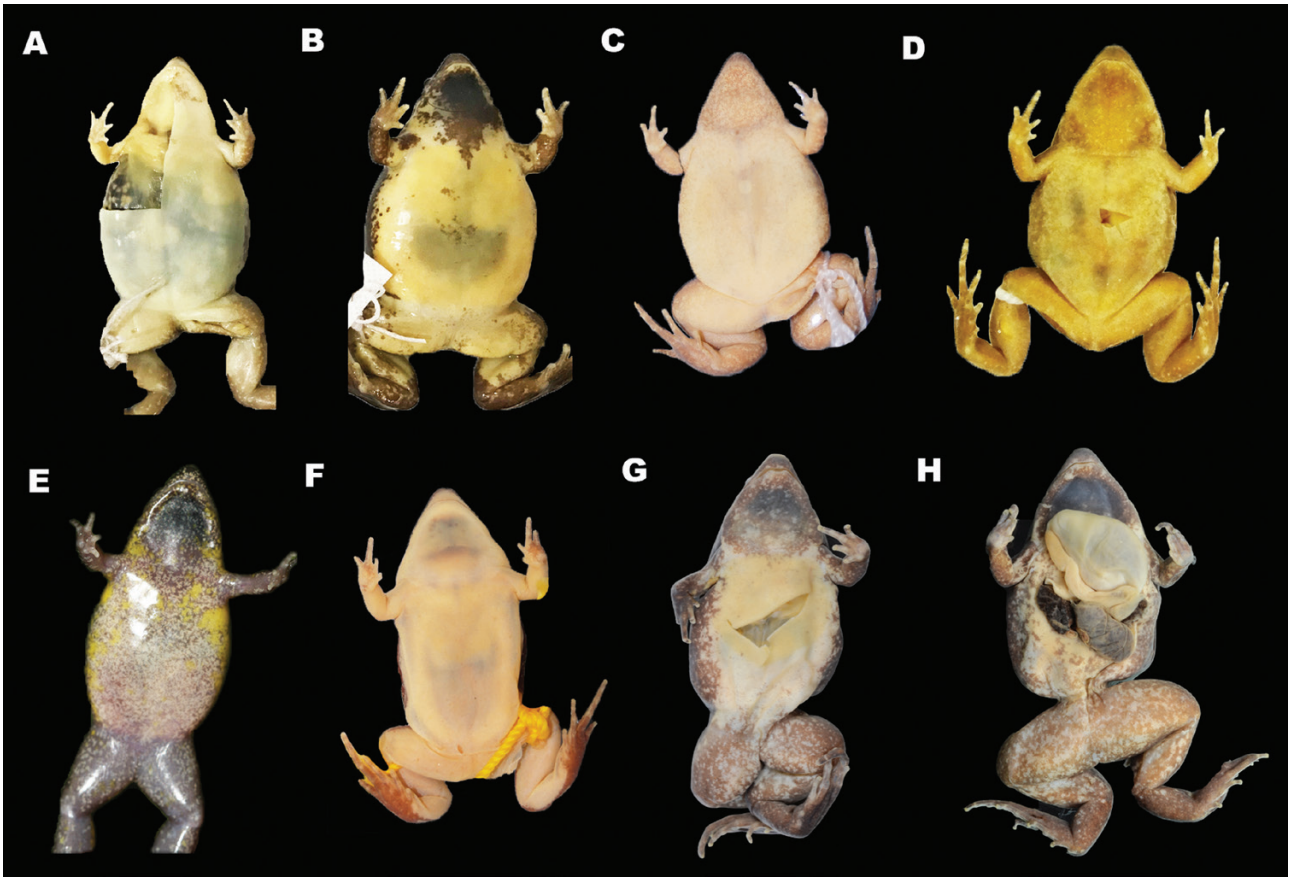

Figure 4. Variation of ventral colour pattern among sample vouchers of different species of *Elachistocleis* of the *bicolor* group. A, B, *E. helianneae*; MPEG 29416, 29419. C, *E. haroi*; IIBPH 2947. D–F, *E. bicolor*; CFBH 10117, AAGUFU 5953, IIBPH 0285. G, H, *E. nigrogularis*; CAS 231811, 245101.

gathered here is sufficient to solve some taxonomic problems, but certainly not all. Below, we discuss some of these problems and, where possible, propose solutions to obvious and immediately fixable taxonomic issues.

