

Phylogeny and biogeography of paradoxical frogs (Anura, Hylidae, Pseudis) inferred from 12S and 16S mitochondrial DNA

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Abstract

We used mitochondrial DNA sequences of 12S and 16S ribosomal RNA genes to reconstruct phylogenetic relationships of the nine species of South American aquatic hylids known as paradoxical frogs. *Pseudis minuta* and *P. cardosoi* form the sister-group to a clade comprising *Lysapsus* and the remaining *Pseudis*. We suggest the resurrection of *Podonectes*, including *P. minutus* and *P. cardosoi*, to avoid the nonmonophyly of *Pseudis*. Some doubt is cast on the species status of *P. cardosoi*. *Lysapsus limellum*, *P. bolbodactyla*, and *P. paradoxa* each may comprise more than one species, but lack of comprehensive geographic and morphological appraisals precludes taxonomic changes. Biogeographic implications of the phylogeny are discussed. The correlation between hydrographic basins and *Pseudis* species is not fully supported, and a recent contact between Amazon populations in eastern Bolivia and western Brazil (Rondônia) and Paraná basin populations in the Pantanal is suggested. A dispersal-vicariance analysis showed that major diversification of *Pseudis* and *Lysapsus* species occurred in the Brazilian Shield, and that the presence of *P. paradoxa* and *L. limellum* in the Amazon and Paraná basins is due to recent dispersals. Evidence suggests a vicariant event, most likely caused by Miocene marine incursions, as the cause for the restricted distribution of *L. laevis* in the Guiana Shield.

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1. Introduction

Paradoxical frogs (*Pseudis* and *Lysapsus*) are aquatic and semi-aquatic anurans restricted to South America, east of the Andes, from Trinidad to northern Argentina (Duellman and Trueb, 1986; IUCN, Conservation International, and NatureServe, 2005) (Figs. 1 and 2). These frogs occur in ponds associated with river floodplains and have several morphological adaptations to aquatic life, such as large and protuberant eyes, robust hindlimbs, and highly webbed feet. The giant tadpoles of *Pseudis*, which metamorphose into relatively small adults, are a contradiction for which it is commonly known (Emerson, 1988).

Besides the requirement of lentic environments for reproduction and development, these frogs also need open areas. They are widely distributed in the Cerrados of central Brazil, Pantanal floodplains, and savanna fragments within the Amazon forest, but are absent from dense forests (Lynch, 1979). This distribution and specific habitat requirements make paradoxical frogs an ideal group to test biogeographic hypotheses on the evolution and speciation of the South American semi-aquatic fauna.

Phylogenetic reconstructions of fishes (Lovejoy et al., 1998; Montoya-Burgos, 2003), birds (Grau et al., 2005), reptiles (Glor et al., 2001), and mammals (da Silva and Patton, 1998; Smith and Patton, 1999) have shown that the major diversification of extant South American vertebrate fauna occurred in late Miocene/early Pliocene. These results have shifted attention away from the Pleistocene Refugia theory (Haffer, 1969), which states that repeated

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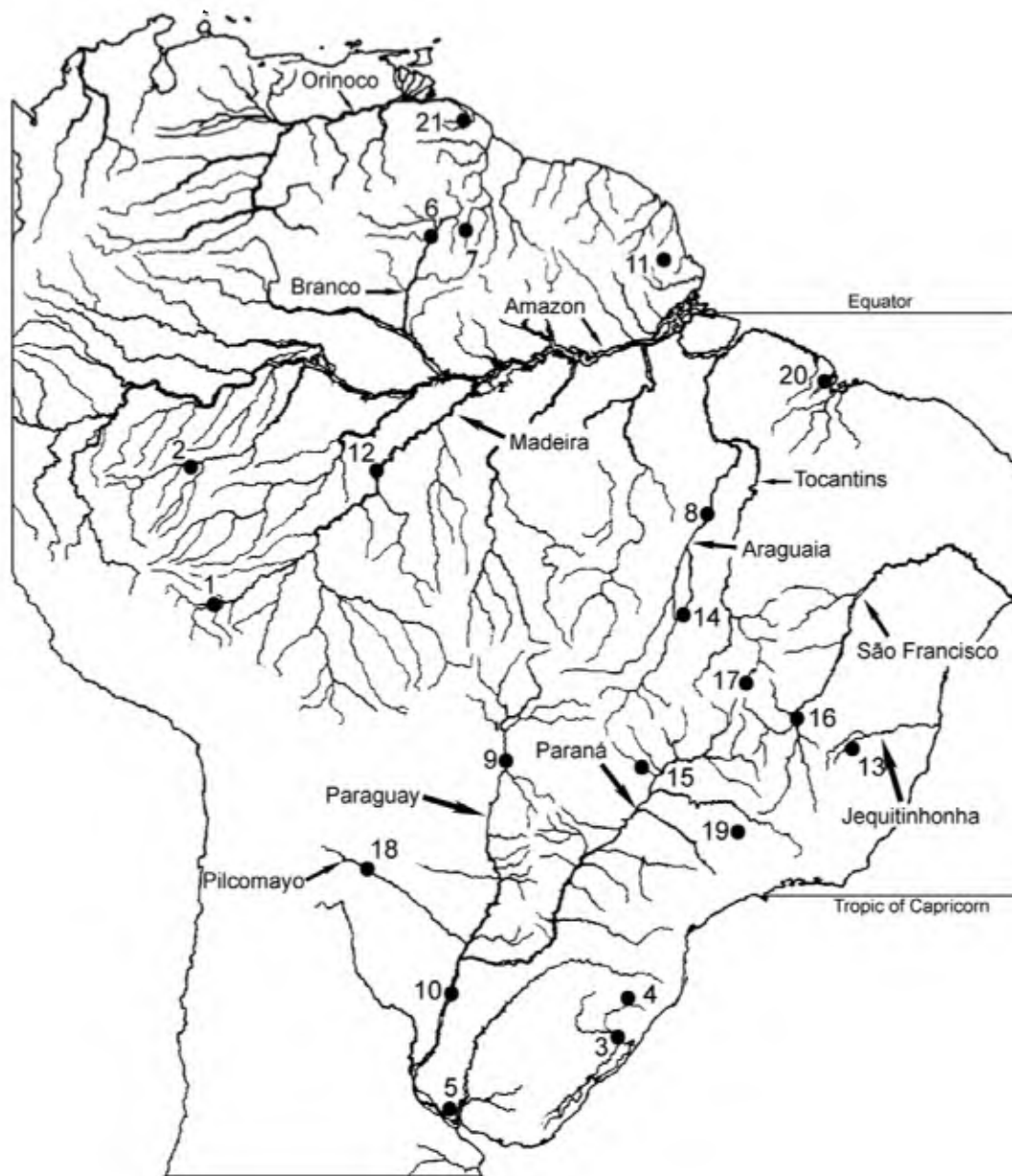


