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The phylogenetic relationship of geographically separated "*Flectonotus*" (Anura: Hemiphractidae), as revealed by molecular, behavioral, and morphological data

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Abstract

The phylogenetic relationship of geographically separated "*Flectonotus*" (Anura: Hemiphractidae), as revealed by molecular, behavioral, and morphological data. Phylogenetic analyses of data derived from one mitochondrial gene and one nuclear gene show that the five species of small marsupial frogs currently recognized as *Flectonotus* are in fact two distinct and not closely related lineages. This conclusion is strongly supported by reproductive behavior and morphological characters. Thus, we recognize the genus *Fritziana* Mello-Leitão for the three species in southeastern Brazil and *Flectonotus* Miranda-Ribeiro for the two species in northern South America.

Keywords: Anura, Hemiphractidae, *Flectonotus, Fritziana,* molecular phylogenetics, reproductive behavior, morphology.

Resumo

Relações filogenéticas entre espécies de "*Flectonotus*" (Anura: Hemiphractidae) isoladas geograficamente reveladas por dados moleculares, de comportamento e morfológicos. Análises filogenéticas de dados derivados de um gene mitocondrial e um gene nuclear mostram que as cinco espécies de pererecas-marsupiais de pequeno porte atualmente incluídas no gênero *Flectonotus* pertencem, na verdade, a duas linhagens distintas e não intimamente aparentadas. Essa conclusão é fortemente sustentada por caracteres morfológicos e características do comportamento reprodutivo. Dessa forma, reconhecemos os gêneros *Fritziana* Mello-Leitão, para as três espécies do sudeste do Brasil, e *Flectonotus* Miranda-Ribeiro, para as duas espécies do norte da América do Sul.

Palavras-chave: Anura, Hemiphractidae, *Flectonotus, Fritziana,* comportamento reprodutivo, filogenética molecular, morfologia.

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Introduction

For more than a quarter of a century, the systematics of the small egg-brooding hemiphractid frogs has been unstable. Duellman and Gray (1983) recognized two genera, *Flectonotus* and *Fritziana,* based on differences in morphology, development, and karyology. In a paper dealing with the reproductive behavior of *Fritziana goeldii,* Weygoldt and Carvalho e Silva (1991) argued that *Fritziana* should be considered a synonym of *Flectonotus*. Their argument is based on the discussion of phylogenetic relations provided by Duellman and Gray (1983) that posited that *Fritziana* might be paraphyletic with respect to *Flectonotus.* However, Duellman and Gray (1983) discussed only one of five equally parsimonious trees. Re-analysis of the data matrix presented by Duellman and Gray (1983) reveals that their data do not resolve the relationships of three species of *Fritziana* (Figure 1). In the most comprehensive molecular phylogeny of the Hemiphractidae (Wiens *et al.* 2007), no samples were included of the three Brazilian taxa previously assigned to *Fritziana*. However, the two species that Duellman and Gray (1983) recognized as *Flectonotus* (*F. fitzgeraldi* and *F. pygmaeus*) formed a well-supported clade sister to all other hemiphractids. The phylogenetic analysis of Wiens *et al.* (2007) largely supports earlier work (e.g., Duellman and Hillis 1987, Duellman *et al.* 1988) suggesting that direct development is the basal condition in hemiphractid frogs and that the presence of free-living tadpoles is a derived reproductive mode, possibly as a result of arrested development (Wassersug and Duellman 1984). Herein we report on the results of the first molecular phylogenetic analysis to incorporate data from all three Brazilian species formerly referred to *Fritziana.* Our molecular phylogeny is complemented by detailed observations on the reproductive behavior of one species of *Flectonotus* and one of *Fritziana,* and a brief review of morphological differences between the Venezuelan and Brazilian species. Taken together, these phylogenetic, reproductive, and morphological data support the recognition of two genera. For ease of comparison, throughout this work we use the generic names *Flectonotus* (for *F. fitzgeraldi* and *F. pygmaeus*) and *Fritziana* (for *F. fissilis*, *F. goeldii*, and *F. ohausi*).

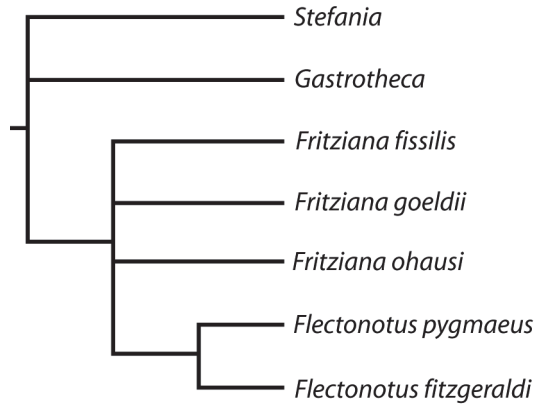

Figure 1. Strict consensus of five equally parsimonious trees (7 parsimony-informative characters; 14 steps; for each tree, consistency index = 1.00, retention index = 1.00) that explain the distribution of character states observed by Duellman and Gray (1983). Parsimony analysis conducted using a heuristic search with starting tree obtained via stepwise addition and tree-bisection-reconnection in PAUP v. 4.0b10 (Swofford, 2003).

Materials and Methods

We determined the phylogenetic relationships of the Brazilian taxa through analysis of DNA sequence data. We obtained data from specimens of all three recognized species of *Fritziana.* We collected data for one mitochondrial (16S ribosomal RNA) and one nuclear locus (proopiomelanocortin, or POMC). Genomic DNA was extracted using a guanidine thiocyanate method (Esselstyn *et al.* 2008) and the genomic regions of interest were amplified using polymerase chain reaction (PCR). Primers pairs used are as follows: mitochondrial 16S—16Sc and 16Sd (Darst and Cannatella 2004); POMC—POMC-1

and POMC-7 (Wiens *et al.* 2005, Smith *et al.* 2007). Both mitochondrial and nuclear genes were amplified with the following PCR conditions: 95˚C (3 min); 35 cycles of 95˚C (30 sec), 55˚C (30 sec), 72˚C (1 min); 72˚C (5 min). Purification and sequencing follows Esselstyn *et al.* (2008), and all newly collected sequence data are accessioned in GenBank. Resulting sequence lengths and GenBank accession numbers are provided in Table 1.