As stated above, we recognize two species groups in *Elachistocleis*: the *E. bicolor* and the *E. surinamensis* groups. At this point, *E. araios*, *E. panamensis*, *E. corumbaensis* and *E. erythrogaster* are left unassigned to either group, whereas the status of the former as a member of *Elachistocleis* is questionable.

The *Elachistocleis bicolor* group

We recognize four nominal species in the *E. bicolor* group: *E. bicolor*, *E. haroi*, *E. helianneae* and *E. nigrogularis*. Except for *E. nigrogularis*, species of this group predominantly have an immaculate belly, but see comments below.

Elachistocleis matogrosso *Caramaschi, 2010 as junior subjective synonym of* Elachistocleis bicolor *(Guérin-méneville, 1838)*

The two topotypical samples of *E. matogrosso* were recovered in *E. bicolor* (Fig. 1). Although some SDM analyses grouped those samples as a different putative species (see ASAP-16S-splitter and mPTP, Fig. 1), their morphological variation falls in what we observed for *E. bicolor*, as delimited herein. Caramaschi (2010) distinguished *E. bicolor* and *E. matogrosso* by subtle differences in four morphological traits: head proportions (HL/HW about 0.92 in *E. matogrosso* vs. HL/HW below 0.90 in *E. bicolor*); the mid-dorsal white line (present in *E. matogrosso* vs. absent in *E. bicolor*); loreal region colouration (same grey colour of dorsum in *E. matogrosso* and white in *E. bicolor*); and shape of the femoral line (broad in *E. matogrosso* vs. thin in *E. bicolor*). None of these differences listed by Caramaschi (2010) holds for the voucher specimens we examined. All are in the range of variation of those characters: the HL/HW ratio varies significantly from 0.76 to 0.93 (N = 24), and the specimens from Cuiabá (type locality of *E. matogrosso*) have the ratio of 0.84 and 0.88; the mid-dorsal line may be present or absent (Fig. 1); all vouchers have the loreal region with the same colour of the dorsum, which varies

from shades of grey to brown; the femoral line is usually thin, but at least one voucher from Argentina (LGE 10782) has a broad line, as does one voucher from Cuiabá originally assigned to *E. matogrosso* (AAG-UFU 5954). Therefore, due to its phylogenetic position, and genetic and morphological similarity, we formally propose that *Elachistocleis matogrosso* Caramaschi, 2010 is a junior subjective synonym of *Elachistocleis bicolor* (Guérin-Méneville, 1838).

Elachistocleis bicolor has a long and complex nomenclatural history (for a summary and references see: Frost, 2022). Currently, *E. bicolor* is recognized as a species with an immaculate yellow venter and dorsum lacking the mid-dorsal white line (Lavilla *et al.*, 2003; Caramaschi, 2010). Lavilla *et al.* (2003) argued that the type locality of *E. bicolor* is Buenos Aires, Argentina; although it is not clear if they were referring to the city of Buenos Aires or the Province of Buenos Aires. Our conclusions would not change either way, as all the nearest samples to both regions belong to the same species (Figs 1, 3).

It is important to remark that, although most of the vouchers we examined indeed fit into the current understanding of *E. bicolor* morphology, some of them do not. Several specimens have the mid-dorsal line, others have the venter with a finely spotted pattern and one specimen (AAG-UFU 5953) has both a dorsal line and a maculate belly (Figs 1, 4E). Even though the ventral colour pattern of this specimen is not as evident as the pattern seen in specimens of the *E. surinamensis* group, it is certainly not equal to the uniformly immaculate pattern historically described for *E. bicolor*. What is even more remarkable is that another syntopic and genetically identical (p-distance = 0.0%) specimen (AAG-UFU 5954) has the classical immaculate yellow belly (Marinho *et al.*, 2018: fig. 6). We note that ontogenetic variation has been recorded for the ventral colour pattern in at least one species (*Elachistocleis haroi*; Bueno-Villafañe *et al.*, 2020). Juveniles and subadults of *E. haroi* present a venter translucent grey with small black specks and white stains, while the yellow colouration develops gradually with growth and sexual maturation of the individuals (see: Bueno-Villafañe *et al.*, 2020: fig. 1). Curiously, the three vouchers of *E. bicolor* with spotted venters are one juvenile and two small-sized males (SVL ≈ 23 mm). This, of course, is not indisputable evidence of a consistent pattern (the other two smaller males have uniform immaculate yellow venter), but certainly requires further investigation.

