Phylogenetic relationships and cryptic species diversity in the Brazilian egg-brooding tree frog, genus *Fritziana* Mello-Leitão 1937 (Anura: Hemiphractidae)

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ABSTRACT

The genus *Fritziana* (Anura: Hemiphractidae) comprises six described species (*F. goeldii, F. ohausi, F. fissilis, F. ulei, F. tonimi,* and *F. izecksohni*) that are endemic to the Brazilian Atlantic Forest. Although the genus has been the subject of studies dealing with its taxonomy, phylogeny, and systematics, there is considerable evidence for cryptic diversity hidden among the species. The present study aims to understand the genetic diversity and phylogenetic relationships among the species of *Fritziana*, as well as the relationships among populations within species. We analyzed 107 individuals throughout the distribution of the genus using three mitochondrial gene fragments (*12S, 16S,* and *COI*) and two nuclear genes (*RAG1* and *SLC8A3*). Our data indicated that the species diversity in the genus *Fritziana* is underestimated by the existence of at least three candidate species hidden amongst the group of species with a closed dorsal pouch (i.e. *F. fissilis* and *F. ulei*). We also found four species presenting geographical population structures and high genetic diversity, and thus require further investigations. In addition, we found that two candidate species show a new arrangement for the *tRNA-Phe* gene, unique in Anura so far. Based on our results, we suggest that the conservation status of the species, as well as the species diversity in the genus *Fritziana,* needs to be reviewed.

1. Introduction

Amphibians are the class of vertebrates in which the number of described species has increased the most in recent years (Köhler et al., 2005). In 1985, there were approximately 4014 species of amphibians formally described and considered valid (Glaw and Köhler, 1998; Hanken, 1999; Frost, 2017), but by the end of 2017 the number of species has increased by nearly 93% (7763 species; Frost, 2017, accessed 18 December 2017). This increase in the number of species is the result of the exploration of poorly known or remote areas (e.g., Köhler et al., 2005; Padial et al., 2012; Peloso et al., 2016), as well as the growing application of molecular tools for taxonomic and systematic analyses, and the understanding that small differences revealed by a combination of independent lines of evidence (e.g., advertisement calls and external morphological variation), are sufficient to characterize new species (Fouquet et al., 2007; Vieites et al., 2009; Padial et al., 2010; Jansen et al., 2011). Molecular analyses have been especially important in revealing many morphologically cryptic and sibling species within taxa that had previously been recognized as a single species

(Hanken, 1999; Bickford et al., 2007; Stuart et al., 2006).

Despite the high number of new species descriptions in the last three decades, suggesting that the diversity of amphibians is still highly underestimated, amphibians are the most endangered group of vertebrates, with nearly one-third of evaluated species (32.4%) globally threatened or extinct (IUCN, 2016). Furthermore, there are around 24.5% of species in which information regarding threat status is presently not available (IUCN Category Data Deficient, DD), and a significant proportion of these species are likely to be globally threatened (IUCN, 2016). Currently, the conservation status of a species is supported by decision rules based on parameters such as distribution range, population size, population history, and risks of extinction (Akçakaya et al., 2000; Stuart et al., 2004). Therefore, a proper understanding of taxonomy and the distribution range of a species is necessary for a thorough threat assessment (Stuart et al., 2004; Gehara et al., 2013).

The frogs of the Hemiphractidae family are referred to as egg-brooding frogs because the females carry their eggs on their back (Del Pino, 1980; Duelmann and Gray, 1983). This family includes six genera distributed in Central and South America: *Cryptobatrachus, Flectonotus,*

Fritziana, *Gastrotheca*, *Hemiphractus*, and *Stefania*. The monophyly and relationship of hemiphractids have been constantly questioned and controversially discussed in the last few years (e.g., Haas, 2003; Darst and Cannatella, 2004; Faivovich et al., 2005; Wiens et al., 2005; Frost et al., 2006; Wiens et al., 2007; Frost et al., 2008; Guayasamin et al., 2008; Pyron and Wiens, 2011; Schmid et al., 2012). Castroviejo-Fisher et al. (2015) made a more inclusive analysis of the family considering both molecular and phenotypic data and recovered the family as monophyletic, being the sister group of Athesphatanura. These authors also suggested that the species diversity in Hemiphractidae is underestimated and that the genera *Cryptobatrachus*, *Hemiphractus,* and *Fritziana* may hide cryptic species.

The genus *Fritziana* Mello-Leitão, 1937 is endemic to the southeastern and southern Brazilian Atlantic Forest and its transitional areas (Duelmann and Gray, 1983; Franz and Mello, 2015; Peixoto et al., 2016; Frost, 2017), and is composed of six known species: *Fritziana goeldii* (Boulenger, 1895), *F. ohausi* (Wandolleck, 1907), *F. fissilis* (Miranda-Ribeiro, 1920), *F. ulei* (Miranda-Ribeiro, 1926), *F. tonimi* Walker et al., 2016, and *F. izecksohni* Folly et al., 2018. All these species, except *F. tonimi*, were described from the Serra dos Órgãos mountain range, Rio de Janeiro state, Brazil, where they are sympatric. The genus was originally described to include *F. goeldii* and *F. ohausi*, species with an incomplete dorsal pouch where the lateral skin just borders the eggs without covering them (Miranda-Ribeiro, 1920). Many changes were made to the contents of the genus since its description (Bokermann, 1966; Duelmann and Gray, 1983; Weygoldt and Carvalho-e-Silva, 1991) and only recently, based on molecular phylogenies (Duellman et al., 2011; Blackburn and Duellman, 2013), were the Brazilian species with open and closed dorsal pouches included in this genus.

Pouch morphology has been used as an important morphological character for the description of species within *Fritziana* (Duelmann and Gray, 1983). This character is also used for species identification, which often makes the identification of males, juveniles, and females without a pouch a challenge, even for well-trained taxonomists. In this sense, the use of molecular tools can be very useful for a better understanding of the limits of species and population distribution within this genus.

There are only a few studies that provide analyses on the molecular data of *Fritziana* (Duellman et al., 2011; Schmid et al., 2012; Blackburn and Duellman, 2013; Castroviejo-Fisher et al., 2015; Walker et al., 2016), and these studies have small sample sizes and restricted geographical sampling. Nevertheless, all these previous studies have highlighted that this genus may contain unknown species diversity and show that molecular evidence can improve the delimitation of species within the genus. Castroviejo-Fisher et al. (2015), for example, suggested that among the seven samples used in their study, three might represent new species hidden under the name *Fritziana fissilis*.

Here we provide an overview of DNA sequence diversity within the genus, using three mitochondrial and two nuclear markers, compiling data from almost all localities in which the genus is known to occur. We discuss the underestimated species diversity within this genus and the implications of these results for species conservation. We also inferred, for the first time, the phylogenetic relationships among almost all the species of *Fritziana*.

2. Materials and methods

2.1. Taxon sampling and sequence analyses

We gathered from various collections 107 samples from five of the currently six recognized species of *Fritziana*, representing almost the entire geographical distribution along with new locality records (Appendix A; Fig. 1). We could not include samples from the recently described *F. izecksohni*, because we do not have reliable tissue to include in molecular analyses. For phylogenetic inferences, we also included 13 taxa of Hemiphractidae representing all genera

(*Cryptobatrachus*, *Flectonotus*, *Gastrotheca*, *Hemiphractus,* and *Stefania*), and one sample of *Pristimantis cruentus* to root the tree (Appendix B).

Genomic DNA was extracted from ethanol-preserved tissues (muscle or liver), using standard high-salt protocols (Maniatis et al., 1982) adapted for microcentrifuges (Lyra et al., 2016), or with the DNeasy Blood & Tissue kit (QIAGEN Inc.) following the manufacturer's protocol. We collected DNA sequence data for three mitochondrial gene fragments: the 12S ribosomal RNA (*12S*), 16S ribosomal RNA with adjacent *tRNA-Val* (*16S*), and Cytochrome *c* oxidase subunit 1 (*COI*); and two nuclear protein-coding genes: the Recombination Activating gene 1 (*RAG1*) and the Solute Carrier family 8 member 3 (*SLC8A3*). PCR primers used for both amplification and sequencing are shown in Appendix C along with reaction conditions. PCR products were purified using enzymatic reaction following Lyra et al. (2016), and the resulting amplified fragments were sequenced by Macrogen Inc. of Seoul, South Korea or in the "Centro de Estudos de Insetos Sociais" of the Instituto de Biociências, Universidade Estadual Paulista (Rio Claro, São Paulo, Brazil).

Chromatographs were checked manually, assembled, and edited using CodonCode Aligner 3.5 (Codon Code Corporation) or Geneious v.6 (Biomatter Ltd.). Coding gene fragments (*COI*, *RAG1*, and *SLC8A3*) were translated into amino acids and no stop codons were observed, suggesting that the sequences were functional. DNA sequences were aligned independently for each fragment using the online version of MAFFT v.7 (Katoh and Standley, 2013). We checked and excluded potential contaminations, and a combined matrix was generated using SequenceMatrix software (Vaidya et al., 2011). All sequences were submitted to GenBank, and accession numbers are provided in Appendix A. Sequences and sample metadata (as voucher number and localities) were also submitted to the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert, 2007) under the project name "FRITZ".

For some samples, we recovered different lengths for the *12S* fragment when amplified with the primer MVZ59, anchored in the *tRNA-Phe* (see Section 3.1). These samples were investigated for mitochondrial DNA rearrangement using tRNAscan-SE 1.21 (Schattner et al., 2005). The *12S* initial position for all of the samples was also determined by comparison to published anuran mitogenomes, and fragments upstream of *12S* were excluded from the phylogenetic inferences, to avoid incorrect alignment of the non-homologous fragment.

For the nuclear genes *RAG1* and *SLC8A3*, we identified the polymorphic positions corresponding to heterozygous individuals and coded according to the IUPAC ambiguity codes. The sequences were then resolved using the PHASE 2.1.1 software (Stephens et al., 2001) and run ten independent times, using a 0.90 probability threshold and a parent-independent mutation model. The online web tool SeqPHASE (Flot, 2010) was used to generate input and the final fasta files. Phased nuclear sequences were used for network reconstruction, and the unphased sequences coded with IUPAC were used for the phylogenetic inference. Nuclear allele networks were constructed with HapView (Salzburger et al., 2011), using maximum parsimony trees inferred using the Phylip 3.695 package (Felsenstein, 1989) with the DNApars extension. Some sequences were excluded from haplotype-network reconstruction because of small fragment size, due to bad sequencing results, or because the nuclear phase could not be resolved.

We estimated the maximum intraspecific and minimum interspecific uncorrected p-distances for the mitochondrial fragments *COI* and *16S* (only for the fragment between primers 16Sar-L/16Sbr-H), using the Spider package in R v 3.2.2 (Brown et al., 2012; R Core Team, 2015), considering major genetic lineages recovered in phylogenetic inferences and the population lineages within species (see Section 3).