To test the phylogenetic relationships of the Brazilian taxa, we assembled a dataset with broad taxonomic coverage based on available data in GenBank. Rather than assume the monophyly of Hemiphractidae, we sampled throughout extant anuran diversity for relevant data that were available. We attempted to sample most genera for which data were available for the nuclear gene POMC. For these same taxa, we then compiled sequences of the mitochondrial 16S ribosomal RNA gene. In a few cases, we included 16S sequences for taxa for which data for POMC were unavailable. When the information was available, we used 16S data for the same individual from which POMC data were collected. Details on the taxa and the corresponding GenBank sequences used are provided in the Appendix I.

Multiple alignments of 16S and POMC data were generated using MUSCLE (Edgar 2004) with minor adjustments made by eye; the alignments used for analysis are deposited in Dryad (doi 10.5061/dryad.qq877). We analyzed the data using a single partition for 16S and partitioned by codon position for POMC (i.e., 3 partitions). Using the Akaike information criterion (AIC) and jModeltest v.0.1.1 (Posada 2008), we selected the following as the best-fit models of sequence evolution for these four partitions: 16S, GTR + Γ (lnL = –23923.04; AIC = 48208.07; vs. next best, GTR + I + Γ: lnL = –23924.41, AIC = 48212.82); POMC—position 1, GTR + Γ (lnL = –1781.25, AIC = 3936.50; vs. next best, GTR + I + Γ: lnL = –1786.36, AIC = 3938.72); POMC—position 2, GTR + Γ (lnL = –5789.74, AIC = 11953.47; vs. next best, GTR + I + Γ: lnL = –5803.50, AIC = 11982.99); POMC—position 3, GTR + Γ (lnL = –2210.48, AIC = 4794.97; vs. next best, GTR + I + Γ: –lnL = 2214.19, AIC = 4804.37).

We estimated phylogenetic relationships using both maximum-likelihood (ML) and Bayesian methods. We conducted a single analysis that combined the 16S and POMC data (4 partitions total). ML analyses were conducted on the aligned sequence data in RAxML ver. 7.0.4 (Stamatakis 2006) using a random starting tree, the faster rapid hill-climbing algorithm proposed by Stamatakis *et al.* (2007), and the GTR + Γ model of sequence evolution for each partition.

Table 1. Newly collected data of *Fritziana* analyzed in the phylogenetic analysis.

Species	Collection No.	16S	GenBank–16S	POMC	GenBank–POMC	Country
F. fissilis	CTMZ 02119 (MZUSP 135461)	826 bp	JN157630	431 bp	JN157628	Brazil
F. fissilis	CTMZ 01563 (MZUSP 133700)	835 bp	JN157634	490 bp	JN157627	Brazil
F. goeldii	MNRJ 34921	868 bp	JN157631	n/a	n/a	Brazil
F. goeldii	MNRJ 34922	799 bp	JN157632	n/a	n/a	Brazil
F. goeldii	MNRJ 34923	866 bp	JN157633	n/a	n/a	Brazil
F. ohausi	CTMZ 04627 (MZUSP 139225)	824 bp	JN157635	451 bp	JN157629	Brazil

ML analyses used 1000 search repetitions and we used the phylogenetic estimate with the smallest –ln likelihood score as the preferred ML phylogeny. We performed 1000 nonparametric bootstrap replicates in RAxML with the same model of sequence evolution with one search replicate per bootstrap replicate and a random starting tree; branch lengths and model parameters were optimized during the bootstrap analysis. Split support was calculated using SumTrees (Sukumaran and Holder 2008). We obtained a Bayesian estimate of phylogenetic relationships using MrBayes ver. 3.1.2 and the GTR + Γ model of sequence evolution for each partition. Bayesian analyses used four runs of four MCMC chains run for 12 million, sampled every 2000 generations, and using a temperature of 0.2 and default priors. Following examination of trends and distributions of log-likelihoods and parameter values using Tracer ver. 1.5 (Rambaut and Drummond 2009a) and convergence in AWTY (Nylander *et al.* 2008), we discarded the first six million generations; estimated sample sizes (ESS) from the four combined runs were all above 300. The phylogenies were rooted using the salamander *Plethodon cinereus* as an outgroup.

Reproduction and larval behavior of *Fritziana goeldii* and *Flectonotus pygmaeus* were observed in captive specimens housed in different terraria about 80 × 55 × 80 cm in size, equipped with twigs and plants, especially bromeliads containing water in their leaf axils, at 19–24°C. Individuals of *Fritziana goeldii* were captive-bred offspring of frogs used by Weygoldt (1989) and Weygoldt and Carvalho e Silva (1991) originating from Rio de Janeiro, Estado Rio de Janeiro, Brazil. *Flectonotus pygmaeus* were from the Maracay–Ocumare de la Costa Road, 650 m asl, Estado Aragua, Venezuela, and their captive-bred offspring. Egg laying in *Fritziana goeldii* was observed more than eight times, that of *Flectonotus pygmaeus* five times, one filmed with a Sony camcorder DCR-TRV 120E in infrared ("Night-Shot") mode.

The following acronyms are used for Brazilian collections: CFBH = Célio F. B. Haddad, Rio Claro, MNRJ = Museu Nacional Río de Janeiro, MZUSP = Museu de Zoologia, Universidade de São Paulo.

Results

Molecular Phylogenetic Analysis

The ML phylogeny based on analysis of the combined 16S and POMC data resolves Hemiphractidae as a monophyletic lineage (Figure 2), though with low support (bootstrap [BS] = 21%; posterior probability [PP] = 0.77). Species referred to *Flectonotus* from northern South America (*F. fitzgeraldii* and *F. pygmaeus*) are not resolved as sister to *Fritziana* from southeastern Brazil (*F. fissilis*, *F. goeldii*, and *F. ohausi*). Support for the monophyly of a clade containing all five species is low (BS = 1%, PP = 0.04). However, clades corresponding to *Flectonotus* (*F. fitzgeraldii* and *F. pygmaeus*) and *Fritziana* (*F. fissilis*, *F. goeldii*, and *F. ohausi*), sensu Duellman and Gray (1983), are strongly supported; *Flectonotus:* BS = 100%, PP = 1.00; *Fritziana:* BS = 100%, PP = 1.00. Mean uncorrected pair-wise divergence between these two clades is high for both loci: 19.3% for 16S; and 7.1% for POMC.