THE *ELACHISTOCLEIS SURINAMENSIS* GROUP

We recognize nine nominal species (*E. cesarii*, *E. magna*, *E. muiraquitan*, *E. pearsei*, *E. piauiensis*, *E. sikuani*, *E. skotogaster*, *E. surinamensis* and *E. tinigua*) and three candidate new species in the *E. surinamensis* group. All of them have predominantly maculate bellies, except *E. muiraquitan*.

Elachistocleis surumu *Caramaschi, 2010 as a junior subjective synonym of* Elachistocleis surinamensis *(Daudin, 1802)*

Although we do not have samples from the type locality of *E. surumu* (Vila Surumu, Municipality of Pacaraima, State of Roraima, Brazil), we sampled many specimens from nearby localities surrounding the type locality (Fig. 3) and all of them are recovered in a single lineage that is widespread in the eastern portion of the Guiana Shield (Figs 1, 3). The proposed morphological differences between *E. surumu* and *E. surinamensis* are subtle. Caramaschi (2010) differentiated *E. surumu* from *E. surinamensis* based on the alleged presence of a light vertebral stripe in *E. surinamensis* (absent in *E. surumu*) and minor details in the ventral colour patterns. However, neither the original description of *E. surinamensis* (Daudin, 1802) nor the recent species redescription and neotype designation (Jowers *et al.*, 2021) stated that *E. surinamensis* has any kind of dorsal stripe or line. Caramaschi (2010) did not list any voucher specimens of *E. surinamensis* in his list of examined material. Ventral colour patterns of *E. surumu* are contained in the variation found herein for specimens of *E. surinamensis* (see also: Jowers *et al.*, 2021). Therefore, we consider *Elachistocleis surumu* Caramaschi, 2010 as a junior subjective synonym of *Elachistocleis surinamensis* (Daudin, 1802).

Elachistocleis bumbameuboi *Caramaschi, 2010 and* Elachistocleis carvalhoi *Caramaschi, 2010 as junior subjective synonyms of* Elachistocleis magna *Toledo, 2010*

Caramaschi (2010) distinguished *E. bumbameuboi* and *E. carvalhoi* from *E. magna* based on subtle differences in the dorsal and ventral colour patterns, as follows: regarding the dorsal pattern, *E. magna* has 'dorsum uniform dark greyish with scarce minute brighter dots in the outer boundaries', whereas *E. bumbameuboi* and *E. carvalhoi* have, respectively, 'dorsum uniformly dark grey or black without marks' and 'dorsum uniformly brown or dark grey without marks'; regarding the ventral pattern, *E. magna* has 'venter grey with minute scattered white spots, mainly on the belly and ventral surfaces of legs', whereas *E. bumbameuboi* and *E. carvalhoi* have, respectively, 'venter grey with minute anastomosed whitish spots, producing a salt-and-pepper pattern' and 'venter greyish with large anastomosed yellow or whitish