Fig. 1. Major South American river drainages and distribution of the samples used in the present study. Rivers mentioned in the discussion are shown. Points 1, 2, 5, 7, 10, and 18 are the localities for samples downloaded from GenBank. See Fig. 3 for species identities for each locality and Table 1 for specific locality information.

cycles of forest contraction and expansion during Pleistocene climatic oscillations isolated and rejoined populations, creating conditions for repeated speciation events in the Amazon. Alternatively, recent attention has been given to the effects of marine incursions and the uplift of the Brazilian and Guiana Shields (Aleixo, 2004; Grau et al., 2005; Lovejoy et al., 1998). Marine incursions have been implicated in the isolation of the Brazilian Shield during the Miocene through the formation of a seaway connecting the Amazon and Paraná basin (Webb, 1995). Several cycles of sea level rise (Hallam, 1992), furthermore, could have caused repeated extinctions of floodplain-dependent species and colonization of the region by species from areas isolated during sea level upraise. Periodic incursions of

100m above the present level have been reported (Haq et al., 1987). The effects of a major recent sea incursion during the Pliocene, starting 5mya and lasting about 800,000 years (Haq et al., 1987), would have been the extinction of lowland species followed by recent re-colonization by populations isolated in uplands. The evaluation of the influence of such events on the evolution of neotropical fauna requires both robust phylogenetic hypotheses for groups with different requirements and a thorough understanding of the taxonomy of these poorly studied organisms.

Until recently, the higher phylogenetic relationships of paradoxical frogs were unclear. Savage and de Carvalho (1953) elevated the group to a distinct family, Pseudidae,

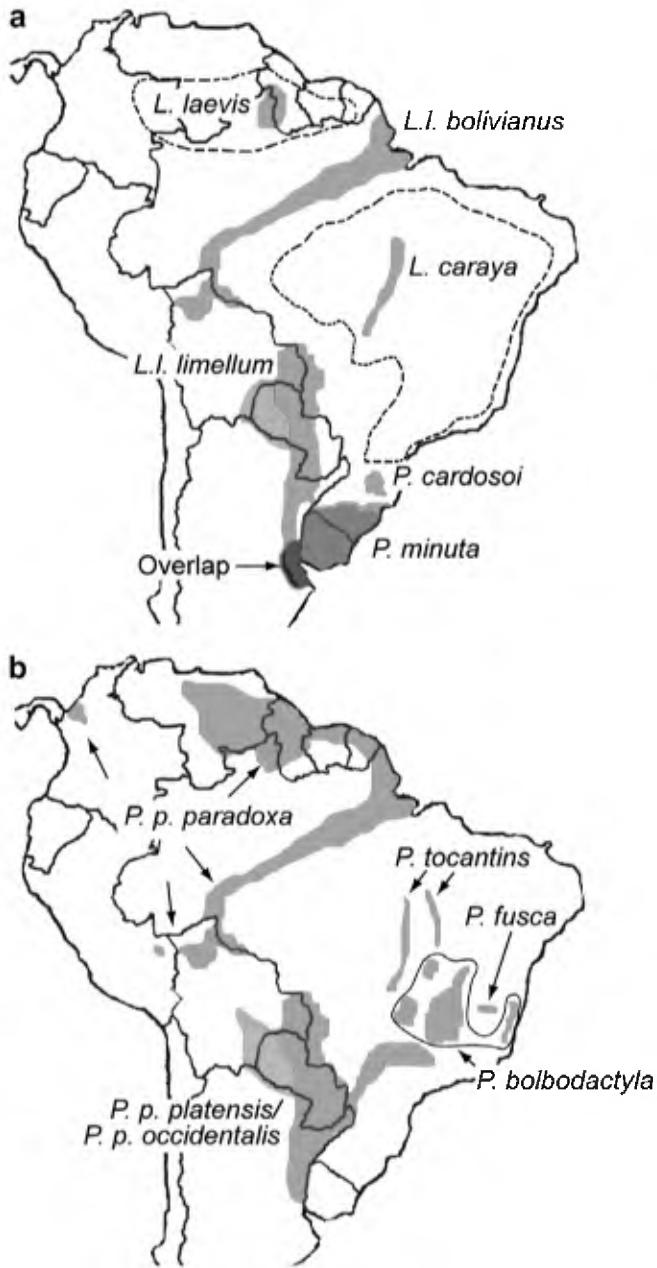


Fig. 2. *Pseudis* species ranges in South America, east of the Andes. The overlap in (a) corresponds to the co-occurrence of *P. minuta* and *L. l. limellum*. The western part of the distribution of *P. tocantins* in (b) overlaps with that of *L. caraya* in (a) and corresponds to the Araguaia River floodplain. *Pseudis bolbodactyla*, *P. cardosoi*, *P. fusca*, *P. tocantins*, and *L. caraya* are endemic to Brazil. The Guiana (above) and Brazilian Shield (below) limits contours are indicated by dashed lines in (a).

but they have been grouped within Ranidae (Günther, 1858), Leptodactylidae (Noble, 1922), and Hylidae (Duellman and Trueb, 1986; Parker, 1935). da Silva's (1998) morphological phylogenetic analysis placed pseudids within Hylidae, and subsequent mitochondrial and nuclear DNA phylogenies have strongly supported this arrangement (Darst and Cannatella, 2004; Faivovich et al., 2005; Hoegg et al., 2004; Wiens et al., 2005). However, the newest taxonomies (Faivovich et al., 2005; Frost et al., 2006) abandoned the names Pseudinae or Pseudidae because they

render the more inclusive rank name (Hylidae or Hylinae) paraphyletic. We feel it is informative to refer to this well-supported clade by a single taxon name, and thus we use the name Pseudae Fitzinger 1843. It is used here as a proper taxon name without rank.