Our analysis resolved Hylidae, Bufonidae, Centrolenidae, and Terrarana as monophyletic (Figure 2). We note that the Ceratophryidae and Dendrobatidae were not resolved as monophyletic. However, our analysis includes very low taxon sampling within these families and we did not design our analyses to explicitly evaluate the monophyly of these relationships. Thus, we place little importance in the lack of monophyly for Ceratophryidae and Dendrobatidae.

Reproduction

Egg deposition, female post-mating behavior, and larval behavior in Fritziana goeldii.—The dorsal surfaces of the bodies of both male and female *Fritziana goeldii* are smooth. Longitudinal folds become apparent on the female's back only during mating. Egg deposition as described by

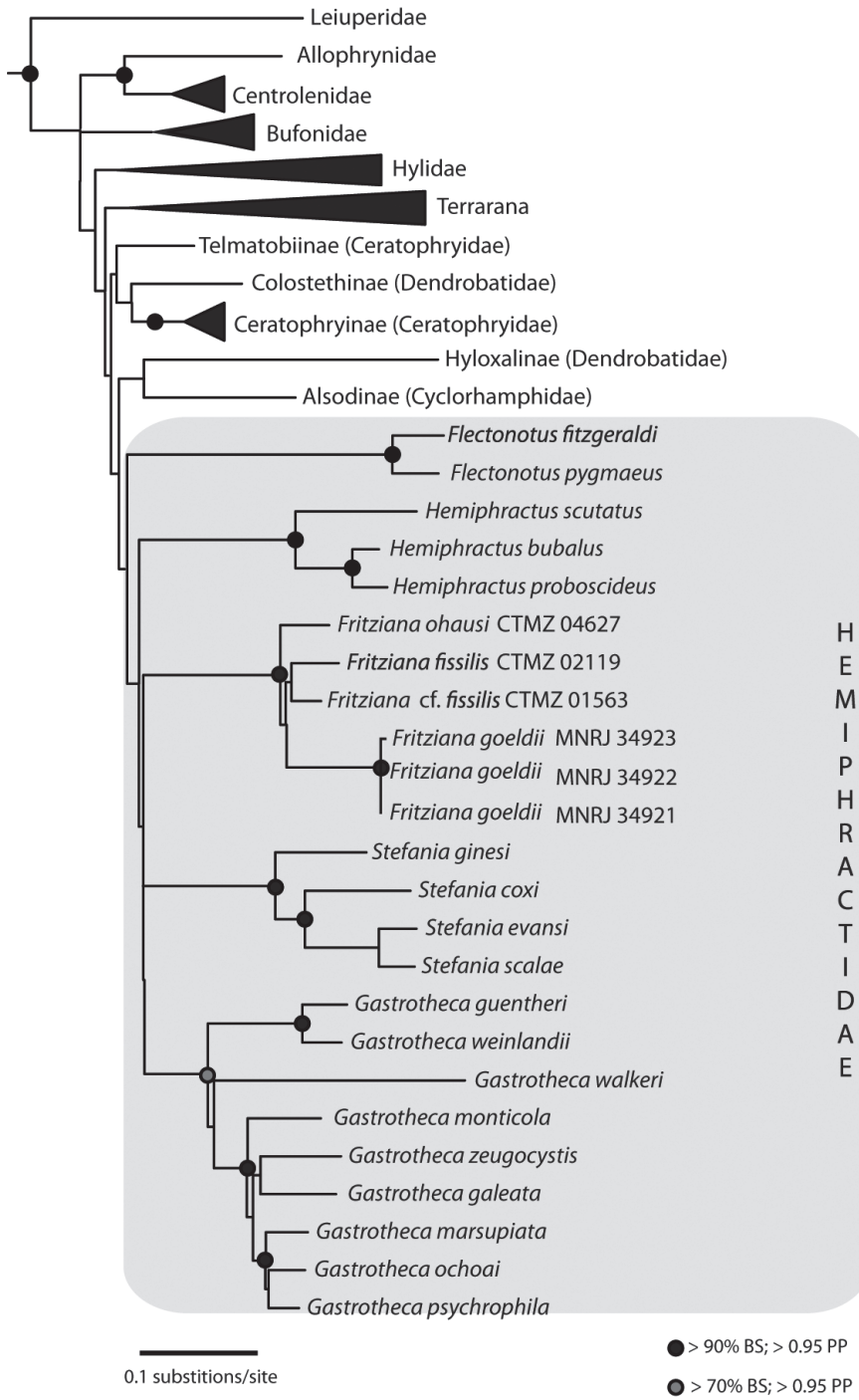

Figure 2. Maximum-likelihood phylogram estimated from nuclear (POMC) and mitochondrial DNA sequences (16S ribosomal RNA genes) depicting the phylogenetic relationship of *Flectonotus* and *Fritziana*.

Weygoldt and Carvalho e Silva (1991) agrees with our observations. We add information on what happens before the first egg is deposited, and summarize their and our observations for comparative purposes.

After amplexus, the pair rests for more than 2 hr; if not disturbed during that time, mating begins. The pair usually sits on a horizontal or slightly sloping surface, such as a leaf, with the female's head slightly lower than her cloacal opening. She arches her body so that her head and posterior part of her body are elevated. The male also arches his body and places one or both of his feet at the level of, or posterior to, the female's cloaca (Figure 3A). A mucous secretion emerges from the female's cloaca; the male pushes the mucus anteriorly below his abdomen with vigorous pedaling movements of his feet. The more the male's feet reach forward, the more he arches his back, performing a "pelvic thrust" (Figure 3B). This procedure takes about 15 sec; then both frogs rest in their initial position. The procedure is repeated 19–29 times at intervals of about 1–5 min, but sometimes at longer intervals. With each pedaling pelvic thrust, the male beats the mucous secretion into a foamy mass. The female's dorsal skin gradually widens; sometimes small folds are visible dorsolaterally posterior to the male's forearms. Eventually, the female raises her cloaca higher than before and extrudes an egg. With the same movement as before, the male grasps the egg with his foot and moves it anteriorly with a pelvic thrust, continuously pedaling with his feet. Within about the next minute, the next egg is laid and deposited in the foamy mass (Figure 3C). As the number of eggs increases, the pelvic thrust is less intense, because the eggs are pushed a shorter distance, inasmuch as previously laid eggs cover the anterior part of the female's back. However, the male continues pedaling. In at least one pair, the last two bouts did not contain an egg, and the female flexed her back less than before. The female indicates the end of oviposition by raising the anterior part of her body and arching her head upward. Usually this motion is repeated a few times before the male deserts her by climbing forward over her head. The female rests for several minutes with the eggs embedded in the foamy matrix (Figure 3D). We have observed clutch sizes of 9–19 eggs ($\bar{x}$ = 13.5, n = 11); Weygoldt and Carvalho e Silva (1991) counted 10–22 ($\bar{x}$ = 16.1, n = 11) eggs.