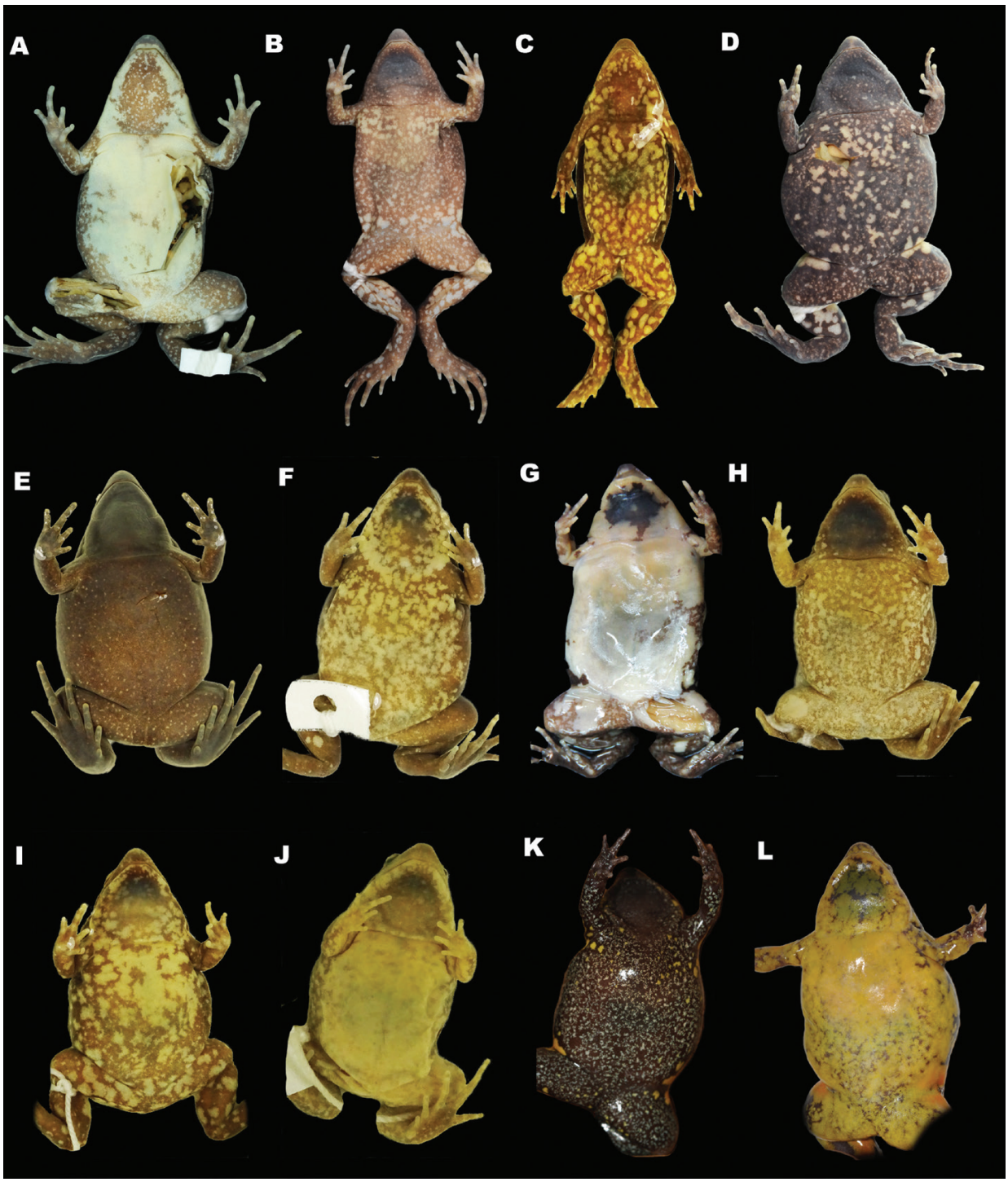

Figure 5. Variation of ventral colour pattern among sample vouchers of different species of *Elachistocleis* of the *surinamensis* group. A, *E. muiraquitan*; KU 215552 (image KUDA 013705; photo by Ana Motta). B, *E. pearsei*; USNM 572735. C, *E. sikuani*; CFBH 36120. D, *E. surinamensis*; CAS 257681. E, F, *E. cesarii*; CFBH, 31875, 38066. G, *E.* cf. *piauiensis* 1; URCAH 5474. H, *E.* cf. *piauiensis* 2; CFBH 20565. I, *E.* cf. *piauiensis* 3; CFBH 16998. J, *E.* cf. *piauiensis* 4; CFBH 31629. K, L, *E. magna*; AAGUFU 5766, 6264. Photos not to scale.

yellow blotches, producing a coarse marbled pattern, mainly in the chest area.