Currently *Pseudae* comprises two genera, the small-sized *Lysapsus* (reaching 2.4 cm snout-vent length) and the larger *Pseudis* (attaining 7.5 cm snout-vent length), with three and six species, respectively (Frost, 2004). Gallardo (1961) named subspecies within both genera, recognizing *L. limellum limellum* (incorrectly using the name as *limellus*), *L. l. bolivianus*, *L. l. laevis*, and later *L. l. caraya* (Gallardo, 1964). He subdivided *Pseudis* into *P. paradoxa paradoxa*, *P. p. bolbodactyla*, *P. p. fusca*, *P. p. platensis* and *P. p. occidentalis*. Cochran and Goin (1970) described *P. p. nicefori* from Colombia. Since then, *L. laevis* and *L. caraya* were recognized as species (Klappenbach, 1985), as were *P. bolbodactyla* and *P. fusca*, along with the description of *P. tocantins* (Caramaschi and da Cruz, 1998). The latest taxonomic addition was the description of *P. cardosoi* from the Araucaria Plateau of southern Brazil (Kwet, 2000).

Herein, we reconstruct phylogenetic relationships among currently recognized species of *Lysapsus* and *Pseudis* using 12S, tRNAval, and 16S mitochondrial DNA sequences and evaluate taxonomic, biogeographic, and evolutionary implications based on the resultant phylogenetic hypothesis.

2. Materials and methods

2.1. Taxa sampled

We sampled all nine species of *Pseudis* and *Lysapsus* from 16 populations in Brazil and 1 in Guyana (Fig. 1). We also included five sequences from Argentine populations (Faivovich et al., 2005) and one from an additional Brazilian population (Darst and Cannatella, 2004) from GenBank. Morphological and molecular phylogenetic reconstructions consistently recovered *Scarthyia goinorum* as the sister group to *Pseudis* and *Lysapsus* (da Silva, 1998; Darst and Cannatella, 2004; Faivovich et al., 2005; Wiens et al., 2005), and therefore we included two *S. goinorum* sequences from GenBank as outgroups. Museum numbers, field numbers, and localities are listed in Table 1, and localities where samples were collected appear in Fig. 1. Tissues were preserved in 99% ethanol and stored at -20°C or directly frozen in liquid nitrogen and stored at -80°C prior to use.

2.2. Data collection

We extracted DNA with Viogene Genomic DNA Mini-prep System and amplified 12S, tRNAval, and 16S rRNA mitochondrial genes (totalling about 2.4 kb) with PCR reactions using the following primers: MVZ59, tRNAval, 12L1, 16SH, 16SM, 16SA, 16SC, 16SD (Goebel et al., 1999). Standard polymerase chain reactions were used with the

Table 1
Details of specimens used in the present work

Taxon	Field number	Museum number	GenBank number	Locality, state, country
<i>Lysapsus caraya</i>	AAGARDA 299	CHUNB 43138	EF152999	Couto Magalhães, TO, Brazil
<i>L. laevis</i>	AAGARDA 0600	CHUNB 43075	EF152998	Boa Vista, RR, Brazil
<i>L. laevis*</i>	—	AM-CC 101720	AY843696	Southern Rupununi Savanah, Aishalton, Guyana
<i>L. limellum bolivianus</i>	AAGARDA 0558	CHUNB 42978	EF153001	Tartarugalzinho, AP, Brazil
<i>L. limellum bolivianus</i>	GRCOLLI 12556	CHUNB 32411	EF153002	Humaitá, AM, Brazil
<i>L. limellum limellum</i>	AAGARDA 453	CHUNB 42784	EF153000	Corumbá, MS, Brazil
<i>L. limellum limellum*</i>	—	MACN 38645	AY843697	Bella Vista, Corrientes, Argentina
<i>Pseudis bolbodactyla</i>	AAG 017	CHUNB 42658	EF153006	Alvorada do Norte, GO, Brazil
<i>P. bolbodactyla</i>	AAGARDA 496	CHUNB 42764	EF153005	Aporé, GO, Brazil
<i>P. bolbodactyla</i>	AAGARDA 187	CHUNB 42879	EF153007	Pirapora, MG, Brazil
<i>P. cardosoi</i>	AAG 036	CHUNB 42610	EF152997	Jaquirana, RS, Brazil
<i>P. fusca</i>	AAGARDA 041	CHUNB 42625	EF153003	Araçuaí, MG, Brazil
<i>P. minuta</i>	AAG 053	CHUNB 34687	EF152996	Porto Alegre, RS, Brazil
<i>P. minuta*</i>	—	MACN 37786	AY843739	Depto Islas del Ibicuy, Entre Ríos, Argentina
<i>P. paradoxa paradoxa</i>	AAGARDA 551	CHUNB 42928	EF153009	Tartarugalzinho, Amapá, Brazil
<i>P. paradoxa paradoxa</i>	AAGARDA 583	CHUNB 43032	EF153010	Pinheiro, Maranhão, Brazil
<i>P. paradoxa paradoxa</i>	AAGARDA 604	CHUNB 43002	EF153011	Boa Vista, Roraima, Brazil
<i>P. paradoxa paradoxa</i>	BPN 517	UTA 53104	EF153012	Mabaruma, Barima-Waini, Guyana
<i>P. paradoxa platensis*</i>	DCC 3284	—	AY326032	18 km S Luiz Antonio, SP, Brazil
<i>P. paradoxa platensis</i>	AAGARDA 418	CHUNB 42848	EF153008	Corumbá, MS, Brazil
<i>P. paradoxa platensis*</i>	—	MACN 38642	AY843740	Departamento Bella Vista, Corrientes, Argentina
<i>P. paradoxa occidentalis*</i>	—	MACN 38584	AY549364	Laguna Yema, Formosa, Argentina
<i>P. tocantins</i>	AAGARDA 392	CHUNB 42943	EF153004	Sandolândia, Tocantins, Brazil
<i>Scarthyia goinorum*</i>	—	QULC 2340	AY843752	Igarapé Nova Empresa, AM, Brazil
<i>S. goinorum*</i>	WED 55411	KU 205763	AY326035	Madre de Dios, Cusco Amazonico, Peru