2.2. Species assessment

To assign genetic lineages to 'species' or 'candidate species', we first identified well-supported haplotype clades on the mtDNA tree (Miralles
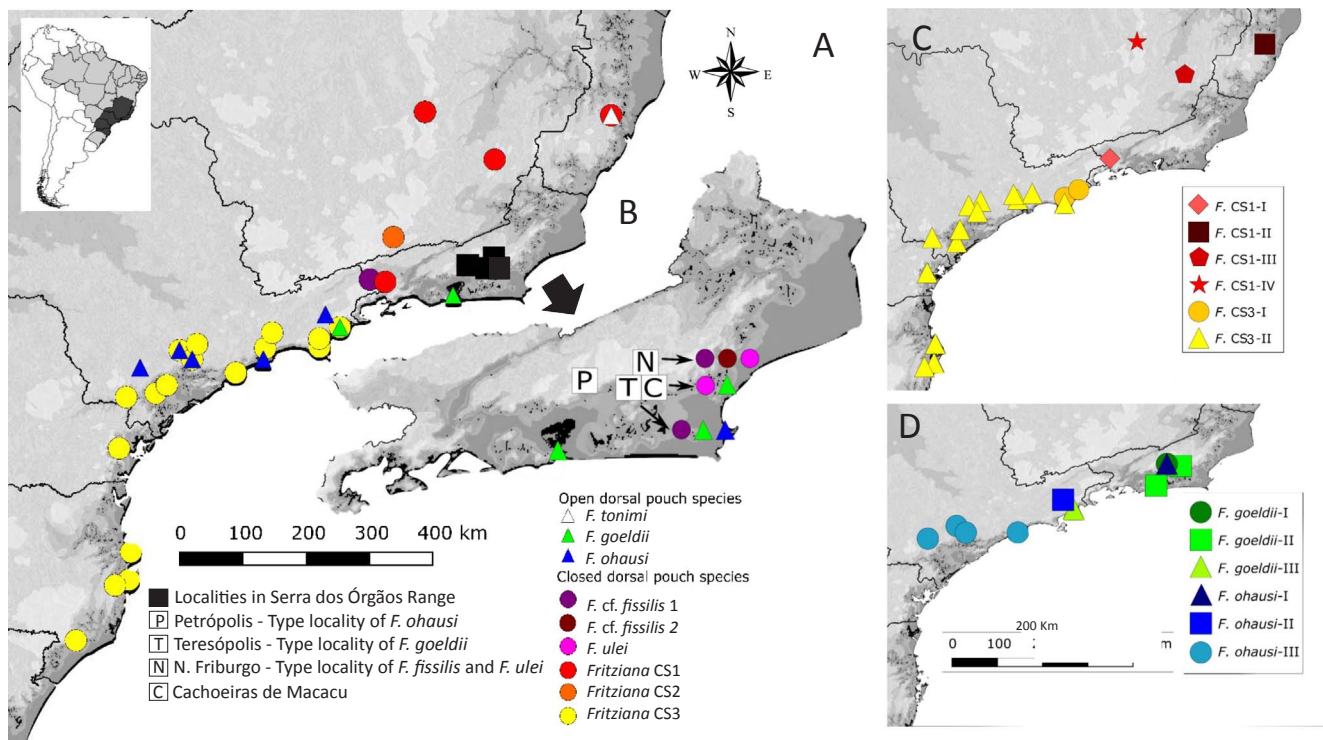
Fig. 1. Distribution of species in the genus *Fritziana* based on the samples used for molecular analyses. (A) Distribution of specimens analyzed and (B) details of Rio de Janeiro state, where many species occur in sympatry. Circles show species with closed dorsal pouch, and triangles show species with open dorsal pouch. (C) Distribution of *Fritziana* CS1 (red) and *F.* CS3 (yellow) internal population lineages and (D) *F. goeldii* (green) and *F. ohausi* (blue) internal population lineages. CS denotes candidate species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and Vences, 2013). Next, we analyzed the nuclear allele networks looking for congruence among the mtDNA and nucDNA. Once we found no evidence of introgression between the genetic lineages, we proceeded with the phylogenetic inferences using the combined dataset. Then, we associated each of the genetic lineages to a described species based on morphological characters of the voucher specimens and on type localities. When we could not associate a genetic lineage to a described species (in the case of *Fritziana fissilis*, see Section 3.1), we used the terminology "confer" (cf.). We considered 'candidate species' (CS) to be the lineages that did not correspond to a currently recognized species.

Haplotype clades in some species revealed subdivision into 'population lineages', with high genetic distances between some of these lineages (higher than 3% for *16S* or 10% for *COI*; Supplementary Table S1). This subdivision accommodates the idea that a species might be genetically and geographically structured in its distribution range, but it may lead to an underestimation of the species diversity within the genus. We chose this conservative approach to minimize wrongly delimiting a group of individuals as a distinct species (see Padial et al., 2010), since in the present study we have no other line of evidence that supports these population lineages as candidate species.

2.3. Phylogenetic inferences

Phylogenetic analyses were conducted using Bayesian inference (BI) with MrBayes v. 3.2.3 (Ronquist et al., 2012) for both the mitochondrial and combined datasets (mitochondrial + nuclear genes). We used PartitionFinder (Lanfear et al., 2012), under the Akaike Information Criterion (AIC; Akaike, 1974) to estimate the best partitioning scheme and models of evolution: *12S* (GTR + I + G), *16S* (GTR + I + G), 1st position of *COI* (GTR + G), 2nd position of *COI* (F81 + I), 3rd position of *COI* (TrN + G), 1st position of *RAG1* (TIM + G), 2nd position of *RAG1* (HKY + I), 3rd position of *RAG1* (K81uf + G), 1st position of

SLC8A3 (GTR + I), 2nd position of *SLC8A3* (HKY), and 3rd position of *SLC8A3* (TrN + G). The final alignment contained a total of 4120 base pairs (bp): 502 bp for *12S*, 1612 bp for *16S*, 645 bp for *COI*, 855 bp for *RAG1*, and 506 bp for *SLC8A3*.

The analyses consisted of two independent runs, each with eight chains, for 100 million generations; parameters and trees were sampled every 1000 generations. The analyses were done using the CIPRES Science Gateway (Miller et al., 2010). We verified the effective sample size (ESS) and convergence between the runs in Tracer 1.6 (Rambaut et al., 2009) and discarded the first 25% samples from each run before summarizing the trees. We generated a majority-rule consensus tree, visualized it in FigTree 1.4.3 (Rambaut and Drummond, 2009) and considered values of Bayesian posterior clade probabilities (PP) higher than 0.95 as evidence of monophyly (Huelsenbeck and Rannala, 2004).

3. Results

3.1. Phylogenetic inference and species assessment

The resulting Bayesian trees for the mitochondrial and combined dataset were very similar, only differing in the node support for the relationships among species (Figs. 2 and 3). We found a total of 44 alleles for *RAG1* and 27 for *SLC8A3*, and no nuclear allele sharing among the major lineages, suggesting no evidence for introgression.

The trees resolve *Fritziana* as a monophyletic genus with at least nine highly supported genetic lineages that were considered species. The species *Fritziana goeldii*, *F. ohausi*, and *F. tonimi* were recovered as monophyletic, being that the first two presented geographically structured and genetically divergent populations. On the other hand, the specimens with closed dorsal pouches, usually recognized as *F. fissilis/F. ulei*, were recovered as paraphyletic in relation to *F. tonimi* and *F. ohausi* and comprising six independently evolving lineages.

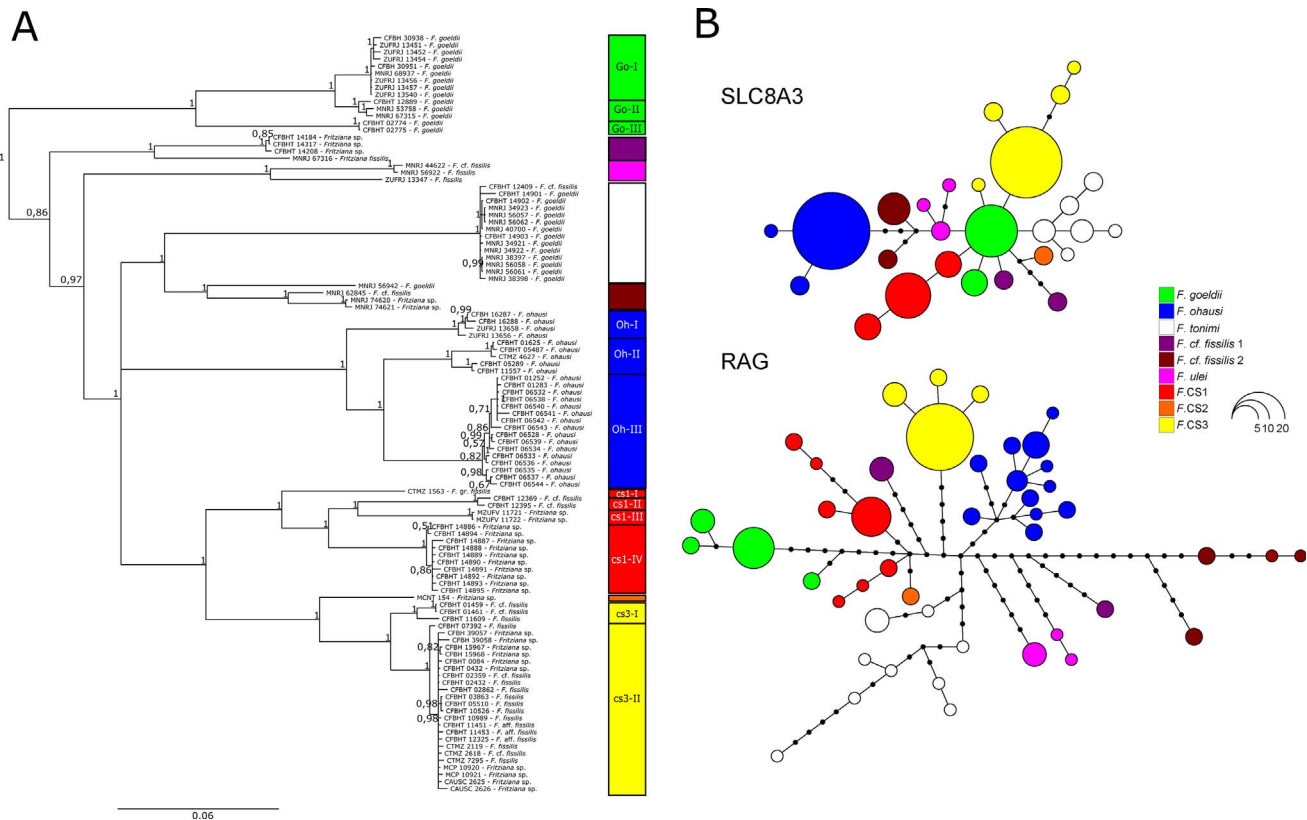Samples from Nova Friburgo (Rio de Janeiro state), the type locality

Fig. 2. A – Bayesian tree for *Fritziana* inferred for mitochondrial data. The numbers at nodes are Bayesian posterior probabilities. B – Haplotype networks of the nuclear genes reconstructed in HapView using a parsimony tree. *RAG1*: Recombination Activating gene 1; *SLC8A3*: Solute Carrier family 8 member 3. Circles represent haplotypes, with size being proportional to the number of individuals; dots represent intermediate, unsampled haplotypes; colors represent current recognized species and candidate species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of *F. fissilis* and *F. ulei*, are distributed in three genetic lineages. One of the lineages could be assigned to *F. ulei* based on the voucher specimen ZUFRJ 13347 used to revalidate *F. ulei* (Folly et al., 2014) and on the morphology of other specimens of the clade. *Fritziana fissilis sensu stricto* could not be assigned to one of the two remaining lineages, since the few specimens available in both lineages present morphological diagnostic characters of *F. fissilis* (Miranda-Ribeiro, 1920; Bokermann, 1950; Duellman and Gray, 1983; Folly et al., 2014). Due to this, we identified these lineages as *Fritziana* cf. *fissilis* 1 and *Fritziana* cf. *fissilis* 2.