Subsequent to oviposition, the female remains concealed and inactive for 4–8 days. The foam bubbles in the egg matrix disappear within 2–3 days (Figure 4A). The egg matrix and eggs, together termed "egg sac" by Weygoldt and Carvalho e Silva (1991), form a unit that cannot be removed from the female's back without injuring her. Likewise, single eggs cannot be removed. Occasionally, unfertilized eggs remain in the matrix (Figure 4B). In one instance, several unfertilized eggs were present in an egg sac and became infested by a spreading fungus. When more than half of the eggs had been infested, the entire egg sac was sloughed.

Embryonic development is completed after 17 (Weygoldt and Carvalho e Silva 1991) or 20–23 days (K.-H. J., pers. obs.), at which time the female enters a water-filled bromeliad leaf axil vent. In females that were offered only water-filled jars, tadpoles left the eggs while the egg sac was submerged, but still on the female's back, or soon after the whole egg sac had been sloughed off and left in the water. Immediately after sloughing, skin folds are still visible (Figure 4C), but disappear within a few hours. Upon escaping the egg sac, the tadpoles are in Gosner's (1960) Stages 30–33 (Weygoldt and Carvalho e Silva 1991). The intestines of the robust-bodied tadpoles are completely filled with yolk (Figure 4D). Within a few hours after hatching, the tadpoles ate the remains of the egg sac, as well as any unfertilized eggs or dead embryos, if present. They also ate commercial fish food, if offered. Metamorphosis was completed within 21–25 days in the water, or 38–42 days after oviposition. Tadpoles that ate nothing reached metamorphosis at the same time as those that fed.

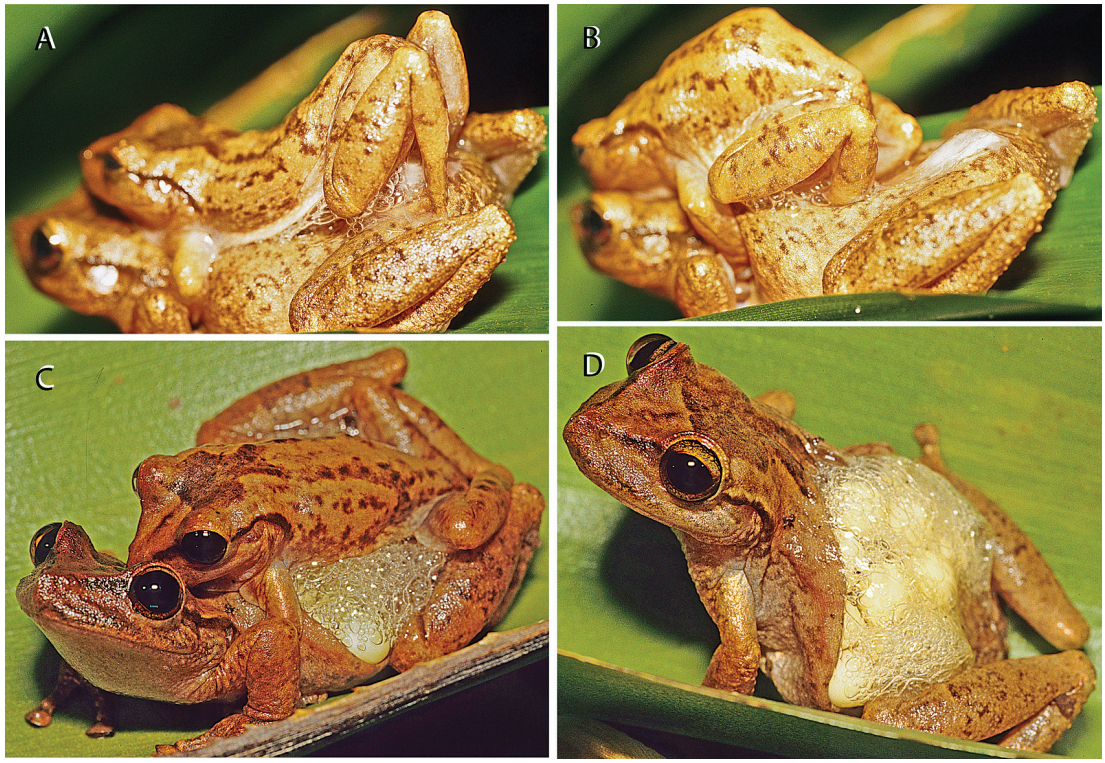

Figure 3. Egg-sac formation and oviposition in *Fritziana goeldii* (**A**) Male gathering mucous secretion from female's cloaca with his feet. (**B**) With a pelvic thrust the male pushes the mucous anteriorly and beats it into a foamy mass with pedaling movements of his feet. (**C**) Male pushes eggs into the foamy mass with his feet; lateral skin folds have formed lateral to the eggs on the female's back. (**D**) Female with foamy egg sac immediately after separation from the male.

Egg deposition, female post-mating behavior, and larval behavior in Flectonotus pygmaeus.—The dorsum is smooth in male *Flectonotus pygmaeus,* whereas females have two longitudinal flaps of skin that either meet along the midline, thereby closing the dorsal pouch (Figure 5), or leave a gap of 1–2 mm in females that are not reproductively active. The pouch is always closed prior to mating. The oviposition behavior described by Duellman and Maness (1980) agrees with our observations. For comparative purposes we summarize their and our observations, especially by using a filmed sequence; we also add information on female post-mating behavior and tadpole behavior.