Asterisks indicate species for which sequences were downloaded from GenBank. Collection acronyms: AM-CC, Ambrose Monell Cryo Collection; CHUNB, Coleção Herpetológica da Universidade de Brasília; KU, University of Kansas, Museum of Natural History; MACN, Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”; QULC, Queen’s University Laboratory Collection, Kingston, Canada; UTA, University of Texas at Arlington. Field numbers: AAGARDA and AAG, Adrian A. Garda; BPN, Brice P. Noonan; DCC, David C. Cannatella; GRCOLLI, Guarino Rinaldi Colli; WED, William E. Duellman.

following thermocycler conditions, with slight variations on annealing temperatures to improve products: 2 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 46 °C for 30 s, and 72 °C for 60 s. We used Viogene Gel-M™ Extraction System to clean products. ABI Prism Big Dye System was used in sequencing reactions with the following conditions for 25 cycles: 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Clean products were sequenced in an ABI 3100 PRISM sequencer.

2.3. Phylogenetic analysis

Sequencher (version 4.5) was used to assemble contiguous sequences for each species from individual overlapping fragments. Sequences were aligned and manually adjusted with MacClade 4.06 (Maddison and Maddison, 2000). Regions where the alignment remained ambiguous after inspection were excluded from analyses. Because the divergence among these species is low, it was necessary to exclude only a small region of about 10 bases. A total of 2377 characters were included in the final dataset.

We performed parsimony analysis using PAUP* 4.0b10 (Swofford, 2000). We used an heuristic search with TBR and 1000 random-addition sequence replicates under equal weighting, and also using a step matrix with transitions:transversions weighted 1:6 (gaps were not scored as characters). To evaluate clade support, a nonparametric

bootstrap resampling was used with 1000 replicate datasets and 100 random-addition sequences per dataset.

Modeltest (Posada and Crandall, 1998) was used to determine the best model of sequence evolution. A general time-reversible model with gamma distribution of substitution rates at variable sites and a proportion of invariant sites was selected. A maximum likelihood search was performed with a GTR+ Γ +I model in PAUP using an heuristic search and random starting trees.

We conducted a Bayesian analysis using MrBayes 3.1.1 assuming the model of sequence evolution determined by Modeltest. We used two identical searches with six chains and one million generations each, sampled every 1000 generations. The first 10,000 generations were discarded as burn-in using diagnostics provided in MrBayes. Default uniform priors were used.

2.4. Dispersal-vicariance analysis

To infer the geographic distribution of major ancestral nodes, we used DIVA version 1.1 (Ronquist, 1997). We used four areas in the analysis, corresponding to two main South American Cratons (Guiana Shield and Brazilian Shield) and two hydrographic basins (Amazon River basin and Paraná River basin) (Figs. 2 and 4). These four areas correspond to the South American platform (Almeida et al., 1981, 2000).

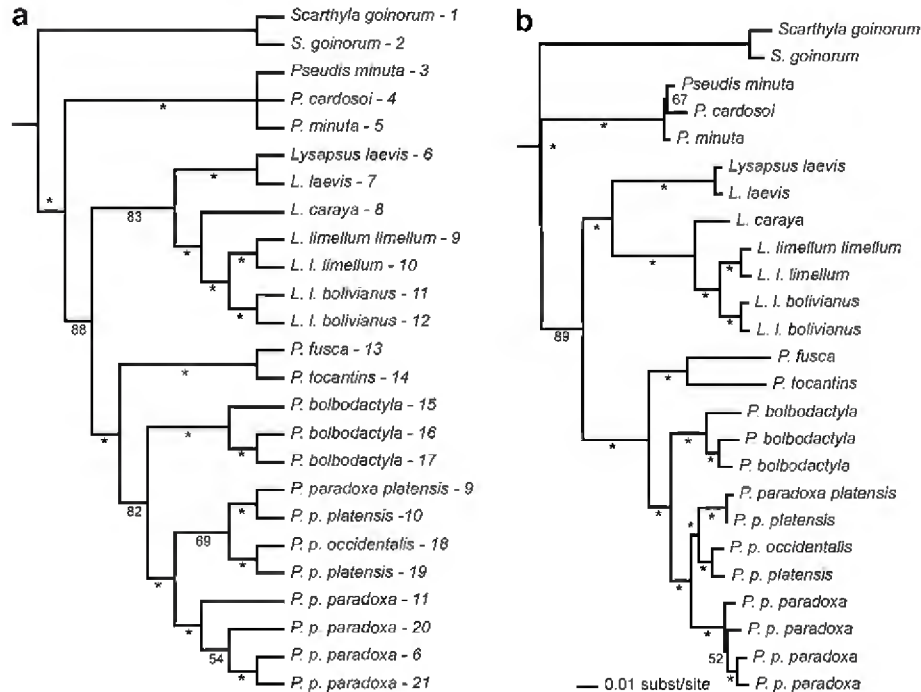


Fig. 3. (a) Maximum parsimony tree with bootstrap support values. Numbers to the right of each species name correspond to localities in Fig. 1. (b) Maximum likelihood tree with Bayesian posterior probability (bpp) values. Asterisks indicate bootstrap values equal or above 95% and bpp equal to or above 0.99.