Photographs of the holotypes of *E. carvalhoi* (Caramaschi, 2010: fig. 3) and *E. bumbameuboi* (Caramaschi, 2010: fig. 4) and a photograph of a paratype of *E. bumbameuboi* (Nunes *et al.*, 2010: fig. 1) depict specimens with dorsa scattered with minute white dots. Thus, our examination of specimens of *E. bumbameuboi*, *E. carvalhoi* and *E. magna* show a broad overlap among the various shades of dorsal colour and the size and distribution pattern of ventral markings (e.g. see Fig. 5).

Based on our phylogenetic hypothesis, on the broad geographic sampling, which included a paratype of *E. bumbameuboi*, topotypes of *E. carvalhoi* and *E. magna*, and on the examination of several voucher specimens, we consider that *E. bumbameuboi* Caramaschi, 2010 and *E. carvalhoi* Caramaschi, 2010 are junior subjective synonyms of *E. magna* Toledo, 2010.

The status of Elachistocleis pearsei, E. surinamensis, E. sikuani *and* E. tinigua

Elachistocleis surinamensis and *E. pearsei* are the oldest available names in this group. The former was named by Daudin (1802) as *Bufo surinamensis*, and the latter was named by Ruthven (1914) as *Hypopachus pearsei*. Both were considered synonyms of *Elachistocleis ovalis* by Parker (1934). Dunn (1944; 1949) commented on *H. pearsei* under the new combination *Elachistocleis pearsei*. Carvalho (1954) placed it into the new genus *Relictivomer* Carvalho, 1954 based on the presence of the posterior part of the prevomer and, recently, de Sá *et al.* (2012) reallocated it into *Elachistocleis* based on DNA data. Kenny (1969) redescribed *E. surinamensis* from specimens from Trinidad and cited a personal communication from A. Grandison, who had found no differences between specimens of *E. surinamensis* from Trinidad and a paratype of *E. pearsei*. Duellman (1997) also compared specimens of *E. pearsei* from Panama and specimens of *Elachistocleis* sp. from Gran Sabana, south-eastern Venezuela (which he did not assign to any species, but which falls in the range of occurrence of *E. surinamensis*; see Fig. 3) and did not find any noticeable difference in the external morphology of adults or in their advertisement calls. Jowers *et al.* (2021) recently redescribed and designated a neotype for *E. surinamensis* from Trinidad but, unfortunately, provided no comparisons of the species with *E. pearsei*.

While the present paper was in review, two new species from Colombia were described and named: *Elachistocleis sikuani* and *E. tinigua* (Acosta-Galvis *et al.*, 2022). Although we did not access the vouchers and did not include the new sequences generated therein in our analyses, we confirmed that two lineages already represented in our tree correspond to them (based on genetic similarity, 99.8%). The authors made a brief comparison of the new species with *E. pearsei* and *E. surinamensis*, mentioning some differences in body size, quantity and colour of the blotches in live specimens, degree of conspicuousness of the post-commissural glands and presence/absence of the mid-dorsal white line (Acosta-Galvis *et al.*, 2022). The latter feature was reported as absent in *E. sikuani* and *E. tinigua* but present ('evident') in *E. surinamensis*. However, as mentioned above in discussion about the variation of *E. surumu*, the presence of a mid-dorsal white line in *E. surinamensis* was not reported in its original description (see: Daudin, 1802) or in its redescription and neotype designation (see: Jowers *et al.*, 2021). On the contrary, Jowers *et al.* (2021) used the absence of the mid-dorsal line in *E. surinamensis* to distinguish it from *E. nigrogularis*. We also have not observed a mid-dorsal white line in any examined specimens of *E. surinamensis* (Fig. 1).

Acosta-Galvis *et al.* (2022) did not provide a comparison between *E. pearsei* and *E. surinamensis*. Instead, they added more pieces to the puzzle by giving new names to two allopatric lineages. They followed the prevailing tradition in the taxonomy of *Elachistocleis* of creating more and more nomina rather than conducting a thorough review addressing the validity and application of available names. We reiterate that a comprehensive morphological revision of *Elachistocleis* is needed and should take priority over the naming of additional new *Elachistocleis* species. Unfortunately, our sampling of specimens of those species is limited, and a reassessment of the taxonomic status of those species is beyond the scope of the present work. Therefore, for time being, we recognize *E. pearsei*, *E. surinamensis*, *E. sikuani* and *E. tinigua* as valid species.