We considered the remaining three lineages in *F. fissilis* as candidate species (CS) according to their phylogenetic relationship with other species, non-occurrence in the type locality of *F. fissilis* and *F. ulei*, and because sequence analyses revealed a mitochondrial rearrangement in the position of the *tRNA-Phe* in relation to *tRNA-Pro* in *F.* CS2 and *F.* CS3 (Fig. 4). Although these two candidate species presented the same rearrangement and are clustered in a well-supported clade, we considered them to be two distinct candidate species because each of them presents a different size in the non-coding sequence between *12S* and the *tRNA-Pro* (Fig. 4), they occur in different geographical areas (Fig. 1), and they present high genetic distances for both *16S* and *COI* (Table 1). In addition, there is no nuclear allele shared among the three different candidate species (Fig. 2), adding evidence that each one is an independently evolving lineage.

The species *Fritziana goeldii* was recovered as the sister group of all the other species, and *Fritziana* cf. *fissilis* 1 is the sister species of the clade composed of *F. ulei* and of all the remaining species (PP = 0.97; Fig. 3); *Fritziana tonimi* was recovered as the sister taxon of *F.* cf. *fissilis* 2; and *Fritziana* CS1 is the sister taxon of *F.* CS2 + *F.* CS3. The relationships between the clades of *F.* CS1 + (*F.* CS2 + *F.* CS3), *F. tonimi* + *F.* cf. *fissilis* 2, and *F. ohausi* could not be resolved in the present analyses (PP = 0.76).

3.2. Genetic distances

The genetic distances obtained with *COI* and *16S* are summarized in Table 1. The smallest intraspecific uncorrected p-distance value was found for *F. tonimi* (*COI* = 0%; *16S* = 0.4%) and the highest for *F. goeldii* (*COI* = 15.3%; *16S* = 7.2%). Among the species, the highest value was found between *F. tonimi* and *F. goeldii* (*COI* = 18.9%; *16S* = 11.2%), and the smallest between *F.* CS2 and *F.* CS3 (*COI* = 12.3%; *16S* = 2.6%).

Fritziana goeldii comprises three population lineages, which encompass the population from the mountains of Rio de Janeiro state (Serra dos Órgãos and surroundings – Go-I), which is the sister lineage of the population from the coastal area of Rio de Janeiro municipality (Go-II) (Figs. 1–3), and both of these form the sister group of the lineage from the north of São Paulo state (Go-III) ((Go-I + Go-II) + Go-III). The uncorrected p-distances among these population lineages are in the supplementary material Table S1, and varied from 3.6% to 14.7% for *COI* and 0.8% to 6.8% for *16S*.

Fritziana ohausi is structured into one population lineage distributed throughout Rio de Janeiro state (Oh-I), which is the sister lineage of the groups formed by samples from São Paulo state. These samples from São Paulo state are divided into two internal lineages: from the north (Oh-II) and the south (Oh-III) of the state (Figs. 1–3). The distances between these populations varied from 9.3% to 11.1% for *COI* and 2.8% to 3.8% for *16S* (Table S1).

Fritziana CS1 is structured into four population lineages (Figs. 1–3). The population of the Serra do Espinhaço mountain range (CS1-IV), which is the geographical limit between the Cerrado and Atlantic Forest in Minas Gerais state is the sister lineage to the Minas Gerais' Atlantic Forest lineage (CS1-III), and both lineages together form the sister group of the population from Espírito Santo state (CS1-II), usually
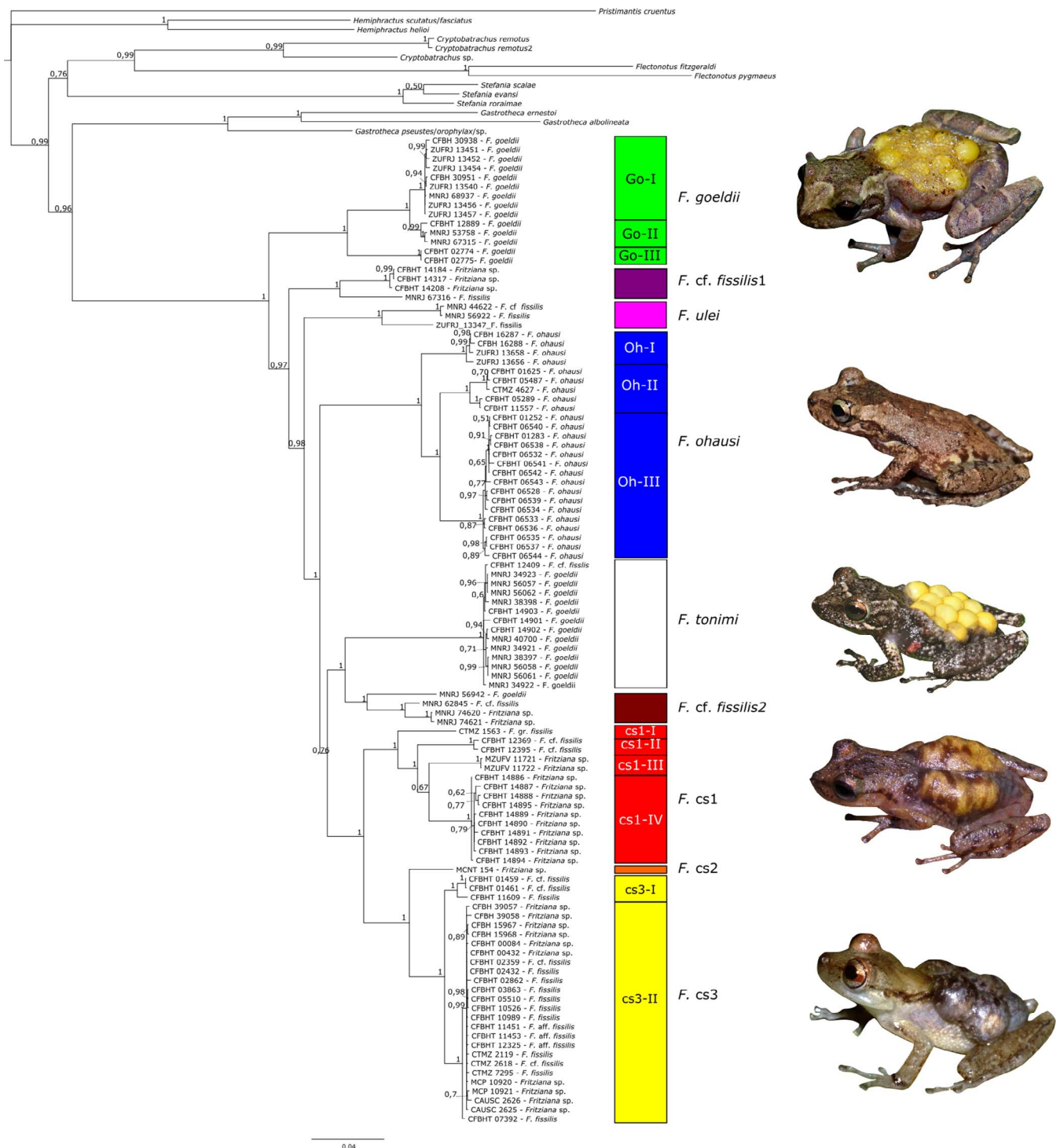
Fig. 3. Bayesian tree for *Fritziana* inferred for concatenated mitochondrial and nuclear data. The colors represent each described or candidate species. Numbers at nodes are Bayesian posterior probabilities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identified in collections as *F. fissilis*. One single sample from São Paulo state (CS1-I) is the sister lineage of the others (CS1-I + (CS1-II + (CS1-III + CS1-IV))). The minimum genetic distances found among these lineages varied from 11.7% to 15% for *COI* and 4.2% to 5.2% for *16S* (Table S1).

Similar to *Fritziana ohausi*, *Fritziana* CS3 also presents population lineages geographically distributed in the north (CS3-I) and south of São Paulo, but also includes the southern states of Paraná and Santa Catarina (CS3-II). This lineage represents the southern distribution of

the genus (Fig. 1). The genetic distances between these lineages were 6.29% for *COI* and 0.6% for *16S* (Table S1).

4. Discussion

4.1. Phylogenetic relationship among Fritziana species

Our analysis, including an expanded geographic and taxonomic sampling of *Fritziana*, revealed that the genus hides a number of
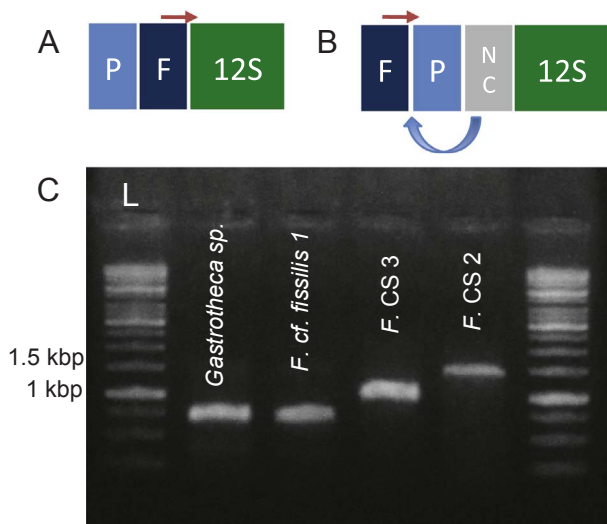
Fig. 4. Graphical representation of the tRNA translocation found for *Fritziana* CS2 and *F.* CS3. A: graphical representation of the most common neobatrachian gene order; B: new tRNA arrangement found for *Fritziana* CS2 and *F.* CS3 including the non-coding region, which differs in size between these species; the red arrow represents the annealing position of primer MVZ59. C: 1% Agarose gel showing difference in fragment size amplified for different species using primers MVZ59 and FH (for primers references see Appendix C); L: Ladder GeneRuler 1 Kb DNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

probable undescribed species, and suggests that the species diversity in this genus has remained underestimated. We also provide a new hypothesis for the phylogenetic relationships among the species of *Fritziana*, including for the first time a representation of almost all nominal species. Unfortunately we were unable to sample tissues of *F. izecksohni* (Folly et al., 2018), but the morphological characteristics of this new species do not match those of the individuals from the genetic lineages we sampled here (data not shown), which give additional evidence that the species diversity of this genus is still unknown.

Different proposals have been made about the relationships among the species in the genus *Fritziana* (Duellman et al., 2011; Schmid et al., 2012; Castroviejo-Fisher et al., 2015; Walker et al., 2016), but most of these studies were not intended to resolve the relationships within *Fritziana*. The previous results have been summarized in Fig. 5, including the re-identification of terminals used in previous work based on our more inclusive dataset (see Appendix A for details). The tree topology that we found differs from previous proposals, being the result of the larger sample size, inclusion of different molecular markers, and analytical approaches. Despite this, the relationships among genetic lineages *F.* CS1 + (*F.* CS2 + *F.* CS3), *F. tonimi* + *F.* cf. *fissilis* 2, and *F. ohausi* still lack sufficient support, as in previous studies, suggesting that we need to focus on collecting more data in order to resolve relationships within *Fritziana*. Here, we have taken the first step towards a better understanding of genetic diversity in this group, and we hope

that this knowledge allows us to identify species limits in this genus from a new perspective.

4.2. The challenge of the "closed dorsal pouch" species

The cryptic species diversity that we found in the genus is mainly hidden among specimens with closed dorsal pouches and associated with two valid names, *F. fissilis* and *F. ulei*. These two species were described from the municipality of Nova Friburgo, Rio de Janeiro state, and morphologically similar specimens collected in this location belong to three different lineages, making the association between molecular clades and species names a challenge.