3. Results

3.1. Phylogenetic analyses

We recovered one most parsimonious tree, which was identical to the tree recovered in the maximum likelihood and Bayesian analyses (Fig. 3). Pseudae is monophyletic with respect to *Scarthyla*, and three major lineages were recovered within them: the clade containing *Pseudis minuta* and *P. cardosoi* is the sister taxon to the group containing *Lysapsus* and the remaining *Pseudis*. Most branches have high bootstrap and posterior probability values, the only exceptions being the placement of *P. minuta*+*P. cardosoi*, the relationships within this clade, and the relationships among *P. paradoxa* populations.

Support for the species status of *P. cardosoi* is relatively low because of its low divergence from *P. minuta*. Support for the monophyly of *Lysapsus* is high, with *L. laevis* being the sister taxon to the remaining *Lysapsus*. *Lysapsus caraya* is the sister taxon to Paraná River basin populations of *L. limellum* (*L. limellum limellum*, sensu Gallardo (1961)) and Amazon River basin populations of *L. limellum* (*Lysapsus limellum bolivianus* sensu Gallardo (1961)).

The clade of *Pseudis fusca*+*P. tocantins* is the sister taxon to the *bolbodactyla*+*paradoxa* clade (Fig. 3). A relatively high degree of divergence is observed among *P. bolbodactyla* populations, with São Francisco River basin populations clustering with the northeast Goiás (Paraná valley in the Tocantins River basin) population, but not with the southeast Goiás clade (Paraná River basin). Within *P. paradoxa*, two major lineages are present: Cor-

rientes (Argentina) and Corumbá (Brazil) populations from the Paraguay River are the sister taxon to Formosa (Argentina) populations from the Pilcomayo River and São Paulo (Brazil) populations in the Paraná River basin, corresponding to Gallardo's (1961) *P. p. platensis* and *P. p. occidentalis*, respectively (Figs. 2 and 3). The second group contains east Amazon populations of *P. p. paradoxa* from Brazil (Roraima, Amapá, and Maranhão) and Guyana (Mabaruma). Gallardo (1961) considered this subspecies restricted to Guyana and Surinam, but later suggested that populations along the Amazon basin would probably belong to this subspecies (Gallardo, 1964).

A likelihood ratio test (Huelsenbeck and Crandall, 1997) to evaluate a clock-like evolution rejected the null hypothesis of a molecular clock ($-\ln L$ clock = 10910.18240, $-\ln L$ non-clock = 10883.80020, $\delta = 52.7644$, $df = 23$, $p < 0.003$).

3.2. Dispersal-vicariance analysis

The DIVA analysis found two equally most parsimonious reconstructions that required seven dispersal events (Fig. 4). Both solutions require six dispersals from the Brazilian Shield to the other areas (Fig. 4a and b). The difference between these reconstructions is whether the ancestor to *Scarthyla*+Pseudae was present in the Guiana Shield. This ambiguity stems from the distribution of *L. laevis*, which can be explained either by dispersal (Fig. 4a) or vicariance (Fig. 4b). The first solution assumes a more restricted ancestral distribution for *Scarthyla*+Pseudae, while suggesting the dispersal of a *Lysapsus* ancestor to the Guiana Shield. Conversely, the second reconstruction