In captivity mating usually started 5–7 hr after the initiation of amplexus. A mating pair sits on a horizontal or sloping surface, so that the posterior part of the body is higher than the head in both individuals. Shortly prior to mating the female exerts a few single push-ups with both fore- and hind legs, as if to alert the male who starts breathing vigorously. Then the female lowers her head and raises her cloaca. The male positions his feet above the female's cloacal opening, which is situated dorsally, or slightly posterior to it and pushes his feet anteriorly with a pelvic thrust and pedaling movements (Figure 6A). It has not been observed if the flaps are open by the time of the first egg being pushed

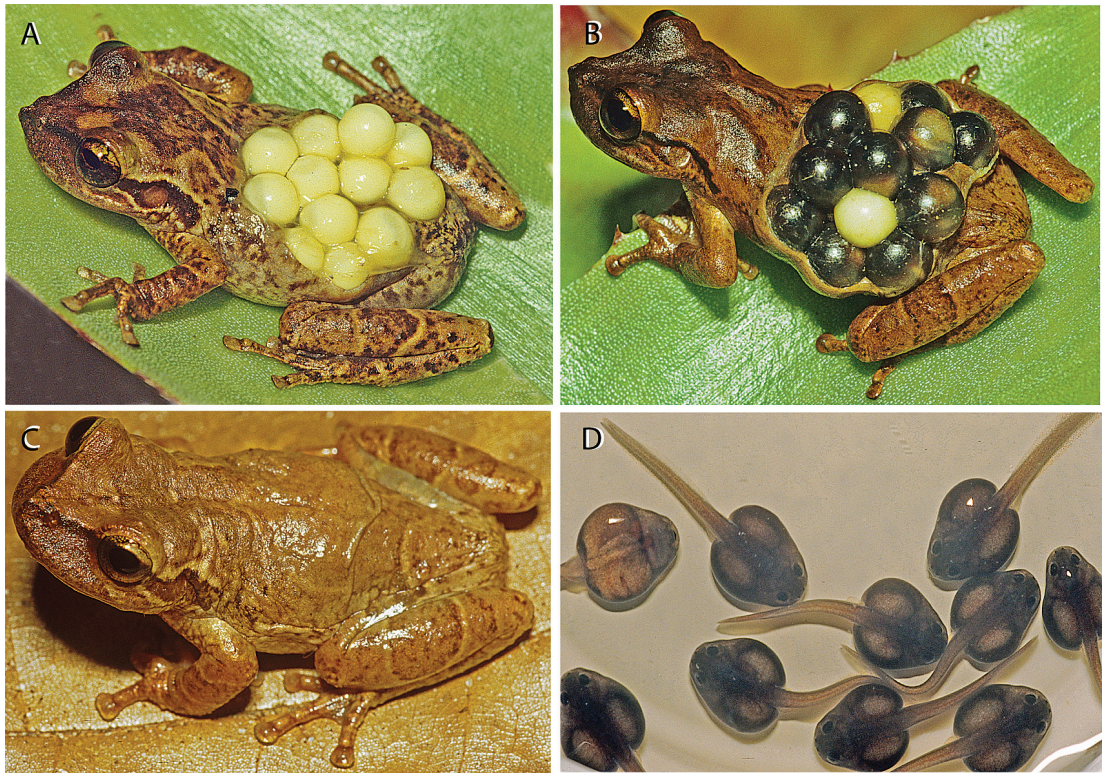

Figure 4. Egg brooding and larval development in *Fritziana goeldii*. (**A**) Female with an egg sac with 12 eggs seven days after mating; the foam has disintegrated, and the matrix is a clear, firm, but somewhat flexible mass.(**B**) Female with 12 eggs 19 days old, four days prior to hatching; the pigmented bell-shaped gills have completely enveloped the embryos. Note two pale unfertilized eggs and the posterior rim of the egg sac. (**C**) Female immediately after sloughing the egg sac; skin folds disappear within a few hours after sloughing. (**D**) Tadpoles a few hours after hatching; individual in the upper left is on its back, showing the abdomen filled with yolk.

anterior, or if the male actually opens them with his feet. Raising the cloaca and pedaling movements usually take 5–8 sec, after which the pair rests. The male's feet remain inserted in the female's skin flaps.

The same procedure is repeated about 10–20 times. On one occasion, there were 13 bouts at intervals of 49 sec to 3:56 min ($\bar{x}$ = 87.3 sec); each bout lasts 5–21 sec ($\bar{x}$ = 10.2 sec). In each bout, the male pushes some mucous fluid from the female's cloaca into the pouch. This is especially apparent in later bouts when the female's posterior dorsum is visibly moist and a small bubble sometimes appears above the cloacal opening. While pedaling, the male occasionally removes one leg from the skin flap and quickly moves it backwards completely outstretched. Then he inserts his foot again. During the intervals, the female continues exerting single push-ups from time to time.

Oviposition begins when the female elevates her cloaca higher than before and the snout almost touches the surface. An egg appears, moves anteriorly between the male's tarsi and is taken with one foot and pushed forward with pedaling movements that are more intensive, but

Figure 5. *Flectonotus pygmaeus,* non-brooding female.

slower than previously (Figure 6B). In one instance, when six eggs were laid, intervals were 42–82 sec ($\bar{x}$ = 53.6), and bouts lasted 12–18 sec ($\bar{x}$ = 15.8). During the last bout, an egg did not appear, but the male continued his movements. During egg laying, the female does not raise her head during intervals between bouts. At the end of oviposition, the female elevates her head and lowers her cloaca. The male quickly removes his feet and raises his shanks (Figure 6C). After the female has raised her body higher once or twice again, the male scrambles forward over her head and departs. At this time, the pouch is closed except for the posterior one fifth or so, where usually one egg is visible. Two to four minutes after termination of amplexus, the female starts inhaling in intervals. By inhaling and exhaling, she positions the eggs. This is repeated several times at least during the next 15 min. The shapes of the eggs, invisible immediately after the end of amplexus, become visible. During this time, the female slowly moves in a circle with a diameter about 3–4 times her snout–vent length; sometimes she changes direction and stops to flex her body and to press her shanks dorsolaterally onto the posterior part of the body. By this time, the aperture to the brood pouch closes completely (Figure 7). Clutches contained 5–13 eggs ($\bar{x}$ = 9.1, n = 14).