The status of Elachistocleis cesarii, E. piauiensis *and* E. magna

Miranda-Ribeiro (1920) described two subspecies of *Engystoma ovale* based on maculate-bellied specimens from the state of São Paulo, Brazil, which were later synonymized by Parker (1934) with *Elachistocleis ovalis*. Toledo *et al.* (2010) recently resurrected and redescribed them under the new combination *Elachistocleis cesarii*. Herein we recognize *E. cesarii* as a species distributed in south-eastern and central-western Brazil (Fig. 3) and closely related to *E. piauiensis* and *E. magna* (Fig. 1).

Elachistocleis piauiensis was described and named by Caramaschi & Jim (1983) from Picos, Piauí. We found four lineages distributed in north-eastern Brazil, but

we could not confidently assign any to *E. piauiensis*, because we did not have access to data from the type locality. Specimens assigned to each of these four lineages are morphologically similar to each other and to the original species description of *E. piauiensis*. The ventral colour pattern varies widely and similarly along the clade composed of *E. cesarii*, *E. magna* and the four candidate species *E.* cf. *piauiensis* 1–4, with no clear difference between them. The presence/absence of the mid-dorsal line also varies, except for *E.* cf. *piauiensis* 3, in which all examined vouchers lack the line. The holotype of *E. piauiensis* also lacks the line, and this could be evidence to assign the name to this specific clade. However, we cannot rule out that the consistent absence in *E.* cf. *piauiensis* 3 is not an effect of our sampling, given the high variability of this character state in several of our delimited species (Fig. 1).

Elachistocleis cesarii was distinguished from *E. piauiensis* by differences in the advertisement call, size of the post-commissural gland and details of the inguinal region colouration (Toledo *et al.*, 2010) – phenotypic traits that we have not explored for the present contribution. On the other hand, *E. magna* was distinguished from *E. cesarii* and *E. piauiensis* solely by its size (male SVL above 31 mm in *E. magna*, against SVL below 29 mm in *E. cesarii* and *E. piauiensis*; Toledo, 2010). Among examined specimens, we observed a consistent difference in size between *E. magna* and *E.* cf. *piauiensis* 1–4, but not between *E. magna* and *E. cesarii*. Males of *E. cesarii* can have SVL up to 31 mm, whereas some males of *E. magna* have SVL below 29 mm. Therefore, a thorough evaluation of phenotypic data allied with a denser molecular sampling is needed to verify if the differential diagnoses among *E. cesarii*, *E. piauiensis* and *E. magna* will hold true with additional data.

Species not sampled

The (mysterious) status of Elachistocleis corumbaensis

Unfortunately, we have no samples confidently assignable to *E. corumbaensis*. We have two samples from two localities near (around 20 km and 80 km) but not from the type locality of the species (Parque Municipal de Piraputangas, Corumbá municipality; Piva *et al.*, 2017) (Fig. 3). For one of these tissue samples (CFBHT 00078), we were unable to find the voucher specimen. The other (MAPT 1363) was identified by the collectors as *E. matogrosso* (see: Koroiva *et al.*, 2020). So we assume, albeit tentatively, that it has an immaculate venter. Both samples fall with *E. bicolor* in our species delimitation (Fig. 1). However, *E. corumbaensis* was described as having a maculate belly (Piva *et al.*, 2017). The closest locality that we have a sample of a maculate-bellied species (*E. cesarii*) is Campo Grande, state of Mato Grosso do Sul, Brazil, 357 km from the type locality of *E. corumbaensis*. It is also worth remarking that *E. corumbaensis* has several similarities with *E. haroi*, particularly the dorsal pattern with a dark mark in the shape of a pine tree (Pereyra *et al.*, 2013; Piva *et al.*, 2017). Our closest sample of *E. haroi* is from Puerto Carmelo Peralta, Department of Alto Paraguay (Paraguay), 275 km south of the type locality of *E. corumbaensis*. For the moment, we consider *E. corumbaensis* a valid species, although it is unclear if this species belongs to the *E. bicolor* group or to the *E. surinamensis* group.