Fritziana ulei was described in 1926 by Miranda-Ribeiro, synonymized with *F. fissilis* by Bokermann (1966), and recently revalidated by Folly et al. (2014). The specimen used by Folly et al. (2014) for the revalidation (ZUFRJ 13347) is included in our analyses, so we associate this lineage with *F. ulei*. We also carefully examined the external morphology of the specimen MNRJ 56922, which clusters with ZUFRJ 13347, and it matches the original description of *F. ulei*. Nonetheless, Folly et al. (2014) considered specimens from the Campo de Fruticultura, in the Parque Nacional da Serra da Bocaina, municipality of São José do Barreiro, São Paulo state, (MZUSP A-75903, A-75844, A-128089; not Ubatuba, São Paulo state, as in Folly et al., 2014) to be *F. ulei*. However, all our samples from São José do Barreiro cluster in lineage *F.* cf. *fissilis* 1. Menegucci et al. (2017) also registered *F. ulei* in Ubatuba, São Paulo state, but based on the study of Folly et al. (2014). We analyzed the photos from this publication and assume that the specimens collected by these authors belong to *Fritziana* CS3. Based on these data, we suggest that the distribution of *F. ulei* should be restricted to its type locality in the Serra dos Órgãos mountain range, Rio de Janeiro state, and the morphological diagnosis suggested by Folly et al. (2014) needs to be reviewed, since these authors might have used a mixed series for the revalidation. Characters used as diagnostic for *F. ulei* occur in other species, such as the interorbital bronze pentagon/hexagon-shaped mark that is also present in *F.* CS3 (MNRJ 90351, for example).

Based on our data, we were unable to assign a molecular lineage to *F. fissilis*. Although Castroviejo-Fisher et al. (2015) tentatively considered the sample MNRJ 62845 (*F.* cf. *fissilis* 2) as *F. fissilis*, we found one additional molecular lineage present in the type locality not sampled by them. These two lineages from Nova Friburgo include specimens with morphological characters normally associated with *F. fissilis*, such as bifid subarticular tubercles in the fingers and toes, present in both males and females. Therefore, we named the lineages *F.* cf. *fissilis* 1 and *F.* cf. *fissilis* 2. This classification needs to be reviewed again once more evidence is available, especially more specimens with DNA sequence data. If in the future we can associate one of the lineages to the holotype, the other lineage will need a formal description.

We considered the other three lineages (CS1, CS2, and CS3), which include females with a closed dorsal pouch, as candidate species, based on their phylogenetic position and the presence of the rearrangement in

Table 1
Maximum intraspecific and minimum interspecific uncorrected p-distances (%) among genetic lineages of *Fritziana*. Upper diagonal: *COI* distances; Below diagonal *16S* distances. N: number of specimens analyzed.

|	N	Intra-*16S*	Intra-*COI*	goe	oha	ton	ulei	cf1	cf2	CS1	CS2	CS3
Fritziana goeldii	14	7.2	15.3		16.2	18.9	16.2	12.3	13.5	15.0	15.3	16.8
Fritziana ohausi	25	4.8	11.7	9.2		16.2	14.7	13.8	15.3	14.1	12.9	13.8
Fritziana tonimi	14	0.4	0.0	11.2	9.2		18.6	14.7	15.9	16.8	17.1	15.3
Fritziana ulei	5	5.2	13.2	9.2	8.8	11.2		13.8	14.1	16.8	14.7	18.0
Fritziana cf. fissilis 1	4	6.0	10.8	7.8	6.6	8.8	5.8		12.6	14.7	12.0	15.0
Fritziana cf. fissilis 2	3	3.0	12.9	7.6	8.4	10.4	7.2	6.8		15.3	15.0	16.2
Fritziana CS1	15	5.6	15.0	9.4	6.0	9.8	7.8	4.6	7.6		12.6	13.8
Fritziana CS2	1	NA	NA	10.0	7.2	10.2	8.2	5.6	8.2	6.6		12.3
Fritziana CS3	27	2.6	9.0	8.4	7.8	9.2	7.8	5.4	7.2	6.0	2.6	
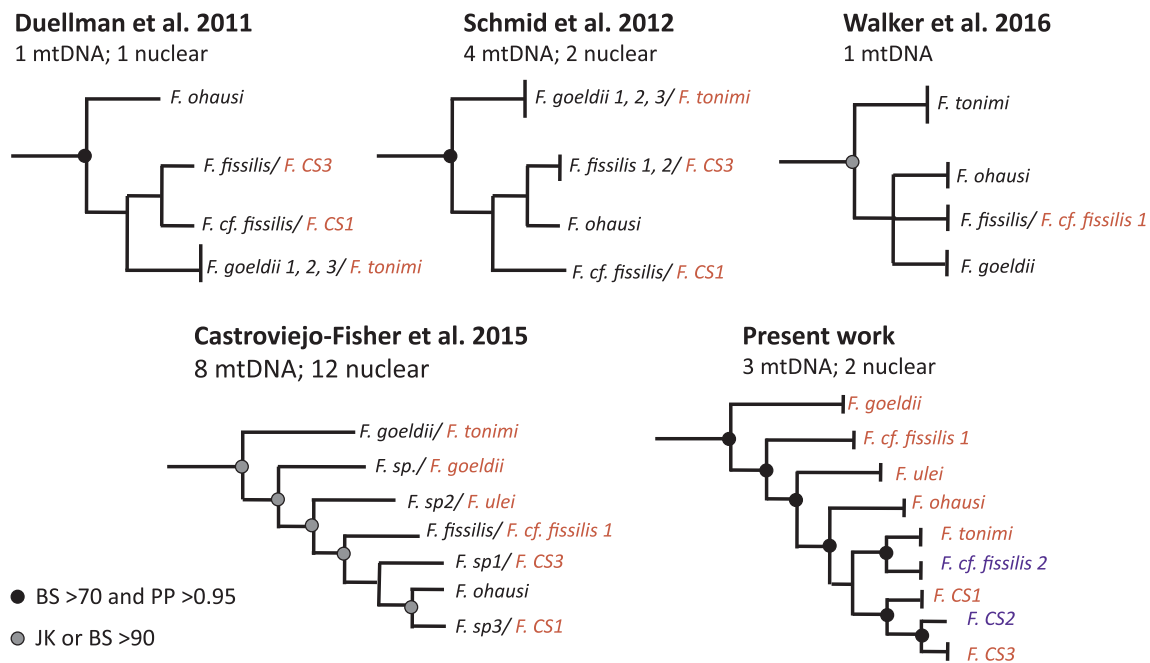
Fig. 5. Summary of phylogenetic hypotheses for *Fritziana*. Names in black are identifications according to the original papers; names in red, new identifications of terminals based in the present study; in blue, new lineages identified only in present work. Black dots: well-supported node using both maximum likelihood and Bayesian analyses; gray dots: well-supported node using parcimony. JK: jackknife; BS: bootstrap; PP: Bayesian posterior probability. Samples used in more than one study are identified in Appendix A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the order of *tRNA-Pro* and *tRNA-Phe* in the mitochondrial DNA (only present in CS2 and CS3), along with some morphological evidence (data not shown). *Fritziana* CS1 includes samples from states of Espírito Santo, which were historically considered *F. fissilis* (Duelmann and Gray, 1983; Frost, 2017), Minas Gerais, and São Paulo, occurring in the Atlantic Forest and transitional areas between the Atlantic Forest and the Cerrado. *Fritziana* CS2 is known from only four specimens (MCN-PUC-MG 12338-12341) from Bom Jardim de Minas, state of Minas Gerais and only one tissue sample. *Fritziana* CS3 includes samples from the Serra do Mar mountain range (from the states of São Paulo to Santa Catarina). The population from Rio Grande do Sul state, named *F.* aff. *fissilis* by Franz and Mello (2015), most likely belongs to this species.

Although we found *Fritziana* CS1 as the sister species of *F.* CS2 + *F.* CS3, the last two lineages presented a translocation on *tRNA-Phe* position, which is a new tRNA order for Anura (Zhang et al., 2013; Xia et al., 2014). This variation can be identified by PCR using a primer anchored in the *tRNA-Phe* and electrophoresis, yielding a different size of the amplified fragment (Fig. 4). *Fritziana* CS2 presents an additional non-coding region with about 300 bp, which differentiates this species from *F.* CS3. We identified *F.* CS2 as a candidate species considering this variation, but a better sampling is needed to allow a proper comparison of both species. The formal descriptions of *F.* CS1 and *F.* CS3, based on morphological characters and considering this molecular variation, are in progress and will be presented in a future study.

The organization of the mitochondrial genome was considered highly conservative for animals (Boore, 1999), but in the last few years different gene rearrangements have been described for anurans (Kurabayashi et al., 2008; Zhang et al., 2013; Xia et al., 2014). Among Hemiphractidae, only *Cryptobatrachus* sp. and *Gastrotheca pseustes* have been analyzed for mitochondrial gene order, and *Cryptobatrachus* sp. presents a modification in the WANCY region (Zhang et al., 2013). Here we found a difference between closely related species of *Fritziana*, suggesting that mitochondrial DNA may be useful to understand molecular variation and evolution among sister species, genera, or families, not only to characterize major groups such as Batrachia and Neobatrachia. Our findings also suggest that gene arrangement might be a diagnostic character for some species in the Hemiphractidae

family, but for a better understanding of the biological and evolutionary significance of the gene rearrangements, it is important to have more data on the complete mitochondrial genomes.

4.3. Species distribution and conservation status

Considering that species in the genus *Fritziana* are small to medium size (*sensu* Duellman, 1970) and are reproductively dependent on water accumulated in bromeliad or bamboo, we expected that they would present low dispersal capability, and thus low gene flow among populations, as observed for other tropical species of anurans (e.g., Rodríguez et al., 2015). Indeed, we found geographic genetic structure in *Fritziana ohausi*, *F. goeldii*, *F.* CS1, and *F.* CS3. The other species or candidate species in our study have restricted geographic distribution and/or small sample size, thus precluding assessment of geographic genetic variation within them.

The genetic distance is equal or larger among cryptic lineages than some values found for interspecies distances of *16S* and *COI* (Table 1 and Supplementary Table 1). Considering only genetic diversity, it is possible that *F. goeldii*, *F. ohausi*, and *F.* CS1 also hide some unknown species diversity, but in this first taxonomic revision, we decided to take a more conservative position in suggesting candidate species. Population lineages based on haplotype clade support and genetic distances are a starting point for further investigations on species limits using additional sources of information, such as morphology, bioacoustics, or cytogenetics (Padial et al., 2010).

It is interesting to note that differences in the number and the size of chromosomes were already found for the genus and should be investigated in more detail. For example, *F. goeldii* has 2N = 26 and fundamental number (FN) = 42; *F. ohausi* has 2N = 28 and FN = 44; and *F. fissilis* has 2N = 30 and FN = 50 (Beçak, 1968; Bogart, 1973; Schmid et al., 2012). The data for all species are tentative and should be reviewed considering the species diversity revealed by our analyses. For example, Bogart (1973) did not include information on the localities of samples or voucher numbers for specimens of *F. fissilis* analyzed and, because of that, they might be any of the six lineages identified in the present work.