The night after mating, the female forages actively. After 23–26 days ($\bar{x}$ = 24.2, n = 16), the skin flaps begin to gape slightly. The female then seeks a water-filled bromeliad leaf axil and releases the tadpoles at night. When offered only a jar of water, the female was observed to submerge only the posterior third of her body. After several minutes, the tadpoles exit the pouch rapidly, one after another. The female does not deposit an egg sac or remains of an egg matrix, but occasionally, an unfertilized egg or a dead embryo is extruded. In a few instances, clutches in the pouch showed no development at all. In these cases, the skin folds retreated toward the sides of the body and the pouch opened (Figure

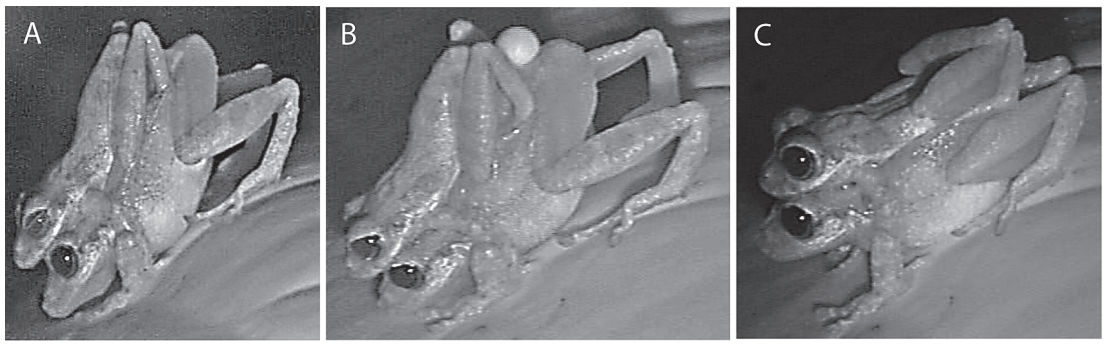

Figure 6. Mating in *Flectonotus pygmaeus;* photos taken from an infrared film sequence. (**A**) During the first bouts the male takes up mucous secretion from the female's cloacal opening and pushes it forward with a pelvic thrust and pedaling with his feet. (**B**) Subsequently, the eggs are pushed beneath the skin folds in the same manner. (**C**) The female indicates the end of egg deposition by raising her head and lowering the posterior part of her body; the male raises his legs and soon departs by scrambling forward over the female's head.

8). The eggs appeared to be closely packed. No egg matrix was visible, and the eggs broke off singly or in small groups, and did not seem to be connected to one another.

The larval intestines were filled with yolk. The tadpoles neither ate dead eggs or embryos nor any other kind of common tadpole food offered. They metamorphosed without feeding and left the water 11–17 days ($\bar{x}$ = 13.8, n = 96) after being released from the pouch and 34–43 days ($\bar{x}$ = 38.0, n = 89) after mating.

Morphology

Flectonotus and *Fritziana* differ in the size and shape of the nasal bones; in *Flectonotus,* the nasals are small, slender, and widely separated, whereas in *Fritziana,* they are much larger and nearly abut anteriorly (Duellman and Gray 1983). Embryos of hemiphractid frogs have one or two pairs of gills. Large, bell-shaped gills completely cover the embryos in *Gastrotheca, Hemiphractus,* and *Stefania,* whereas the gills are notably smaller and only partially cover the embryos in *Cryptobatrachus, Flectonotus,* and *Fritziana.* In *Fritziana,* two pairs of gills are present; they are derived from the first and second branchial arches. In contrast, *Flectonotus* has only one pair of gills, which are derived from the first branchial arch.

The tadpoles of both genera lack labial denticles; the beaks in tadpoles of *Fritziana* have small, keratinized beaks, whereas the beaks of tadpoles of *Flectonotus* are weakly cornified. The tadpoles of *Fritziana fissilis* and *F. goeldii* have a complete ventral velum in the floor of the mouth; the ventral velum is small and present only laterally with the resulting gap equal to about half the width of the buccal floor in *Flectonotus pygmaeus* (Wassersug and Duellman 1984).

Figure 7. Brooding female *Flectonotus pygmaeus.*

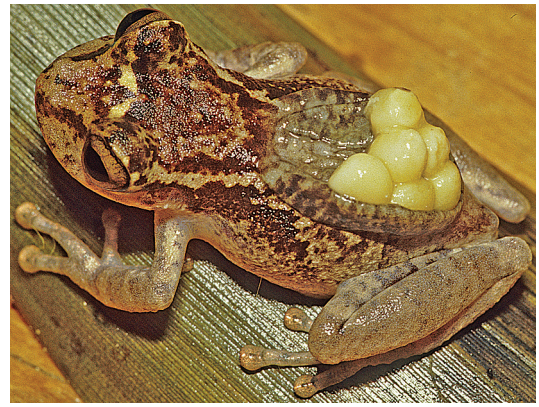

Figure 8. A female *Flectonotus pygmaeus* discarding an unfertilized clutch. The skin flaps retreat. The anterior eggs have already broken off. Note the absence of an egg matrix.

Discussion

Our phylogenetic analysis of DNA sequence data supports the recognition of two distinct lineages that correspond to *Flectonotus* and *Fritziana* as recognized by Duellman and Gray (1983). Each genus is resolved as monophyletic with high support. However, there is little support that *Flectonotus* and *Fritziana* are sister taxa as posited by Duellman and Gray (1983) and Wassersug and Duellman (1984), and implicit in the previous taxonomies for these genera (for review, see Duellman and Gray 1983). Our analysis generally agrees with several previous phylogenetic analyses that resolved the Hemiphractidae as monophyletic (Wiens 2007, Guayasamin *et al.* 2008, Heinicke *et al.* 2009), but differs from several earlier studies with low taxon sampling of hemiphractids that did not

find evidence of monophyly (Darst and Cannatella 2004, Faivovich *et al.* 2005, Frost *et al.* 2006).

Our results suggest *Flectonotus* and/or *Fritziana* to be the earliest branching lineages within the Hemiphractidae. This is consistent with the results of Wiens *et al.* (2007) and Wiens (2007) and the analysis of nuclear loci by Guayasamin *et al.* (2008), but differs from the results of Heinicke *et al.* (2009). Unfortunately, the relationships of *Flectonotus* in the analysis of Heinicke *et al.* (2009) are difficult to interpret because these authors used a composite terminal taxon for "*Flectonotus*" that combined data from Wiens *et al.* (2005, 2007) for *F. fitzgeraldi* with data collected by Faivovich *et al.* 2005 (also used in Frost *et al.*, 2006) for a specimen (CFBH 5720) designated *Flectonotus* sp. from Santo Amaro da Imperatriz in Santa Catarina, Brazil, most certainly a species of *Fritziana,* not *Flectonotus.* Thus, the analysis by Heinicke *et al.* (2009) combined data from *Flectonotus* and *Fritziana*, two deeply divergent lineages.