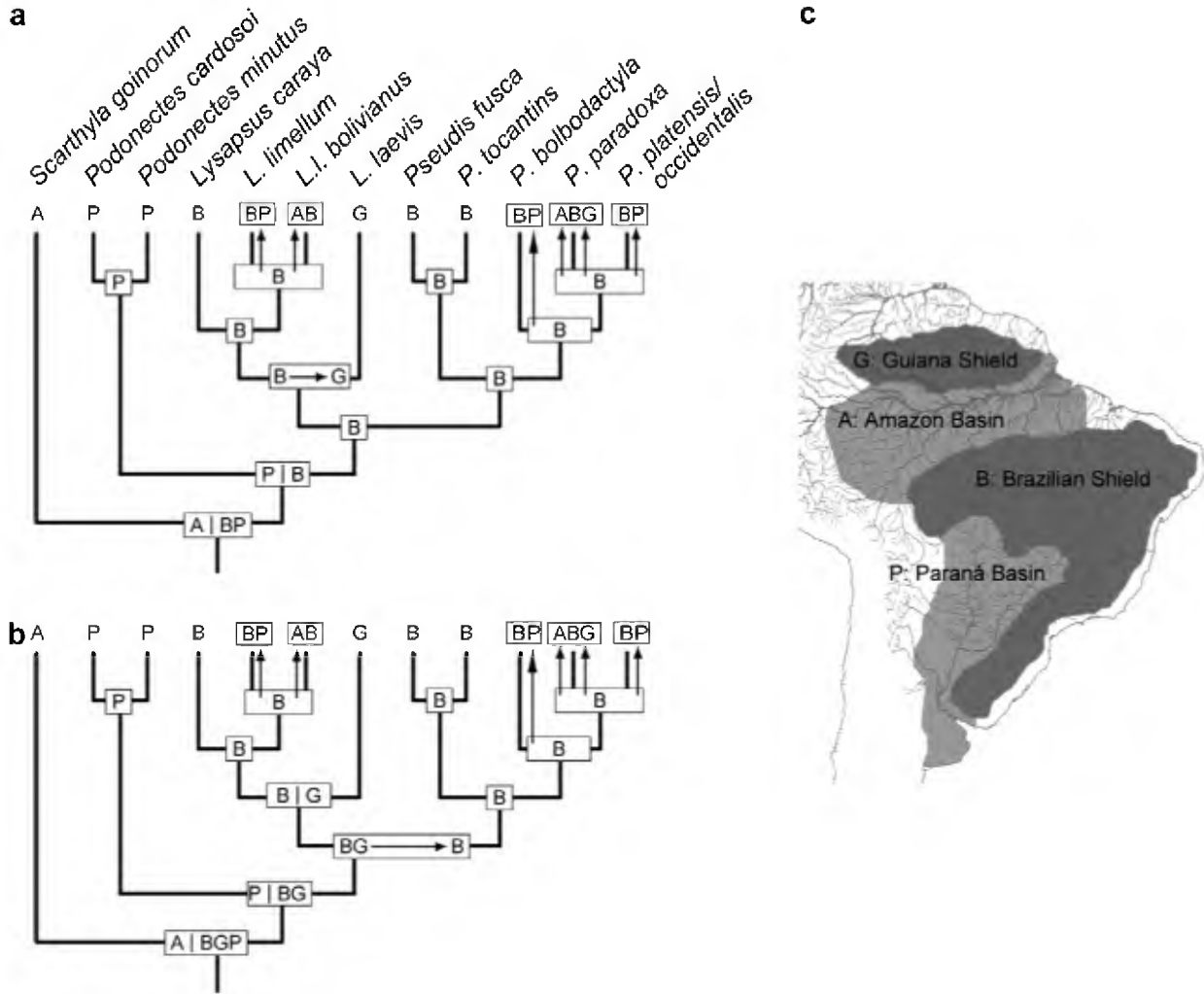


Fig. 4. (a) Four major geographic areas in the South American platform used in the dispersal-vicariance analysis. (b) Reconstructed ancestral distributions for each node on the two most parsimonious solutions obtained. Arrows indicate dispersals and vertical bars indicate vicariance events. (c) Geographic areas of South America used in the dispersal-vicariance analysis.

assumes a widespread ancestor and a within-area speciation event in the ancestor of *Pseudis*+*Lysapsus*, yielding two daughter lineages within a single area (Brazilian Shield), analogous to the splitting of gene lineages within a species lineage before a speciation event occurs (Fig. 4b). The presence of *L. laevis* in the Guiana Shield is then explained by vicariance.

4. Discussion

4.1. Taxonomy

The genera *Pseudis* and *Lysapsus* show remarkable morphological specialization to aquatic environments and are distinct from other hylids. This is why monophyly of the combined genera was never challenged but their relationship to other anurans long remained obscure. To continue recognition of these frogs by an informative name we suggest the unranked name Pseudae Fitzinger 1843 for the group formed by *Pseudis*, *Lysapsus*, and *Podonectes* (see below) species. At the time of Fitzinger, the use of plural names, often ending in

–ae, for suprageneric groups was common. As the nomenclatural practices became formalized, the use of this ending was abandoned in favor of –idae and –inae for families and sub-families. Rather than coin a new name, we use the older form, but without implication of rank.

Relationships within the group have not been addressed in detail until now. Partial surveys of external morphology (Caramaschi and da Cruz, 1998; Klappenbach, 1985; Savage and de Carvalho, 1953), sperm ultrastructure (Garda et al., 2004), and molecular data (Faivovich et al., 2005) were available for some species, and tentative synapomorphies were presented in these assessments. Faivovich et al. (2005) argued that current data do not unambiguously separate the two genera and that their monophyly warrants corroboration. Based on carpal tubercles, Caramaschi and da Cruz (1998) considered *P. bolbodactyla*, *P. fusca*, and *P. tocantins* to form a clade (with reduced or vestigial tubercles), differing from *P. paradoxa* subspecies (with well developed carpal tubercles). *Pseudis bolbodactyla*, *P. fusca*, and *P. tocantins* are easily separated from *P. minuta* because of their single vocal sac (paired in *P. minuta*).

Phylogenetic relationships shown in Fig. 3 support monophyly of *Lysapsus*, but *Pseudis*, as currently recognized, is not monophyletic. When assigning populations of *Pseudis* and *Lysapsus* to subspecies, Gallardo (1961) considered the placement of *P. minuta* problematic. Klappenbach surveyed 19 morphological characters in *P. p. platensis*, *P. minuta*, *L. limellum*, and *L. laevis*. Ten characters grouped *P. minuta* and *P. p. platensis*, 3 grouped *Lysapsus* and *P. minuta*, and 6 were exclusive to *P. minuta*. He considered the paired vocal sac and the absence of carpal tubercles in *P. minuta* important for generic relationships, but preferred to place *minuta* in *Pseudis* because of the remaining ten characters that it shared with this genus. Garda et al. (2004) studied Pseudae sperm ultrastructure and found *P. minuta* distinct from *Lysapsus* and *Pseudis*, with a large subacrosomal cone and a distinct multilaminar structure above the nucleus. Hence, a considerable number of distinguishing morphological characters exist for *P. minuta* and *P. cardosoi*.

The nonmonophyly of *Pseudis* can be solved in two ways: all Pseudae can be considered *Pseudis* (*Lysapsus* would be a synonym of *Pseudis*) or *P. minuta*+*P. cardosoi* can be allocated to a different genus. The placement of these two species as the sister group to remaining *Pseudis*+*Lysapsus* is not as well supported as other branches (Fig. 3), but is the best working hypothesis because is recovered consistently by all methods. The next alternative topology places them as the sister taxon to all *Lysapsus*. Acceptance of this placement also makes *Pseudis* paraphyletic, and some taxonomic change remains necessary. Current morphological and molecular evidence favors placement of *P. minuta* and *P. cardosoi* in a different genus so that all genus-level taxa remain monophyletic.

Podonectes palmatus Steindachner 1864 is a junior synonym of *P. minuta* (Frost, 2004; Savage and de Carvalho, 1953). We therefore suggest resurrection of *Podonectes* and inclusion of two species in it: *Po. minutus* and *Po. cardosoi*. *Podonectes* is presumably derived from *podo-* for foot, and *-nectes*, swimmer. Generic names ending in *-nectes* are generally treated as masculine (e.g., *Eumectes*). See Appendix A for taxonomic details on the usage of generic names for Pseudae.

4.2. Species and subspecies of *Pseudis* and *Lysapsus*

Based only on the degree of genetic divergence, one might suspect that *Po. cardosoi* is not distinct from *Po. minutus*. However, differences in calls, morphology, and geographic distribution contradict these findings (Kwet, 2000). Because several factors can render mtDNA gene trees different from species trees (Funk and Omland, 2003; Nichols, 2001), and because of our small sample sizes, it is premature to challenge the species status of *Po. cardosoi*. Careful consideration of different data sets with a comprehensive geographic coverage (including morphology, nuclear genes, and intensive sampling along distribution contact zones) is needed to confirm that *Po. cardosoi* is distinct from *Po. minutus*.