According to the IUCN (2016), *Fritziana fissilis*, *F. ohausi,* and *F. goeldii* are listed as 'Least Concern' species mainly because of their presumed wide distribution and large populations. The recognition of cryptic species among *F. fissilis/F. ulei* makes their distributions and populations smaller than previously considered, similar to what has happened with *F. goeldii* after the description of *F. tonimi* (Walker et al., 2016). These results point out the necessity to reevaluate the conservation status of all species in the genus. First, it is important to reevaluate *F. fissilis,* beginning with a careful review of the morphology of the specimens, including type series, to try to identify which molecular lineage can be attributed to this species (*F.* cf. *fissilis* 1, *F.* cf. *fissilis* 2 or even a lineage not sampled in our analyses). *Fritziana* cf. *fissilis* 1, *F.* cf. *fissilis* 2, and *F. ulei*, with the recognition of the candidate species, now have narrow distributions and presumed small populations. They occur in well-collected areas, are sparingly sampled in collections, and little is known about their biology. Based on these results, we suggest that *F. fissilis*, *F. ulei*, and *F. tonimi* should be considered "Data Deficient" (sensu IUCN, 2016). *Fritziana goeldii* and *F. ohausi* should be reevaluated, considering that the last evaluation dates are from 2004, and now we have confirmed that they present internal genetic structures. *F. goeldii* has also had its distribution reduced, and the large genetic distances between its populations need to be better evaluated. The candidate species need to be described and then evaluated by the IUCN criteria. While *F.* CS1 and *F.* CS3 have the widest distributions, are common species, and are probably not endangered, the new species must be described before an adequate evaluation of their conservation status can be completed.

5. Conclusions

The amphibian diversity in the Atlantic Forest is still underestimated and the fact that the destruction of the forest is currently quicker than the recognition and description of new species, may cause extinctions before we can comprehend its true diversity (Haddad and Prado, 2005; Crawford et al., 2010). With these molecular results and by better understanding the diversity of *Fritziana*, we find that other sources of data are urgently needed to diagnose and to name the new species of *Fritziana*. It is also important to review the diagnoses and morphological variation of the named species. By using several lines of evidence, such as internal and external morphologies of adults and larvae, bioacoustics, cytogenetics, and ecology, new diagnostic characters may be revealed when re-analyzing mixed series of two or more species.

Appendix A

Samples analyzed: Collection and voucher number, collection and new identification, population lineage, species pouch type, localities and GenBank accession numbers. Samples in bold were included in previous studies (a-Faivovich et al., 2005; b-Duellman et al., 2011; c-Schmid et al., 2012; d-Castroviejo-Fisher et al., 2015). Geographical coordinates are given in the Barcode of Life Data Systems (BOLD) under the project name 'FRITZ'.

Tissue collection number	Voucher number	Colletion identification	New identification	Population lineage	Pouch type	Localities
CFBHT 17729	CFBH 30938	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
CFBHT 17743	CFBH 30951	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
MNRJ 68937	MNRJ 68937	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
ZUFRJ 13451	ZUFRJ 13451	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
ZUFRJ 13452	ZUFRJ 13452	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
ZUFRJ 13454	ZUFRJ 13454	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
ZUFRJ 13456	ZUFRJ 13456	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
ZUFRJ 13457	ZUFRJ 13457	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
ZUFRJ 13540	ZUFRJ 13540	*F. goeldii*	*F. goeldii*	GO-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
MNRJ 53758	MNRJ 53758	*F. goeldii*	*F. goeldii*	GO-II	Open	Cachoeiras de Macacu – RJ
MNRJ 67315	MNRJ 67315	*F. goeldii*	F. goeldii	GO-II	Open	Cachoeiras de Macacu – RJ
CFBHT 12889	CFBH 26981	*F.* cf. *goeldii*	*F. goeldii*	GO-II	Open	Rio de Janeiro – RJ
CFBHT 2774	CFBH 10910	*F. goeldii*	*F. goeldii*	GO-III	Open	Ubatuba – SP
CFBHT 2775	CFBH 10909	*F. goeldii*	*F. goeldii*	GO-III	Open	Ubatuba – SP
CFBH 16287	CFBH 16287	*F. ohausi*	*F. ohausi*	Oh-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
CFBH 16288	CFBH 16288	*F. ohausi*	*F. ohausi*	Oh-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
ZUFRJ 13656	ZUFRJ 13656	*F. ohausi*	*F. ohausi*	Oh-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
ZUFRJ 13658	ZUFRJ 13658	*F. ohausi*	*F. ohausi*	Oh-I	Open	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
CFBHT 11557	CFBH 23920	*F. ohausi*	*F. ohausi*	Oh-II	Open	Santos – SP
CFBHT 5289	CFBH 15950	*F. ohausi*	*F. ohausi*	Oh-II	Open	Santos – SP
CFBHT 1625	CFBH 7620	*F. ohausi*	*F. ohausi*	Oh-II	Open	Pilar do Sul – SP

CFBHT 5487	CFBH 16545	*F. ohausi*	*F. ohausi*	Oh-II	Open	Tapiraí – SP
CTMZ 4627 (c,d)	**MZUSP 139225**	***F. ohausi***	***F. ohausi***	**Oh-II**	**Open**	**Estação Ecológica Xitué – Ribeirão Grande – SP**
CFBHT 1252	CFBH 7611	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 1283	CFBHT 1283	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6528	CFBH 14817	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6532	CFBH 14818	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6533	CFBH 14819	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6534	CFBH 14820	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6535	CFBH 14821	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6536	CFBH 14822	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6537	CFBH 14823	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6538	CFBH 14824	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6539	CFBH 14825	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6540	CFBH 14826	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6541	CFBH 14827	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6542	CFBH 14828	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6543	CFBH 14829	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 6544	CFBH 14830	*F. ohausi*	*F. ohausi*	Oh-III	Open	São Luiz do Paraitinga – SP
CFBHT 12409	CFBH 24809	*F. cf. fissilis*	*F. tonimi*		Open	Santa Teresa – ES
CFBHT 14901	CFBH 30710	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
CFBHT 14902	CFBH 30711	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
CFBHT 14903	CFBH 30712	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
MNRJ 34921 (b,c,d)	**MNRJ 34921**	***F. goeldii***	***F. tonimi***		**Open**	**Santa Teresa – ES**
MNRJ 34922 (b,c,d)	**MNRJ 34922**	***F. goeldii***	***F. tonimi***		**Open**	**Santa Teresa – ES**
MNRJ 34923 (b,c,d)	**MNRJ 34923**	***F. goeldii***	***F. tonimi***		**Open**	**Santa Teresa – ES**
MNRJ 38397	MNRJ 38397	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
MNRJ 38398	MNRJ 38398	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
MNRJ 40700	MNRJ 40700	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
MNRJ 56057	MNRJ 56057	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
MNRJ 56058	MNRJ 56058	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
MNRJ 56061	MNRJ 56061	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
MNRJ 56062	MNRJ 56062	*F. goeldii*	*F. tonimi*		Open	Santa Teresa – ES
MNRJ 56942	MNRJ 56942	*F. goeldii*	*F. cf. fissilis 1*	CF1	closed	Nova Friburgo – RJ
MNRJ 62845 (d)	**MNRJ 62845**	***F. cf. fissilis***	***F. cf. fissilis* 1**	**CF1**	**closed**	**Nova Friburgo – RJ**
MNRJ 74620 (d)	**MNRJ 74620**	***F. sp.***	***F. cf. fissilis* 1**	**CF1**	**closed**	**Parque Nacional da Serra dos Órgãos – Teresópolis – RJ**
MNRJ 74621	MNRJ 74621	*F. sp.*	*F. cf. fissilis 1*	CF1	closed	Parque Nacional da Serra dos Órgãos – Teresópolis – RJ
CFBHT 14184	CFBH 28886	*F. sp.*	*F. cf. fissilis 2*	CF2	closed	Parque Nacional da Serra da Bocaina – São José do Barreiro – SP
CFBHT 14208	CFBHT 14208	*F. sp.*	*F. cf. fissilis 2*	CF2	closed	Parque Nacional da Serra da Bocaina – São José do Barreiro – SP
CFBHT 14317	CFBHT 14317	*F. sp.*	*F. cf. fissilis 2*	CF2	closed	Parque Nacional da Serra da Bocaina – São José do Barreiro – SP
MNRJ 67316	MNRJ 67316	*F. fissilis*	*F. cf. fissilis 2*	CF2	closed	Nova Friburgo – RJ
ZUFRJ 13347	ZUFRJ 13347	*F. fissilis*	*F. ulei*		closed	Nova Friburgo – RJ
MNRJ 44622 (d)	**MNRJ 44622**	***F. cf. fissilis***	***F. ulei***		**closed**	**Cachoeiras de Macacu – RJ**
MNRJ 56922 (d)	**MNRJ 56922**	***F. fissilis***	***F. ulei***		**closed**	**Cachoeiras de Macacu – RJ**
CFBHT 12369	CFBH 24810	*F. cf. fissilis*	*F. CS1*	CS1-I	closed	Santa Teresa – ES
CFBHT 12395	CFBH 24811	*F. cf. fissilis*	*F. CS1*	CS1-I	closed	Santa Teresa – ES
MZUFV 11721	MZUFV 11721	*F. sp.*	*F. CS1*	CS1-II	closed	Parque Estadual Serra do Brigadeiro – Ervália – MG
MZUFV 11722	MZUFV 11722	*F. sp.*	*F. CS1*	CS1-II	closed	Parque Estadual Serra do Brigadeiro – Ervália – MG
CFBHT 14886	CFBH 30747	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14887	CFBH 30748	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14888	CFBH 30749	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14889	CFBH 30750	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14890	CFBH 30751	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14891	CFBH 30752	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14892	CFBH 30753	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14893	CFBH 30754	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14894	CFBH 30755	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CFBHT 14895	CFBH 30756	*F. sp.*	*F. CS1*	CS1-III	closed	Santuário Nossa Senhora da Piedade – Caeté – MG
CTMZ 1563 (b,d)	**MZUSP133700**	***F. gr. fissilis***	***F. CS1***	**CS1-IV**	**closed**	**Estação Ecológica de Bananal – Bananal – SP**
MCNT 154	MCNAM 12341	*F. sp.*	*F. CS2*	CS2	closed	Bom Jardim de Minas – MG
CFBHT 11609	CFBH 19955	*F. fissilis*	*F. CS3*	CS3-I	closed	Ubatuba – SP
CFBHT 1459	CFBH 8273	*F. cf. fissilis*	*F. CS3*	CS3-I	closed	Caraguatatuba – SP
CFBHT 1461	CFBHT 1461	*F. cf. fissilis*	*F. CS3*	CS3-I	closed	Caraguatatuba – SP
CFBH 39057	CFBH 39057	*F. aff. fissilis*	*F. CS3*	CS3-II	closed	Parque das Neblinas – Mogi das Cruzes – SP
CFBH 39058	CFBH 39058	*F. aff. fissilis*	*F. CS3*	CS3-II	closed	Parque das Neblinas – Mogi das Cruzes – SP
CFBHT 10526	CFBH 22113	*F. fissilis*	*F. CS3*	CS3-II	closed	Parque Estadual da Serra do Mar – Itanhaém – SP
CFBHT 3863	CFBH 12205	*F. fissilis*	*F. CS3*	CS3-II	closed	Parque Estadual da Serra do Mar – Itanhaém – SP