Resolving either *Flectonotus* or *Fritziana* as the earliest branching lineage within Hemiphractidae generally supports the pattern of character evolution outlined by Wiens *et al.* (2007). This pattern differs somewhat from that of Wassersug and Duellman (1984) as it implies either that the egg-brooding basin on the female's dorsum is plesiomorphic for Hemiphractidae and was then lost one or multiple times (e.g., *Hemiphractus*, *Stefania*) or that this basin evolved multiple times independently. However, we note that relationships between hemiphractid genera lack strong support in our analysis, and future analyses including *Cryptobatrachus* might alter interpretations of character evolution within marsupial frogs.

Mating, post-mating and tadpole behavior are almost identical in *Flectonotus fitzgeraldi* and *F. pygmaeus* (Proy 1995). As in *F. pygmaeus,* there is a lengthy elapse of time between the commencement of amplexus and actual mating in *F. fitzgeraldi*. The female performs circular movements early, during egg laying. Like *F. pygmaeus*, females of *F. fitzgeraldi* do not release an egg sac when tadpoles are deposited in water and the latter are obligatory non-feeding. Only one ovary produces the eggs of a clutch. This is visible through the translucent ventral skin in *F. pygmaeus* as well, but not through the pigmented skin of *F. goeldii*.

Fritziana goeldii and *Flectonotus* differ in both mating and post-mating behavior and, not surprisingly, this is correlated with morphological differences. In *Fritziana goeldii*, the male constructs a foam nest on the female's back. The foam forms the matrix into which the eggs are embedded and held together. In species of *Flectonotus,* the skin flaps on the back of the female's dorsum functionally replace the egg matrix. A "basin" was observed in which the eggs are placed on the *Fritziana goeldii* female's dorsum. Lateral skin folds may form along the sides of the clutch and deepen the basin, depending on the number of eggs. These lateral folds also form in *Hemiphractus* and *Stefania* in which eggs are deposited on the dorsum (K.-H.J., pers. obs.) if clutches are large. Combined with our phylogenetic data, this implies that egg covers have evolved independently in the shape of skin flaps (*Flectonotus*) or pouches (*Gastrotheca*) in hemiphractids, but *Fritziana*, *Hemiphractus*, and *Stefania* cannot be differentiated by presence or lack of skin folds. The skin flaps covering the eggs in *Flectonotus* seems facilitate foraging by females during brooding, whereas female *Fritziana goeldii*, which lack these flaps, are inactive for several days.

The differences in keratinization of larval beaks is reflected in feeding habits of the two genera—keratinized beaks in the facultative non-feeding tadpoles of *Fritziana,* and the weakly cornified beaks in the obligatory non-feeding tadpoles of *Flectonotus.* By having small external gills that only partially envelop the embryo, *Flectonotus* and *Fritziana* are like *Cryptobatrachus,* in which eggs carried openly on the back of the female undergo direct development. Unlike *Fritziana* and all other genera of hemiphractids, *Cryptobatrachus* and *Flectonotus*

have only one pair of small external gills. *Gastrotheca* also has a single pair of gills, but these are the result of fusion of the gills from the first and second branchial arches (Wassersug and Duellman 1984).

A brief analysis of the advertisement calls of the two species of *Flectonotus* and the three species of *Fritziana* by Duellman and Gray (1983) showed that the call of *Flectonotus pygmaeus* consists of only one note and thereby differs from the multi-noted calls of *F. fitzgeraldi* and of the three species of *Fritziana*. A more detailed analysis of the calls of *Fritziana goeldii* and the two species of *Flectonotus* by Sinsch and Juraske (2006) also revealed that *F. pygmaeus* is unique in having a call consisting of a single note ("pulse group" fide Sinsch and Juraske). These authors (2006:156) suggested that "*F. pygmaeus* vocalizations may indicate that this species is outgroup to a clade formed by the other four members of *Flectonotus* [includes *Fritziana*] which share the calls composed of at least two pulse groups." Our molecular, behavioral, and morphological data do not support their suggestion.

Taxonomic Conclusions

The distinct differences in morphology and reproductive behavior between the Venezuelan *Flectonotus* and the Brazilian "*Flectonotus*" are strongly supported by genomic differences, as well as karyological differences (Bogart 1973). Thus, we recognize two genera of these frogs in the family Hemiphractidae.

Flectonotus Miranda-Ribeiro, 1926

Flectonotus Miranda-Ribeiro, 1926:109. Type species *Nototrema pygmaeus* Boettger, 1893, by monotypy.

Content.—Two recognized species—*F. pygmaeus* (Boettger), *F. fitzgeraldi* (Parker).

Distribution.—Extreme northeastern Cordillera Oriental in Colombia, Cordillera de Mérida, Cordillera de la Costa, and Serranía de Paria in Venezuela, Trinidad, and Tobago.

Fritziana Mello-Leitão, 1937

Fritzia Miranda-Ribeiro, 1920:321. Type species *Hyla goeldii* Boulenger, 1895, by original designation. Preoccupied by *Fritzia* Cambridge (Arachnida).

Coelonontus Miranda-Ribeiro, 1920:327. Type species *Coelonotus fissilis* Miranda-Ribeiro, 1920. Preoccupied by *Coelonotus* Peters (Pisces).

Fritziana Mello-Leitão, 1937:330. Replacement name for *Fritzia* Miranda-Ribeiro, 1920.

Nototheca Bokermann, 1950:217. Replacement name for *Coelonotus* Miranda-Ribeiro, 1920.

Content.—Three species—*F. fissilis* (Miranda-Ribeiro), *F. goeldii* (Boulenger), and *F. ohausi* (Wandolleck).

Distribution.—Mountains and coastal lowlands of southeastern Brazil from Espírito Santo to Santa Catarina.

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Appendix I. GenBank accessions used for phylogenetic anlaysis; "n/a" denotes cases in which data were not available.