Our results support two currently recognized species of *Lysapsus* (*laevis* and *caraya*) and suggest that *L. l. limellum*

and *L. l. bolivianus*, which occur in different hydrographic basins (Fig. 1: 11 and 12, 9 and 10), may be separate evolutionary lineages. In *Pseudis*, the geographically distant *Ps. tocantins* (Tocantins River basin) and *Ps. fusca* (Jequitinhonha River basin) form the sister taxa to the *Ps. bolbodactyla*+*Ps. paradoxa* clade (Figs. 1 and 3). Considerable evidence indicates the recognition of the three subspecies of *Ps. paradoxa* (*paradoxa*, *platensis*, and *occidentalis*) as distinct species. However, such changes should not be made until morphological synapomorphies are identified and the two other subspecies (*P. p. nicefori* and *P. p. caribensis*) are sampled. Populations of *Pseudis* and *Lysapsus* not included in this analysis are known to occur along all of the Amazon River and up its tributaries (whenever open areas or floodplains are present), such as the Beni River in Bolivia and the Guaporé and Madeira Rivers in Brazil. A more comprehensive sampling of morphological and molecular characters is therefore desired before such taxonomic modifications can be endorsed. This is also the case for *Ps. bolbodactyla* populations, which show high interpopulational genetic distances (much higher than between *cardosoi* and *minutus* and *L. l. limellum* and *L. l. bolivianus*, for example; see Fig. 3b). Although morphological differences have been reported in the literature for some of these species (e.g., *P. p. occidentalis* and *P. p. platensis* (Gallardo, 1964)), we do not consider their current diagnosis compelling. Phenotypic variation of *Pseudis* populations is remarkable. Some morphological characters commonly used to separate species, such as lines on the ventral surface of the thighs and size (Caramaschi and da Cruz, 1998; Gallardo, 1961), are hampered by lack of data on geographical and populational variability.

4.3. Pseudae biogeography

This is the first molecular phylogenetic hypothesis for a widespread group of South American frogs for which the effects of continental-scale geological events such as marine incursions and the uplift of the Brazilian Shield can be evaluated. A considerable constraint is the lack of fossils for most South American frogs. Few anuran fossils are available for this region, and no Pseudae are known (Colli, 2005). Because Pseudae are absent from the eastern part of the Andes, it is not possible to use the orogeny of the northern part of the Andes as a calibration of the phylogeny, as used for other groups (Montoya-Burgos, 2003; Noonan and Wray, 2006). Furthermore, a likelihood ratio test (Huelsenbeck and Crandall, 1997) rejected the clock-like evolution hypothesis for the present data set, and the lack of reasonable calibrations and/or constraint for any node precludes the use of relaxed molecular clocks as well. Nevertheless, some predictions derived from such events, such as the monophyly of Brazilian Shield taxa resulting from marine incursions (Aleixo, 2004; Noonan and Wray, 2006), can be evaluated. We discuss some of these hypotheses below in view of the phylogenetic relationships.

4.3.1. One basin—one species hypothesis

Gallardo (1961) suggested that each subspecies of Pseudoeurycea was associated with one hydrographic basin in South America. This one species–one basin hypothesis was later extended to *Bufo granulosus* subspecies (Gallardo, 1965). Most populations from the same hydrographic basin are indeed clustered in the present phylogeny, but not all species are restricted to one basin (Figs. 1, 3, and 4). The exceptions are populations of *Ps. bolbodactyla* (which occur in the São Francisco, Paraná, and upper Tocantins basins) and the *Ps. paradoxa* and *L. limellum* subspecies distributed in the Amazon and Paraná basins. Clustering of Chaco populations of *Ps. paradoxa* along the Pilcomayo River with Paraná populations in São Paulo (Fig. 1, 18 and 19) is difficult to explain. Rather than being associated with specific hydrographic basins, the distributions of these species seems to be better predicted by the occurrence of open formations such as Savanna enclaves and floodplains (Lynch, 1979), as suggested for *B. granulosus* (Narvaes, 2003). Nevertheless, some species are clearly restricted to specific basins, such as *Ps. tocantins* and *L. caraya*. Most of central and northern South America is still poorly sampled, however, and more populations will likely be discovered along major rivers.

4.3.2. Ancestral distributions and marine introgressions

The DIVA reconstruction algorithm assumes that each dispersal between any two areas incurs equal cost, even if the dispersal path must cross an intervening area. Similarly, any pattern of vicariance is possible. DIVA does not explicitly reconstruct extinction events, so these are not considered in the set of possible explanations presented. Both scenarios in Fig. 4 involve six dispersals from the Brazilian Shield to the Amazon and Paraná basins, indicating the general importance of the Brazilian Shield in the historical biogeography and diversification of Pseudoeurycea.

The dispersal events reconstructed for *L. limellum* and *Ps. paradoxa* (Fig. 4a and b) are concordant with the histories of the Amazon and Paraná basins. Only at the end of the Tertiary/early Quaternary did these two basins acquire their current shape, in response to the end of marine introgressions (Nores, 2004), local tectonics (Rossetti et al., 2005), and the epeirogenic uplift of the Brazilian and Guiana Shields and the northern part of the Andes. The uplift of the Brazilian Shield during the late Tertiary compartmentalized the landscape, with depressions such as the Pantanal and Guaporé forming between plateaus in the Brazilian Shield. The Pantanal floodplain in the Paraná basin, for example, is younger than 3 million years (Assine, 2004), while the Amazon basin acquired its current anastomosed shape during the Pliocene/Pleistocene epochs (Rossetti et al., 2005). Geological evidence supports two cycles of marine incursions into the Amazon and Paraná basins in the middle and late Miocene, promoting extensive flooding of lowlands and isolation of three large land blocks, the Brazilian and Guiana Shields and the eastern slope of the Andes (Hallam, 1992; Hernandez et al., 2005; Nores, 2004).