CFBHT 5510	CFBH 11177	*F. fissilis*	*F.* CS3	CS3-II	closed	Parque Estadual da Serra do Mar – Itanhaém – SP
CTMZ 7295	MZUSP 143718	*F. fissilis*	*F.* CS3	CS3-II	closed	Parque Natural Municipal Nascentes de Paranapiacaba – Paranapiacaba, Santo André – SP
CFBHT 7392	CFBH 17496	*F. fissilis*	*F.* CS3	CS3-II	closed	Ilhabela – SP
CFBHT 10989	CFBH 23316	*F. fissilis*	*F.* CS3	CS3-II	closed	Piedade – SP
CFBHT 2359	CFBH 10008	*F.* cf. *fissilis*	*F.* CS3	CS3-II	closed	Pilar do Sul – SP
CFBHT 2432	CFBH 10315	*F. fissilis*	*F.* CS3	CS3-II	closed	Tapiraí – SP
CTMZ 2618	MZUSP 136570	*F.* cf. *fissilis*	*F.* CS3	CS3-II	closed	Parque Estadual Carlos Botelho – São Miguel Arcanjo – SP
CFBH 15967	CFBH 15967	*F.* sp.	*F.* CS3	CS3-II	closed	Parque Estadual Turístico do Alto Ribeira – Iporanga – SP
CFBH 15968	CFBH 15968	*F.* sp.	*F.* CS3	CS3-II	closed	Parque Estadual Turístico do Alto Ribeira – Iporanga – SP
CFBHT 432	CFBH 6301	*F.* sp.	*F.* CS3	CS3-II	closed	Parque Estadual Turístico do Alto Ribeira – Iporanga – SP
CTMZ 2119 (b,c,d)	**MZUSP 135461**	***F. fissilis***	***F.* CS3**	**CS3-II**	**closed**	**Eldorado Paulista – SP**
CFBHT 2862	CFBH 11106	*F. fissilis*	*F.* CS3	CS3-II	closed	Antonina – PR
CAUSC 2626	CAUSC 2626	*F.* sp.	*F.* CS3	CS3-II	closed	Bombinhas – SC
MCP 10920	MCP 10920	*F.* sp.	*F.* CS3	CS3-II	closed	Florianópolis – SC
MCP 10921	MCP 10921	*F.* sp.	*F.* CS3	CS3-II	closed	Florianópolis – SC
CAUSC 2625	CAUSC 2625	*F.* sp.	*F.* CS3	CS3-II	closed	Santo Amaro da Imperatriz – SC
CFBHT 84 (a,c,d)	**CFBH 5726**	***F.* sp.**	***F.* CS3**	**CS3-II**	**closed**	**Santo Amaro da Imperatriz – SC**
CFBHT 11451	CFBH 23726	*F.* sp. (aff. *fissilis*)	*F.* CS3	CS3-II	closed	Siderópolis – SC
CFBHT 11453	CFBH 24277	*F.* sp. (aff. *fissilis*)	*F.* CS3	CS3-II	closed	Siderópolis – SC
CFBHT 12325	CFBH 25723	*F.* sp. (aff. *fissilis*)	*F.* CS3	CS3-II	closed	Siderópolis – SC

Tissue collection number	GenBank accession numbers				
	12S	16S	COI	RAG1	SLC8A3
CFBHT 17729	MG099091	KU991178	MG099296	MG099382	MG099460
CFBHT 17743	MG099092	MG099189	MG099297	MG099383	MG099461
MNRJ 68937	MG099067	MG099168	MG099272	MG099365	MG099446
ZUFRJ 13451	MG099101	MG099198	MG099305	MG099391	MG099470
ZUFRJ 13452	MG099102	MG099199	MG099306	MG099392	MG099471
ZUFRJ 13454	MG099103	MG099200	MG099307	MG099393	MG099472
ZUFRJ 13456	MG099104	MG099201	MG099308		
ZUFRJ 13457	MG099105	MG099202	MG099309	MG099394	MG099473
ZUFRJ 13540	MG099106	MG099203	MG099310	MG099395	MG099474
MNRJ 53758	MG099057	MG099161	MG099262	MG099359	MG099442
MNRJ 67315	MG099065	MG099166	MG099270	MG099363	MG099444
CFBHT 12889	MG099049	KU991179	MG099254	MG099351	
CFBHT 2774	MG099017	MG099125	MG099222	MG099327	
CFBHT 2775	MG099018	MG099126	MG099223	MG099328	
CFBH 16287	MG099113	MG099208	MG099315	MG099401	MG099480
CFBH 16288	MG099114	MG099209	MG099316	MG099402	MG099481
ZUFRJ 13656	MG099107	KU991169	MG099311	MG099396	MG099475
ZUFRJ 13658	MG099108	MG099204	MG099312	MG099397	MG099476
CFBHT 11557	MG099043	MG099151	MG099248		
CFBHT 5289	MG099021	MG099129	MG099226	MG099331	MG099415
CFBHT 1625	MG099014	KU991170	MG099219	MG099324	MG099411
CFBHT 5487	MG099022	MG099130	MG099227	MG099332	MG099416
CTMZ 4627 (c,d)	MG099074	**MG099174**	**MG099279**		**MG099451**
CFBHT 1252	MG099010	MG099119	MG099215	MG099320	MG099407
CFBHT 1283	MG099011	MG099120	MG099216	MG099321	MG099408
CFBHT 6528	MG099024	MG099132	MG099229	MG099334	MG099418
CFBHT 6532	MG099025	MG099133	MG099230	MG099335	MG099419
CFBHT 6533	MG099026	MG099134	MG099231		
CFBHT 6534	MG099027	MG099135	MG099232		
CFBHT 6535	MG099028	MG099136	MG099233	MG099336	MG099420
CFBHT 6536	MG099029	MG099137	MG099234	MG099337	MG099421
CFBHT 6537	MG099030	MG099138	MG099235	MG099338	MG099422
CFBHT 6538	MG099031	MG099139	MG099236	MG099339	MG099423
CFBHT 6539	MG099032	MG099140	MG099237	MG099340	MG099424
CFBHT 6540	MG099033	MG099141	MG099238	MG099341	MG099425
CFBHT 6541	MG099034	MG099142	MG099239		
CFBHT 6542	MG099035	MG099143	MG099240		
CFBHT 6543	MG099036	MG099144	MG099241		
CFBHT 6544	MG099037	MG099145	MG099242	MG099342	MG099426

CFBHT 12409	MG099048	KU991171	MG099253	MG099350	MG099434
CFBHT 14901	MG099086	MG099186	MG099291		
CFBHT 14902	MG099087	KU991172	MG099292	MG099378	
CFBHT 14903	MG099088	KU991173	MG099293	MG099379	
MNRJ 34921 (b,c,d)	MG099050	**MG099156**	**MG099255**	**MG099352**	**MG099435**
MNRJ 34922 (b,c,d)	MG099051	**MG099157**	**MG099256**	**MG099353**	**MG099436**
MNRJ 34923 (b,c,d)	MG099052	**MG099158**	**MG099257**	**MG099354**	**MG099437**
MNRJ 38397	MG099053	KU991175	MG099258	MG099355	MG099438
MNRJ 38398	MG099054	MG099159	MG099259	MG099356	MG099439
MNRJ 40700	MG099055	KU991174	MG099260	MG099357	MG099440
MNRJ 56057	MG099058	MG099162	MG099263		
MNRJ 56058	MG099059	MG099163	MG099264		
MNRJ 56061	MG099060	KU991176	MG099265		
MNRJ 56062	MG099061	KU991177	MG099266		
MNRJ 56942	MG099063	MG099165	MG099268	MG099361	
MNRJ 62845 (d)	MG099064	**KU991167**	**MG099269**	**MG099362**	
MNRJ 74620 (d)	MG099068	**KU991168**	**MG099273**	**MG099366**	**MG099447**
MNRJ 74621	MG099069	MG099169	MG099274	MG099367	MG099448
CFBHT 14184	MG099095	MG099192	MG099300	MG099386	MG099464
CFBHT 14208	MG099096	MG099193		MG099387	MG099465
CFBHT 14317	MG099097	MG099194	MG099301		MG099466
MNRJ 67316	MG099066	MG099167	MG099271	MG099364	MG099445
ZUFRJ 13347	MG099100	MG099197	MG099304	MG099390	MG099469
MNRJ 44622 (d)	MG099056	**MG099160**	**MG099261**	**MG099358**	**MG099441**
MNRJ 56922 (d)	MG099062	**MG099164**	**MG099267**	**MG099360**	**MG099443**
CFBHT 12369	MG099046	MG099154	MG099251	MG099348	MG099432
CFBHT 12395	MG099047	MG099155	MG099252	MG099349	MG099433
MZUFV 11721	MG099098	MG099195	MG099302	MG099388	MG099467
MZUFV 11722	MG099099	MG099196	MG099303	MG099389	MG099468
CFBHT 14886	MG099076	MG099176	MG099281	MG099371	MG099453
CFBHT 14887	MG099077	MG099177	MG099282	MG099372	MG099454
CFBHT 14888	MG099078	MG099178	MG099283	MG099373	
CFBHT 14889	MG099079	MG099179	MG099284	MG099374	MG099455
CFBHT 14890	MG099080	MG099180	MG099285	MG099375	MG099456
CFBHT 14891	MG099081	MG099181	MG099286	MG099376	MG099457
CFBHT 14892	MG099082	MG099182	MG099287	MG099377	MG099458
CFBHT 14893	MG099083	MG099183	MG099288		
CFBHT 14894	MG099084	MG099184	MG099289		
CFBHT 14895	MG099085	MG099185	MG099290		
CTMZ 1563 (b,d)	MG099071	**MG099171**	**MG099276**		
MCNT 154	MG099070	MG099170	MG099275	MG099368	MG099449
CFBHT 11609	MG099044	MG099152	MG099249		
CFBHT 1459	MG099012	MG099121	MG099217	MG099322	MG099409
CFBHT 1461	MG099013	MG099122	MG099218	MG099323	MG099410
CFBH 39057	MG099093	MG099190	MG099298	MG099384	MG099462
CFBH 39058	MG099094	MG099191	MG099299	MG099385	MG099463
CFBHT 10526	MG099039	MG099147	MG099244	MG099344	MG099428
CFBHT 3863	MG099020	MG099128	MG099225	MG099330	MG099414
CFBHT 5510	MG099023	MG099131	MG099228	MG099333	MG099417
CTMZ 7295	MG099075	MG099175	MG099280	MG099370	MG099452
CFBHT 7392	MG099038	MG099146	MG099243	MG099343	MG099427
CFBHT 10989	MG099040	MG099148	MG099245		
CFBHT 2359	MG099015	MG099123	MG099220	MG099325	
CFBHT 2432	MG099016	MG099124	MG099221	MG099326	MG099412
CTMZ 2618	MG099073	MG099173	MG099278		MG099450
CFBH 15967	MG099115	MG099210	MG099317	MG099403	
CFBH 15968	MG099116	MG099211	MG099318	MG099404	MG099482
CFBHT 432	MG099009	MG099118	MG099214	MG099319	MG099406
CTMZ 2119 (b,c,d)	MG099072	**MG099172**	**MG099277**	**MG099369**	
CFBHT 2862	MG099019	MG099127	MG099224	MG099329	MG099413
CAUSC 2626	MG099112	MG099207	MG099314	MG099400	MG099479
MCP 10920	MG099089	MG099187	MG099294	MG099380	MG099459
MCP 10921	MG099090	MG099188	MG099295	MG099381	
CAUSC 2625	MG099111	MG099206	MG099313	MG099399	MG099478
CFBHT 84 (a,c,d)	MG099008	**MG099117**	**MG099213**		**MG099405**
CFBHT 11451	MG099041	MG099149	MG099246	MG099345	MG099429
CFBHT 11453	MG099042	MG099150	MG099247	MG099346	MG099430
CFBHT 12325	MG099045	MG099153	MG099250	MG099347	MG099431

Appendix B

GenBank accession numbers and voucher information of the species used as outgroups.