The (unresolved) status of Elachistocleis erythrogaster

This species is apparently restricted to the Centre of Research and Nature Conservation Pró-Mata, state of Rio Grande do Sul, above 900 m a.s.l. (Kwet & Di-Bernardo, 1998). It was distinguished from *E. bicolor* [treated as *E. ovalis* in Kwet & Di-Bernardo (1998)], which occurs in sympatry, by the unique red belly, larger size and other bioacoustic and reproductive characteristics (Kwet & Di-Bernardo, 1998). We have sampled some individuals from localities close to the type locality – although from the lowland – and they all fall with *E. bicolor*. This species has not been collected for nearly 20 years and is considered to be rare (Kwet *et al.*, 2010).

The (unsettling) status of Elachistocleis ovalis

There has been a long controversy regarding the validity and applicability of this *nomen*, especially regarding whether it should be applied to immaculate or to maculate-bellied specimens of *Elachistocleis* [see Lavilla *et al.* (2003) and Caramaschi (2010) for lengthy discussions on the issue]. While some authors (e.g. Parker, 1927; Dunn, 1949; Kenny, 1969; De la Riva *et al.*, 2000) applied this name to maculate-bellied specimens – thus conveniently distinguishing them from the immaculate-bellied *E. bicolor* – others did not. In a meeting abstract of his unpublished dissertation, Carcerelli (1992) regarded *E. bicolor* a junior synonym of *E. ovalis*; a view that was followed by a few other authors (e.g. Klappenbach & Langone, 1992; Olmos & Achaval, 1997; Kwet & Di-Bernardo, 1998). That decision was mainly grounded on the fact that Schneider (1799) stated that *Rana ovalis* had an '*inferne flavidus*' (yellow venter). Lavilla *et al.* (2003) also remarked that, considering Schneider's description, *E. ovalis* should be applied to the specimens with 'immaculate, yellow, ventral colouration'; but they did not adhere to the idea of synonymizing *E. ovalis* and *E. bicolor*.

Instead, they regarded *E. ovalis* as restricted to the 'northern portion of the generic range', whereas *E. bicolor* would be restricted to the 'southern portion of the generic range'.

Nevertheless, ventral patterns have been recorded idiosyncratically in the literature and 'uniform', 'yellow' and 'immaculate' bellies have often been treated as synonyms, although this is not necessarily the case. Some individuals of typically maculate-bellied species can present the venter almost entirely covered by yellow blotches (e.g. Fig. 5G, J, L) and thus could also fit into the '*inferne flavidus*' description. Thus, assuming that the type of *E. ovalis* had an immaculate venter, because Schneider (1799) stated that it has '*inferne flavidus*', is not solid reasoning in itself. Moreover, as described above for our samples from Cuiabá (AAG-UFU 5953 and AAG-UFU 5954), sympatric specimens may present different ventral patterns and still be genetically similar, increasing the complexity of this puzzle.

The identity of *E. ovalis* is still a conundrum, especially difficult to solve due to the lack of a type locality and the unknown whereabouts of the type specimen. For these reasons, among others, Caramaschi (2010) proposed an operational solution: he considered *Rana ovalis* Schneider, 1799 and the combination *Elachistocleis ovalis* a *nomen dubium* associated with a *species inquirenda*; a decision that was followed by Jowers *et al.* (2021). Since we could not find any new evidence on the contrary, we also adhere to that decision by Caramaschi (2010).

AmphibiaWeb (2022) and Frost (2022), the two largest amphibian taxonomic catalogues, have essentially also followed this suggestion and, although both still include this species in their list of 22 recognized *Elachistocleis* species, the following notes are added for *E. ovalis*: 'Nominally, *Elachistocleis ovalis* is a *nomen inquirenda* (see comment), not applied to a biological population. But, pending revision, this name is applied to populations from Bolivia, although a great deal of confusion surrounds the identification of specimens mentioned from other countries' (Frost, 2022); 'This ancient name (1799) lacks type material and there is no type locality. Despite wide usage it should be considered an invalid name and not used [...] Pending revision, AmphibiaWeb continues to use the name *E. ovalis*' (AmphibiaWeb, 2022).