Populations isolated in these blocks would then differentiate, while lowland species and populations would become extinct (Rossetti et al., 2005; Webb, 1995). Aleixo (2004) showed that woodpecker populations (*Xyphorhynchus spixiilegans* superspecies) in the Brazilian Shield are indeed older than lowland populations, as expected from the marine incursion hypothesis. Similarly, the occurrence of *Lysapsus* and *Pseudis* in the Paraná and Amazon basins is best explained by recent dispersals to these regions, while older species (*L. laevis*, *L. caraya*, *Ps. tocantins* and *Ps. fusca*) are present in the Brazilian and Guiana Shields (Fig. 4). The similar pattern of recent dispersals independently inferred for *Lysapsus* and *Pseudis* further supports this scenario.

The seventh dispersal reconstructed by the DIVA analysis is the source of discordance between the two solutions in Fig. 4. Either an ancestor to all Pseudoeurycea was absent from the Guiana Shield (Fig. 4a) or all the areas were once occupied by the ancestor of *Scarthyla*+Pseudoeurycea (Fig. 4b). The first solution implies an unlikely long-distance dispersal of a *L. laevis* ancestor to the Guiana Shield across the Amazon basin and its subsequent extinction in this region.

The second solution is more consistent with the current restricted distribution of *L. laevis* (Fig. 4b). Several recent phylogenies of South American vertebrates show major lineage splits concordant with the Brazilian/Guiana Shield separation usually attributed to Miocene marine introgressions (Noonan and Wray, 2006; Ribas et al., 2005). These hypotheses, however, can be appropriately tested only when adequate clock calibrations, similar patterns for different species, and appropriate geological data for the region are available.

4.3.3. Amazon and Paraná basins

The close relationship and small genetic distances between populations of *Lysapsus* and *Pseudis* in the Paraná and Amazon basins may be due to gene flow or recent connection between these regions. In Mato Grosso, Brazil, and in southeast Bolivia, headwaters that run in opposite directions to these two basins are less than 100 km apart, and recent contact or conditions for dispersal are not unlikely. Montoya-Burgos (2003) reconstructed and dated vicariant events for South American rivers based on a phylogenetic hypothesis for *Hypostomus* catfishes. According to his analysis, the Amazon and Paraná basin species have been separated for at least 10 million years. Nevertheless, the close proximity of their headwaters and the lower philopatry of frogs may have maintained gene flow between these two basins.

Within the Amazon basin, sites along major tributaries have similar frog assemblages (Azevedo-Ramos and Galatti, 2001), and previous work showed that Amazonian rivers are not barriers for frog dispersal (Gascon et al., 1996, 1998). The low divergence between the geographically distant populations of *L. limellum* from the Amazon basin in the present study (Figs. 1, 11 and 12, about 1600 km) suggests that gene flow is possible not only between opposing

margins, but also between distant points along Amazon tributaries, especially for these aquatic species. Other open-area frog species (such as *Bufo granulatus*, *Hyla multifasciata*, *Leptodactylus fuscus*, and *Elachistocleis ovalis*) are widespread along the Amazon, Paraná, and other Brazilian Shield river systems (Heyer, 1987). If these frogs follow the same pattern as *L. limellum*, it is likely that floodplains and associated open area fragments are facilitating gene flow to such an extent that the implausible hypothesis that several widespread species of frogs occur throughout South America (Heyer and Reid, 2003; Wynn and Heyer, 2001) cannot be dismissed.

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Appendix A.

A.1. The use of generic names for *Pseudae*

There is an apparent discrepancy concerning the synonymy of some available generic names for *Pseudae*. As pointed out by Savage and de Carvalho (1953), Steindachner (1864) created (under the rules of the Code) a new genus and species when he listed *Podonectes palmatus* as a synonym of *Pseudis minuta*. The name apparently came from a manuscript of Fitzinger. Frost (2004), under the account of *Pseudis minuta*, states that *Podonectes palmatus*

Steindachner 1864 is a “Substitute name for *Pseudis minuta* Günther.” However, under the synonymy of *Lysapsus* Cope 1862, it is stated that the type-species of *Podonectes* is “*Podonectes palmatus* Fitzinger, 1864 (= *Lysapsus limellus* Cope, 1862), by monotypy.”

The discrepancy stems from Savage and de Carvalho (1953), who stated that Steindachner “inadvertently proposed a new generic and specific name for Cope’s *limellum* when he cited a manuscript name of Fitzinger’s in the synonymy of *Pseudis minuta* (nec Günther). This generic name, *Podonectes*, is a strict synonym of *Lysapsus* and its monotype, *P. palmatus*, is the same as Cope’s *limellum*.” (p. 194). Steindachner (1864), however, clearly lists *Lysapsus limellum* Cope 1862 (type-species of *Lysapsus*), among others, under the primary heading for *Pseudis minuta* Günther. Thus, the type-species *Podonectes palmatus* should be considered a junior subjective synonym of *Pseudis minuta* and not of *Lysapsus limellum*.

To avoid future ambiguity, we designate the holotype of *Pseudis minutus* (BM 1947.2.25.96), which was examined in 1998 by Axel Kwet (Kwet, 2000), as the neotype of *Podonectes palmatus* Steindachner 1864. This action renders the species *Podonectes palmatus* a junior objective synonym of *Pseudis minutus*, a species whose holotype is extant and whose distribution is known (Kwet, 2000). Because a single specimen is the name-bearing type for two nominal species, the possibility of future confusion is obviated. Also, because the name *Podonectes palmatus* is available due solely to its perfunctory listing in older literature—no voucher specimens, description, or illustration were associated with it—the possibility of associating this name with the wrong extant species is nil. Furthermore, the designation of the neotype ensures that the name *Podonectes* is either unambiguously a junior synonym of *Pseudis*, or is the appropriate valid generic name for the species *minutus* if that species is placed in a genus apart from *Pseudis* (as we propose here).

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