Taxon	Voucher in GenBank	12S	16S	COI	RAG1	SLC
Cryptobatrachus remotus 1	MHNLS17664	KR559913	KR270399; KR270415	–	KR138419	KR270374
Cryptobatrachus remotus 2	MHNLS18853	KR559914	KR270400; KR270416	–	KR138420	KR270375
Cryptobatrachus sp.	TNHCGDC451	JX564861	JX564861	JX564861	–	–
Flectonotus fitzgeraldi	ZSM16102006	–	KR270417	–	KR138421	KR270376
Flectonotus pygmaeus	MHNLS17478		KR270402, KR270418	–	–	–
Gastrotheca albolineata	MNRJ54401		KR270407, KR270425	MG099212	KR138423	KR270380
Gastrotheca ernestoi	MNRJ57129	KR559920	KR270408; KR270427	–	KR138424	KR270381
Gastrotheca pseustes	TNHC62492	JX564866	JX564866	JX564866	–	–
Hemiphractus scutatus/fasciatus	JMP2150/EVACC031	KR559923	KR270432, KR270411	KC014721	KR138426	KR270383
Hemiphractus helioi	MHNCP9063	KR559922	KR270410, KR270431	–	KR138425	KR270382
Stefania evansi	IRSNB14586	–	KR270433	–	KR138427	KR270384
Stefania scalae	MHNLS17152	–	KR270434	–	KR138428	KR270385
Stefania roraimae	USNM49319	MG099110	MG099205	–	MG099398	MG099477
Pristimantis cruentus		EF493697	EF493697	KC129261	AY948935	AY948898

Appendix C

Primers and cycling conditions used to amplify mitochondrial and nuclear genes in *Fritziana* specimens. Each PCR reaction was performed in a final volume of 25 μL and contained 1 μL of genomic DNA, 2.5 μL of 10x PCR buffer, 0.5 μL (10 pmol) of each primer, 0.5 μL (10 mM) of dNTP, 0.5 μL of magnesium (50 mM), 19.4 μL H2O milliQ and 0.125 μL (5 U/μL) of Taq DNA polymerase (Thermo Scientific Inc.).

Gene	Primers		Sequence (5′ → 3′)	PCR reaction protocol	References
16S	16Sar-L	F	CGCCTGTTTATCAAAAACAT	1 cycle: 3 min 94 °C	Palumbi et al. (1991)
16S	16Sbr-H	R	CCGGTCTGAACTCAGATCACGT	35 cycles: 20 s 94 °C, 20 s 50 °C, 1 min 68 °C	Palumbi et al. (1991)
16S	16sTitus	R	GGTGGCTGCTTTTAGGCC	1 cycle: 5 min 68 °C	Titus and Larson (1996)
16S	H10	F	TGCTTACGCTACCTTTGCACGGT		Titus and Larson (1996)
16S	12sL13	F	TTAGAAGAGGCAAGTCGTAACATGGTA		Feller and Hedges (1998)
16S	16SL2a	R	CCAAACGAGCCTAGTGATAGCTGGTT		Hedges (1994)
12S	12S-H978	R	CTTACCRTGTTACGACTTRCCT	1 cycle: 3 min 94 °C	Present work
12S	12S L48	F	ATGCAAGYMTCMGCRYCCNGTGA	35 cycles: 20 s 94 °C, 20 s 50 °C, 1 min:30 s 68 °C	Present work
12S	MVZ59	F	ATAGCACGTAAAAYGCTDAGATG	1 cycle: 5 min 68 °C	Graybeal (1997).
12S	FH	R	CTTGGCTCGTAGTTCCCTGGCG		Goebel et al. (1999)
COI	AnF1	F	ACHAAYCAYAAAGAYATYGG	*UP reaction – Lyra et al. (2016)	Lyra et al. (2016).
COI	AnR1	R	CCRAARAATCARAADARRTGTTG		Lyra et al. (2016)
COI	AnW	F	AGACCAARRGCCTTCAAAG		Lyra et al. (2016)
RAG1	Amp-RAG-1F	F	AGCTGCAGYCARTACCAYAARATGTA	1 cycle: 3 min 94 °C	Wiens et al. (2007)
RAG1	Hemi-RAG1-R	R	CTCTGCAGCATTTCCAATGTCAC	45 cycles: 20 s 94 °C, 20 s 52 °C, 1 min 72 °C; 1 cycle: 5 min 72 °C	Blackburn and Duellman (2013)
SLC8A3	SLC-F1	F	TAACRTCRCAAGAACGRGAAAT	1 cycle: 3 min 94 °C	Castroviejo-Fisher et al. (2015)
SLC8A3	SLC-R1	R	CCATCTAGAAAATGAGAATTCA	45 cycles: 20 s 94 °C, 20 s 52 °C, 1 min 72 °C; 1 cycle: 5 min 72 °C	Castroviejo-Fisher et al. (2015)

Appendix D. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.02.012.

References

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automat. Contr. 19, 716–723. http://dx.doi.org/10.1109/TAC.1974.1100705.

Akçakaya, H., Ferson, S., Burgman, M., Keith, D., Mace, G., Todd, C., 2000. Making consistent IUCN classifications under uncertainty. Conserv. Biol. 14, 1001–1013. http://dx.doi.org/10.1046/j.1523-1739.2000.99125.x.

Beçak, M.L., 1968. Chromosomal analysis of eighteen species of Anura. Caryologia 21, 191–208. http://dx.doi.org/10.1080/00087114.1968.10796299.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K., Das, I., 2007. Cryptic species as a window on diversity and conservation. Trends Ecol. Evol. 22, 148–155. http://dx.doi.org/10.1016/j.tree.2006.11.004.

Blackburn, D.C., Duellman, W.E., 2013. Brazilian marsupial frogs are diphyletic (Anura: Hemiphractidae: Gastrotheca). Mol. Phylogenet. Evol. 68, 709–714. http://dx.doi.org/10.1016/j.ympev.2013.04.021.

Bogart, J.P., 1973. Evolution of anuran karyotypes. In: Vial, J.L. (Ed.), Evolutionary Biology of Anurans. University of Missouri Press, Columbia, pp. 337–349.

Bokermann, W.C.A., 1950. Redescrição e novo nome genérico para *Coelonotus fissilis* Mir.-Rib. 1920. Papéis Avulsos do Departamento de Zoologia 9, 215–222.

Bokermann, W.C.A., 1966. Lista anotada das localidades tipo de anfíbios brasileiros. Impresso pelo Serviço de Documentação, Reitoria da Universidade de São Paulo, São Paulo 183 p.

Boore, J.L., 1999. Animal mitochondrial genomes. Nucl. Acids Res. 27, 1767–1780. http://dx.doi.org/10.1093/nar/27.8.1767.

Boulenger, G.A., 1895. Third report on additions to the batrachian collection in the Natural-History Museum. Proc. Zool. Soc. Lond. 1894, 640–646.

Brown, S.D.J., Collins, R.A., Boyer, S., Lefort, M.C., Malumbres-Olarte, J., Vink, C.J., Cruickshank, R.H., 2012. Spider: an R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. Mol. Ecol. Resour. 12, 562–565. http://dx.doi.org/10.1111/j.1755-0998.2011.03108.x.

Castroviejo-Fisher, S., Padial, J.M., De La Riva, I., Pombal, J.J.P., Silva, H.R., Rojas-Runjaic, F.J.M., Medina-Méndez, E., Frost, D.R., 2015. Phylogenetic systematics of egg-brooding frogs (Anura: Hemiphractidae) and the evolution of direct development. Zootaxa 4004, 1–75. http://dx.doi.org/10.11646/zootaxa.4004.1.1.

Crawford, A.J., Lips, K.R., Bermingham, E., 2010. Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. Proc. Natl. Acad. Sci. 107, 13777–13782. http://dx.doi.org/10.1073/pnas.0914115107.

Darst, C.R., Cannatella, D.C., 2004. Novel relationships among hyloid frogs inferred from 12S and 16S mitochondrial DNA sequences. Mol. Phylogenet. Evol. 31, 462–475. http://dx.doi.org/10.1016/j.ympev.2003.09.003.

Del Pino, E.M., 1980. Morphology of the pouch and incubatory integument in marsupial frogs (Hylidae). Copeia 1980, 10–17. http://dx.doi.org/10.2307/1444129.

Duellman, W.E., 1970. The hylid frogs of Middle America. In: Monograph of the Museum of Natural History, University of Kansas, vol. 1–2, pp. 1–753.

Duellman, W.E., Jungfer, K.H., Blackburn, D.C., 2011. The phylogenetic relationship of geographically separated "Flectonotus" (Anura: Hemiphractidae), as revealed by molecular, behavioral, and morphological data. Phyllomedusa 10, 29–43. http://dx.doi.org/10.11606/issn.2316-9079.v10i1p15-29.

Duelmann, W.E., Gray, P., 1983. Developmental biology and systematics of the egg-brooding hylid frogs genera *Flectonotus* and *Fritziana*. Herpetologica 39, 333–359.

Faivovich, J., Haddad, C.F.B., Garcia, P.C.A., Frost, D.R., Campbell, J.A., Wheeler, W.C., 2005. Systematic review of the frog family Hylidae, with special reference to Hylinae: phylogenetic analysis and taxonomic revision. Bull. Am. Museum Nat. Hist. 294, 1–240. http://dx.doi.org/10.1206/0003-0090(2005) 294[0001:SROTFF]2.0.CO;2.

Feller, A.E., Hedges, S.B., 1998. Molecular evidence for the early history of living amphibians. Mol. Phylogenet. Evol. 9, 509–516. http://dx.doi.org/10.1006/mpev.1998.0500.

Felsenstein, J., 1989. Mathematical evolutionary theory. Science 246, 941–943.

Flot, J.F., 2010. Seqphase: a web tool for interconverting phase input/output files and fasta sequence alignments. Mol. Ecol. Resour. 10, 162–166. http://dx.doi.org/10.1111/j.1755-0998.2009.02732.x.

Folly, M., Hepp, F., Carvalho-e-Silva, S.P., Duellman, W.E., 2014. Taxonomic status and redescription of *Flectonotus ulei* (Anura: Hemiphractidae), with a key for the species of *Fritziana*. Zoologia 31, 393–399. http://dx.doi.org/10.1590/S1984-46702014000400011.

Folly, M., Hepp, F., Carvalho-e-Silva, S.P., 2018. A new bromeligenous species of *Fritziana* Mello-Leitão, 1937 (Amphibia: Anura: Hemiphractidae) from high elevations in the Serra dos Órgãos, Rio de Janeiro, Brazil. Herpetologica 74, 58–72.

Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M., Gemmell, N.J., 2007. Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. PLoS One 2, 1–10. http://dx.doi.org/10.1371/journal.pone.0001109.

Franz, I., Mello, M.H., 2015. *Fritziana* aff. *fissilis* (Miranda-Ribeiro, 1920) (Anura, Hemiphractidae): the first hemiphractid for the state of Rio Grande do Sul, southern Brazil. Check List 11, 3–5. http://dx.doi.org/10.15560/11.2.1594.

Frost, D.R., 2017. Amphibian species of the world: an online reference. Version 6.0. (2017, March 12). American Museum of Natural History, New York, USA. Electronic database accessible at http://research.amnh.org/herpetology/amphibia/index.html.

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., De Sá, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C., 2008. Is the amphibian tree of life really fatally flawed? Cladistics 24, 385–395. http://dx.doi.org/10.1111/j.1096-0031.2007.00181.x.

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., Sá, R.O., Channing, A., Donnellan, S.C., Raxworthy, C.J., Campbell, A.J., Blotto, B.L., Moler, P., Drewes, R.C., Ronald, A., Lynch, J.D., Green, D.M., Wheeler, W.C., Wilkinson, M., 2006. The amphibian tree of life. Bull. Am. Museum Nat. Hist. 297, 1–370. http://dx.doi.org/10.1206/0003-0090(2006) 297.