Adelophryne gutturosa (16S: EU186679; POMC: GQ345262); *Agalychnis callidryas* (16S: AY843563; POMC: EF158395); *Allophryne ruthveni* (16S: AY843564; POMC: AY819077); *Anotheca spinosa* (16S: AY843566; POMC: AY819110); *Ascaphus truei* (16S: DQ283116; POMC: EU275850); *Bokermannohyla astartea* (16S: n/a; POMC: AY819113); *Bombina orientalis* (16S: DQ283432; POMC: AY692246); *Brachycephalus ephippium* (16S: DQ283091; POMC: GQ345256); *Bufo margaritifer* (16S: AF375514; POMC: AY819080); *Caudiverbera caudiverbera* (16S: DQ283439; POMC: AY819090); *Centrolene prosoblepon* (16S: AY843574; POMC: AY819085); *Ceratophrys ornata* (16S: FJ882777; POMC: AY819091); *Ceuthomantis smaragdinus* (16S: GQ345132; POMC: GQ345269); *Charadrahyla nephila* (16S: AY843649; POMC: DQ388712); *Cochranella griffithsi* (16S: n/a; POMC: AY819086); *Colostethus nexipus* (16S: n/a; POMC: AY819089); *Craugastor biporcatus* (16S: n/a; POMC: GQ345265); *Cruziohyla calcarifer* (16S: AY843562; POMC: GQ366035); *Cyclorana manya* (16S: FJ945361; POMC: AY819147); *Diasporus diastema* (16S: n/a; POMC: GQ345261); *Duellmanohyla soralia* (16S: AY843584; POMC: AY819111); *Ecnomiohyla miotympanum* (16S: AY843645; POMC: AY819122); *Eleutherodactylus cooki* (16S: EF493539; POMC: GQ345260); *Eleutherodactylus curtipes* (16S: DQ679379; POMC: n/a); *Exerodonta smaragdina* (16S: n/a; POMC: DQ388716); *Fejervarya limnocharis* (16S: AY843588; POMC: AB526646); *Flectonotus fitzgeraldi* (16S: DQ679381; POMC: AY819104); *Flectonotus pygmaeus* (16S: DQ679382; POMC: DQ679310); *Gastrophryne carolinensis* (16S: X86278; POMC: AY819098); *Gastrotheca galeata* (16S: DQ679392; POMC: DQ679318); *Gastrotheca guentheri* (16S: DQ679393; POMC: DQ679321); *Gastrotheca marsupiata* (16S: DQ679397; POMC: AY819105); *Gastrotheca monticola* (16S: DQ679398; POMC: AY819106); *Gastrotheca ochoai* (16S: DQ679400; POMC: DQ679326); *Gastrotheca psychrophila* (16S: DQ679404; POMC: DQ679329); *Gastrotheca walkeri* (16S: DQ679409; POMC: DQ679332); *Gastrotheca weinlandii* (16S: DQ679410; POMC: DQ679333); *Gastrotheca zeugocystis* (16S: DQ679411; POMC: DQ679334); *Haddadus binotatus* (16S: DQ283092; POMC: GQ345259); *Hemiphractus bubalus* (16S: DQ679412; POMC: DQ679335); *Hemiphractus proboscideus* (16S: DQ679413; POMC: AY819107); *Hemiphractus scutatus* (16S: DQ679414; POMC: DQ679336); *Hyalinobatrachium colymbiphyllum* (16S: FJ784562; POMC: AY819087); *Hyla astartea* (16S: AY549322; POMC: n/a); *Hyla squirella* (16S: AY843678; POMC: AY819128); *Hylomantis hulli* (16S: GQ366226; POMC: GQ366033); *Hyloscirtus palmeri* (16S: AY843650; POMC: AY819158); *Hyloxalus nexipus* (16S: EU342713; POMC: n/a); *Hypsiboas polytaenius* (16S: AY843655; POMC: AY819124); *Itapotihyla langsdorffii* (16S: AY843706; POMC: AY819129); *Kurixalus carinensis* (16S: GQ285670; POMC: GQ285730); *Lepidobatrachus laevis* (16S: DQ283152; POMC: AY819094); *Litoria aurea* (16S: AY843691; POMC: AY819148); *Litoria caerulea* (16S: AY843692; POMC: AY819149); *Notaden bennettii* (16S: n/a; POMC: AY819099); *Nyctimystes foricula* (16S: FJ945442; POMC: AY819150); *Nyctixalus pictus* (16S: DQ283133; POMC: GQ285729); *Nymphargus griffithsi* (16S: EU663062; POMC: n/a); *Osornophryne guacamayo* (16S: U52783; POMC: AY819083); *Osteopilus septentrionalis* (16S: AY843712; POMC: AY819131); *Pachymedusa dacnicolor* (16S: AY843714; POMC: AY819152); *Phasmahyla jandaia* (16S: GQ366233; POMC: GQ366042); *Phrynopus bracki* (16S: EF493709; POMC: GQ345263); *Phrynopus laplacai* (16S: AM039643; POMC: n/a); *Phyllodytes auratus* (16S: DQ403730; POMC: AY819133); *Phyllomedusa tomopterna* (16S: AY843728; POMC: AY819153); *Physalaemus cuvieri* (16S: AY843729; POMC: AY819096); *Plectrohyla chrysopleura* (16S: n/a;

POMC: AY819134); *Plethodon cinereus* (16S: EF107166; POMC: FJ951365); *Pristimantis curtipes* (16S: n/a; POMC: AY819092); *Pristimantis diastema* (16S: EU186682; POMC: n/a); *Proceratophrys melanopogon* (16S: FJ685699; POMC: GQ345270); *Pseudacris nigrita* (16S: FJ685699; POMC: AY819136); *Pseudis paradoxa* (16S: AY843740; POMC: AY819102); *Psychrophrynella wettsteini* (16S: n/a; POMC: GQ345266); *Ptychohyla spinipollex* (16S: AY843748; POMC: AY819138); *Rana catesbeiana* (16S: AY779206; POMC: AY819103); *Rhacophorus nigropunctatus* (16S: EU215533; POMC: GQ285735); *Scinax sugillatus* (16S: n/a; POMC: AY819142); *Smilisca fodiens* (16S: AY843743; POMC: AY819137); *Spea bombifrons* (16S: AY236818; POMC: AY819076); *Sphaenorhynchus lacteus* (16S: AY549367; POMC: AY819144); *Stefania coxi* (16S: DQ679415; POMC: DQ679337); *Stefania evansi* (16S: AY843767; POMC: AY819108); *Stefania ginesi* (16S: DQ679417; POMC: DQ679338); *Stefania scalae* (16S: DQ679418; POMC: DQ679339); *Telmatobius truebae* (16S: DQ679378; POMC: AY819097); *Theloderma asperum* (16S: GQ285677; POMC: GQ285728); *Tlalocohyla smithii* (16S: AY843668; POMC: AY819127); *Trachycephalus jordani* (16S: AY843771; POMC: AY819145); *Triprion petastatus* (16S: AY843774; POMC: AY819146); *Uperodon littlejohni* (16S: n/a; POMC: AY819100); *Xenopus laevis* (16S: AY581639; POMC: AY819075).