Gastrophyninae phylogeny and the paraphyly of *Elachistocleis*

Phylogenetic relationships between the genera of Gastrophryninae recovered here are similar to the results published by Tu *et al.* (2018), Hime *et al.* (2021) and Sánchez-Nivicela *et al.* (2020). The most remarkable exception is undoubtedly the unexpected position of *Elachistocleis araios*. We did not recover *Elachistocleis* as monophyletic, as *E. araios* was placed as the sister-taxon of a clade composed of *Gastrophryne*, *Hypopachus* and *Elachistocleis s.s.*. At a first glance this result seems to contradict previous studies that recovered *E. araios* as sister to all other species of *Elachistocleis*, with high support (Sánchez-Nivicela *et al.*, 2020; Jowers *et al.*, 2021). However, it is important to highlight some key differences between these studies and ours. First, we have a much larger sampling of *Elachistocleis* than either the above-cited studies. Second, we have a much larger outgroup sampling than Jowers *et al.* (2021). Third, inasmuch as we expanded the matrix in taxon sampling, we have a smaller sampling of characters – our dataset lacks the mitochondrial gene 12S and the nuclear gene tyrosinase, both sampled by the above-cited studies. It is well known that both character and taxon sampling may strongly affect the topology and support of phylogenetic trees (Graybeal, 1998; Pollock *et al.*, 2002; Zwickl & Hillis, 2002; Rosenberg & Kumar, 2003; Rokas & Carroll, 2005; Nabhan & Sarkar, 2012; de Sá *et al.*, 2014; Peloso *et al.*, 2015; Grant, 2019). It is also known that extensive character sampling with poor taxon sampling may lead to highly supported incorrect trees (Hillis *et al.*, 2003; Soltis *et al.*, 2004; Hedtke *et al.*, 2006; Peloso *et al.*, 2015). Notice, for instance, that the Sánchez-Nivicela *et al.* (2020: fig. 1) tree with complete dataset (302 terminals) has much less support for *E. araios* + other *Elachistocleis* than the trees with reduced datasets (20 terminals; Sánchez-Nivicela *et al.* 2020: fig. 2). Even though Jowers *et al.* (2021) did have a slightly larger sampling of ingroup (*Elachistocleis*) in comparison with that of Sánchez-Nivicela *et al.* (2020), their outgroup sampling was limited to only two samples of other gastrophrynines (see: Jowers *et al.*, 2021: fig. 2), and rooted in the clade composed of these two samples – it, therefore, cannot be viewed as a meaningful test of the monophyly of *Elachistocleis*.

We highlight that the high support for the monophyly of *Elachistocleis* in those two studies should, therefore, be interpreted with caution. Nevertheless, erecting a new genus to accommodate *E. araios* does not seem reasonable at present, given that our analyses were not designed to test the monophyly of *Elachistocleis*. We believe that an analysis with extensive taxon and character sampling is better suited to address this issue.

CONCLUSION

The phylogenetic hypothesis and a set of species proposed herein challenge the current systematic and

taxonomic knowledge of *Elachistocleis*. Additionally, traditional characters used in the taxonomy of this group for decades (belly colour pattern and dorsal lines) were shown to carry less diagnostic information than previously perceived. It is now clear that, despite some corrections to the current classification, a comprehensive review of phenotypic characters is urgently needed for *Elachistocleis*. Moreover, we recommend that a broad, integrative study must precede any new species descriptions in the group, especially if diagnoses are based on the characters traditionally employed for its taxonomy. Nonetheless, our study suggests that additional species may still need formal recognition in the future.

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DATA AVAILABILITY

Sequences used in this study are available on GenBank. See Table S2 for accession numbers.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Primers used in this study.
Table S2. GenBank accession numbers, locality information and final species delimitation of sequences used in this study. Boldface numbers are newly generated sequences for the present contribution.
Figure S1. Output graphs of ASAP with 16S. A, distribution of pairwise distances. B, ranked pairwise distances. C, partition schemes of species hypothesis and ultrametric clustering tree.
Figure S2. Output graphs of ASAP with *COI*. A, distribution of pairwise distances. B, ranked pairwise distances. C, partition schemes of species hypothesis and ultrametric clustering tree.
Figure S3. Uncollapsed phylogenetic tree of Gastrophryninae. ML tree from IQ-TREE with the complete dataset (see Material and Methods). Numbers on branches are SH-aLRT/UFBoot support values.