Gehara, M., Canedo, C., Haddad, C.F.B., Vences, M., 2013. From widespread to microendemic: molecular and acoustic analyses show that *Ischnocnema guentheri* (Amphibia: Brachycephalidae) is endemic to Rio de Janeiro. Brazil. Conserv. Genet. 14, 973–982. http://dx.doi.org/10.1007/s10592-013-0488-5.

Glaw, F., Köhler, J., 1998. Amphibian species diversity exceeds that of mammals. Herpetol. Rev. 29, 11–12.

Graybeal, A., 1997. Phylogenetic relationships of bufonid frogs and tests of alternate macroevolutionary hypotheses characterizing their radiation. Zool. J. Linn. Soc. 119, 297–338. http://dx.doi.org/10.1111/j.1096-3642.1997.tb00139.x.

Goebel, A.M., Donnelly, J.M., Atz, M.E., 1999. PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I, and cytochromebin bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. Mol. Phylogenet. Evol. 11, 163–199. http://dx.doi.org/10.1006/mpev.1998.0538.

Guayasamin, J.M., Castroviejo-Fisher, S., Ayarzagüena, J., Trueb, L., Vilà, C., 2008. Phylogenetic relationships of glassfrogs (Centrolenidae) based on mitochondrial and nuclear genes. Mol. Phylogenet. Evol. 48, 574–595. http://dx.doi.org/10.1016/j.

ympev.2008.04.012.

Haas, A., 2003. Phylogeny of frogs as inferred from primarily larval characters (Amphibia: Anura). Cladistics 19, 23–89. http://dx.doi.org/10.1016/S0748-3007(03)00006-9.

Haddad, C.F.B., Prado, C.P.deA., 2005. Reproductive modes in frogs and their unexpected diversity in the Atlantic Forest of Brazil. BioScience 55, 207–217 doi:10.1641/0006-3568(2005)055[0207:RMIFAT]2.0.CO;2.

Hanken, J., 1999. Why are there so many new amphibian species when amphibians are declining? Trends Ecol. Evol. 14, 7–8. http://dx.doi.org/10.1016/S0169-5347(98)01534-1.

Hedges, S.B., 1994. Molecular evidence for the origin of birds Proc. Natl. Acad. Sci. USA 91, 2621–2624.

Huelsenbeck, J., Rannala, B., 2004. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. Syst. Biol. 53, 904–913. http://dx.doi.org/10.1080/10635150490522629.

IUCN 2016. The IUCN Red List of Threatened Species. Version 2016-3. Electronic Database accessible at http://www.iucnredlist.org (accessed 12 March 2017).

Jansen, M., Bloch, R., Schulze, A., Pfenninger, M., 2011. Integrative inventory of Bolivia's lowland anurans reveals hidden diversity. Zool. Scr. 40, 567–583. http://dx.doi.org/10.1111/j.1463-6409.2011.00498.x.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. http://dx.doi.org/10.1093/molbev/mst010.

Köhler, J., Vieites, D.R., Bonett, R.M., García, F.H., Glaw, F., Steinke, D., Vences, M., 2005. New amphibians and global conservation: a boost in species discoveries in a highly endangered vertebrate group. BioScience 55, 693–696. http://dx.doi.org/10.1641/0006-3568(2005) 055[0693:NAAGCA]2.0.CO;2.

Kurabayashi, A., Sumida, M., Yonekawa, H., Glaw, F., Vences, M., Hasegawa, M., 2008. Phylogeny, recombination, and mechanisms of stepwise mitochondrial genome reorganization in mantellid frogs from Madagascar. Mol. Biol. Evol. 25, 874–891. http://dx.doi.org/10.1093/molbev/msn031.

Lanfear, R., Calcott, B., Ho, S.Y.W., Guindon, S., 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol. Biol. Evol. 29, 1695–1701. http://dx.doi.org/10.1093/molbev/mss020.

Lyra, M.L., Haddad, C.F.B., Azeredo-Espin, A.M.L., 2016. Meeting the challenge of DNA barcoding Neotropical amphibians: polymerase chain reaction optimization and new COI primers. Mol. Ecol. Resour. 7–126. http://dx.doi.org/10.1111/1755-0998.12648.

Maniatis, T., Fritsch, E., Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual Vol. 545 Cold Spring harbor laboratory, Cold Spring Harbor, NY.

Menegucci, R.C., Gaia, R., Machado, I.F., 2017. *Fritziana ulei* (Miranda-Ribeiro, 1926): geographic extension, with comments on the natural history of this species. Check List 13, 1–3. http://dx.doi.org/10.15560/13.2.2061.

Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, pp. 1–8.

Miralles, A., Vences, M., 2013. New metrics for comparison of taxonomies reveal striking discrepancies among species delimitation methods in *Madascincus* lizards. PLoS ONE 8. http://dx.doi.org/10.1371/journal.pone.0068242.

Miranda-Ribeiro, A., 1920. As *Hyla* coelonotas do Museu Paulista. Revista do Museu Paulista, São Paulo 12, 323–328.

Miranda-Ribeiro, A., 1926. Notas para servirem ao estudo dos Gymnobatrachios (Anura) brasileiros. Arquivos do Museu Nacional do Rio de Janeiro 27, 1–227.

Padial, J.M., Chaparro, J., Castroviejo-Fisher, S., Guayasamin, J.M., Lehr, E., Delgado, A.J., Vaira, M., Teixeira, M., Aguayo, R., De La Riva, I., 2012. A revision of species diversity in the Neotropical genus *Oreobates* (Anura: Strabomantidae), with the description of three new species from the Amazonian slopes of the Andes. Am. Museum Novit. 3752, 1–55. http://dx.doi.org/10.1206/3752.2.

Padial, J.M., Miralles, A., De la Riva, I., Vences, M., 2010. The integrative future of taxonomy. Front. Zool. 7, 16. http://dx.doi.org/10.1186/1742-9994-7-16.

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabawski, G., 1991. The Simple Fool's Guide to PCR, Version 2.0. Privately published, compiled by S. Palumbi. University of Hawaii, Honolulu.

Peixoto, M.A.A., Guimarães, C.S., Da Silva, D.H., Feio, R.N., 2016. The occurrence of *Fritziana* Mello-Leitão, 1937 (Anura, Hemiphractidae) in Minas Gerais state, southeastern Brazil: new records and distribution of the genus. Check List 12, 1947. http://dx.doi.org/10.15560/12.4.1947.

Peloso, P.L.V., Orrico, V.G.D., Haddad, C.F.B., Lima-Filho, G.R., Sturaro, M.J., 2016. A new species of clown tree frog, *Dendropsophus leucophyllatus* species group, from Amazonia (Anura, Hylidae). South Am. J. Herpetol. 11, 66–80. http://dx.doi.org/10.2994/SAJH.

Pyron, R.A., Wiens, J.J., 2011. A large-scale phylogeny of Amphibia including over 2,800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol. Phylogenet. Evol. 61, 543–583. http://dx.doi.org/10.1016/j.ympev.2011.06.012.

R Core Team, 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria http://www.R-project.org/.

Rambaut, A., Suchard, M., Drummond, A. 2009. Tracer v1.6. http://tree.bio.ed.ac.uk/software/tracer/.

Rambaut, A., Drummond, A. 2009. FigTree, ver. 1.4.3. http://tree.bio.ed.ac.uk/software/figtree/.

Ratnasingham, S., Hebert, P.D., 2007. BOLD: the barcode of life data system (http://www. barcodinglife. org). Mol. Ecol. Notes 7, 355–364.

Rodríguez, A., Börner, M., Pabijan, M., Gehara, M., Haddad, C.F., Vences, M., 2015. Genetic divergence in tropical anurans: deeper phylogeographic structure in forest specialists and in topographically complex regions. Evol. Ecol. 29, 765–785. http://dx.doi.org/10.1007/s10682-015-9774-7.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. Mrbayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542. http://dx.doi.org/10.1093/sysbio/sys029.

Salzburger, W., Ewing, G.B., Von Haeseler, A., 2011. The performance of phylogenetic algorithms in estimating haplotype genealogies with migration. Mol. Ecol. 20, 1952–1963. http://dx.doi.org/10.1111/j.1365-294X.2011.05066.x.

Schattner, P., Brooks, A.N., Lowe, T.M., 2005. The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. Nucl. Acids Res. 33, 686–689. http://dx.doi.org/10.1093/nar/gki366.

Schmid, M., Steinlein, C., Bogart, J.P., Feichtinger, W., Haaf, T., Nanda, I., del Pino, E.M., Duellman, W.E., Hedges, S.B., 2012. The hemiphractid frogs – phylogeny, embryology, life history, and cytogenetics. Cytogenet. Genome Res. 138, 2–4.

Stephens, M., Smith, N.J., Donnelly, P., 2001. A new statistical method for haplotype reconstruction from population data. Am. J. Hum. Genet. 68, 978–989. http://dx.doi.org/10.1086/319501.

Stuart, B.L., Inger, R.F., Voris, H.K., 2006. High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. Biol. Lett. 2, 470–474. http://dx.doi.org/10.1098/rsbl.2006.0505.

Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S.L., Fischman, D.L., Waller, R.W., 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306, 1783–1786. http://dx.doi.org/10.1126/science.1103538.

Titus, T.A., Larson, A., 1996. Molecular phylogenetics of desmognathine salamanders (Caudata: Plethodontidae): a reevaluation of evolution in ecology, life history, and morphology. Syst. Biol. 45, 451–472. http://dx.doi.org/10.1093/sysbio/45.4.451.

Vaidya, G., Lohman, D.J., Meier, R., 2011. SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27, 171–180. http://dx.doi.org/10.1111/j.1096-0031.2010.00329.x.

Vieites, D.R., Wollenberg, K.C., Andreone, F., Köhler, J., Glaw, F., Vences, M., 2009. Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. Proc. Natl. Acad. Sci. U. S. A. 106, 8267–8272. http://dx.doi.org/10.1073/pnas.0810821106.

Walker, M., Gasparini, J.L., Haddad, C.F.B., 2016. A new polymorphic species of egg-brooding frog of the genus *Fritziana* from southeastern Brazil (Anura: Hemiphractidae). Salamandra 52, 221–229.

Wandolleck, B., 1907. Einige neue und weniger bekannte Batrachier von Brazilien. Abhandlungen und Berichte des Zool. und Anthropol. Museums zu Dresden, pp. 1–15.

Weygoldt, P., Carvalho-e-silva, S.P., 1991. Observations on mating, oviposition, egg sac formation and development in the egg-brooding frog, *Fritziana goeldii*. Amphibia-Reptilia 12, 67–80.

Wiens, J.J., Fetzner, J.W., Parkinson, C.L., Reeder, T.W., 2005. Hylid frog phylogeny and sampling strategies for speciose clades. Syst. Biol. 54, 778–807. http://dx.doi.org/10.1080/10635150500234625.

Wiens, J.J., Kuczynski, C.A., Duelmann, W.E., Reeder, T.W., 2007. Loss and re-evolution of complex life cycles in marsupial frogs: does ancestral trait reconstruction mislead? Evolution 61, 1886–1899. http://dx.doi.org/10.1111/j.1558-5646.2007.00159.x.

Xia, Y., Zheng, Y., Miura, I., Wong, P.B.Y., Murphy, R.W., Zeng, X., 2014. The evolution of mitochondrial genomes in modern frogs (Neobatrachia): nonadaptive evolution of mitochondrial genome reorganization. BMC Genom. 15, 691. http://dx.doi.org/10.1186/1471-2164-15-691.

Zhang, P., Liang, D., Mao, R.L., Hillis, D.M., Wake, D.B., Cannatella, D.C., 2013. Efficient sequencing of anuran mtDNAs and a mitogenomic exploration of the phylogeny and evolution of frogs. Mol. Biol. Evol. 30, 1899–1915.