



Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

What happened in the South American Gran Chaco? Diversification of the endemic frog genus *Lepidobatrachus* Budgett, 1899 (Anura: Ceratophryidae)

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ARTICLE INFO

Keywords:

Miocene marine introgression
Species tree
Fossil calibration
Lepidobatrachus asper
Lepidobatrachus laevis
Lepidobatrachus llanensis

ABSTRACT

The Chaco is one of the most neglected and least studied regions of the world. This highly-seasonal semiarid biome is an extensive continuous plain without any geographic barrier, and in spite of its high species diversity, the events and processes responsible have never been assessed. Miocene marine introgressions and Pleistocene glaciations have been mentioned as putative drivers of diversification for some groups of vertebrates in adjacent biomes of southern South America. Here we used multilocus data (one mitochondrial and six nuclear loci) from the three species of the endemic frog genus *Lepidobatrachus* (*Lepidobatrachus asper*, *Lepidobatrachus laevis*, and *Lepidobatrachus llanensis*) to determine if any of the historical events suggested as drivers of vertebrate diversification in southern South America are related to the diversification of the genus and if the Chaco is indeed a biome without barriers. Using fossil calibration in a coalescent framework we estimated that the genus diversified in the second half of the Miocene, coinciding with marine introgressions. Genetic patterns and historical demography suggest an important role of old archs and cratons as refuges during floods. In one species of the genus, *L. llanensis*, genetic structure reveals some breaks along the landscape, the main one of which corresponds to an area of the central Chaco that may act as a climatic barrier. Additionally, we found differential effects of the main Chacoan rivers on species of *Lepidobatrachus* that could be related to the time of persistence of populations in the areas influenced by these rivers.

1. Introduction

It has become increasingly clear that the origin of extant Neotropical biodiversity is not restricted to a particular timeframe or mechanism (Rull, 2011). Diversification of extant biodiversity (fauna and flora) in the Neotropics is related to events and processes that have taken place from late Eocene/early Oligocene to Pleistocene (Rull, 2008). However, this understanding was based mainly on studies from tropical forests, such as the Amazon Forest and the Atlantic Forest, neglecting open biomes like those of the “diagonal of open formations” (sensu Vanzolini, 1963), including Caatinga, Cerrado, and Chaco. Collectively, these formations occupy about 4,600,000 km² (Duellman, 1999) of strongly seasonal sub-humid to semiarid formations, which extend from central Argentina to northeastern Brazil, including large portions of Paraguay and Bolivia. However, it is clear that Caatinga, Cerrado, and Chaco are distinct biomes, each with its own identity

(Cabrera and Willink, 1973; Rizzini, 1979; Dinerstein et al., 1995; Olson et al., 2001). Among these biomes, the Chaco is the most neglected in terms of knowledge regarding its biodiversity evolutionary history (Werneck, 2011), because there is an absence of scientific studies addressing putative drivers of Chacoan species diversification, like dispersal barriers or historical events.

The Chaco is a semiarid biome with marked climatic seasonality (Prado, 1993; Cabrera, 1994), and is characterized by xerophytic vegetation formed by a mosaic of grassland, savannas, open woodlands, and xeric thorn forest (Willig et al., 2000). It spans from southeastern Bolivia to central Argentina, occupying more than 60% of Paraguay and a small portion of midwestern Brazil, in the state of Mato Grosso do Sul. The Chaco is one of the most extensive continuous forested areas in South America with an approximate extent of 1,000,000 km² (Bucher, 1982). The Chaco is an extensive sedimentary alluvial plain with soils derived from the accumulation of fine loess and alluvial sediments

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during the Quaternary (Pennington et al., 2000). This biome has historically been considered a continuum, without geographical barriers to organismal dispersion (Bucher, 1982). However, some potential barriers occur throughout the Chaco. Close to its southern and western limits, some elevated areas are present, such as some of the mountains of the Sierras Pampeanas in northern Córdoba, Argentina, and the Sierras Subandinas in Catamarca and La Rioja, also Argentinean provinces. Furthermore, some rivers cross the Chaco in a west-east direction until their confluence with the Paraguay and Paraná rivers. The main Chacoan rivers are the Pilcomayo, Bermejo, Salado, and Dulce. These rivers are allocthonous, having their headwaters in the Andes, and possess flowing water only during the rainy season, with their channels losing water by infiltration during the rest of the year (Iriondo, 1993). Furthermore, these rivers carry large amounts of sediment that eventually fill channels and cause the rivers to change their courses, thereby forming large alluvial fan systems. The Chaco, as currently known, is the result of the Andean uplift (Gregory-Wodzicki, 2000), marine introgressions (Hernández et al., 2005), and several alluvial fan systems (Iriondo, 1993), which have continuously influenced the distribution and the climatic conditions of this biome, since the Paleogene until today. Other geographically important historical events in southern South America are glaciations, many of which contributed to climatic changes in these latitudes (Ortiz-Jaureguizar and Cladera, 2006).

Miocene marine introgressions have been suggested as probable drivers of diversification of some vertebrate groups in southern South America, like some mammals and reptiles (Candela et al., 2012; Delsuc et al., 2012; Morando et al., 2014). At least three extensive marine introgressions have been recorded for this region (Ottone et al., 2013). The most important in extension, the Paranense Sea, occurred between 15 and 13 million years ago (Ma), and covered almost the entire current distribution of the Chaco (Hernández et al., 2005; Candela et al., 2012; Ottone et al., 2013). Like marine introgressions, Pleistocene glaciations with associated refugia have also been proposed as diversification mechanisms for southern South America (Nuñez et al., 2011; Blotto et al., 2013; Langone et al., 2016). The main South American Pleistocene glaciations occurred during the last million years (Rabassa and Clapperton, 1990; Ruzzante et al., 2008).

The frog genus *Lepidobatrachus* is an interesting model for testing hypotheses about drivers of diversification in the Chaco biome. This monophyletic taxon contains three species (*Lepidobatrachus asper*, *Lepidobatrachus laevis*, and *Lepidobatrachus llanensis*) with geographical distributions restricted to the Gran Chaco (Faivovich et al., 2014). These species are mostly aquatic, inhabit temporary ponds, and exhibit several characteristics associated with survival in semiarid environments, such as cocoon formation and short duration of larval development (Faivovich et al., 2014). When ponds dry, these frogs burrow into the humid soil and produce a cocoon of dead skin, which helps to protect against desiccation during the dry period of estivation (Faivovich et al., 2014). In a similar manner, their short larval development is also related to the ephemeral nature of their aquatic habitats by helping to minimize or to prevent larval desiccation. This set of characteristics, which are closely related to the ephemeral water regime of their preferred habitats, would lead us to think that species of *Lepidobatrachus* experience strong barriers to dispersal and consequently a decreased gene flow and high levels of population differentiation, which further contributes to making this genus a great candidate to study patterns of diversification in the Chaco.

The two main events that have been proposed as drivers of diversification of vertebrates of southern South America occurred during two different and widely separated time periods; middle-Miocene marine introgressions between 15 and 13 Ma and Pleistocene glaciations in the last 1 Ma. With the delimitation of a temporal framework we can infer the most plausible diversification driver within *Lepidobatrachus* and study the mechanisms involved in this process. Both marine introgressions and glaciations have been suggested as

drivers of diversification of some groups of species by isolating populations in suitable areas and promoting opportunities for speciation by geographical isolation. If indeed marine introgressions isolated populations of *Lepidobatrachus* in areas protected from flooding, we should be able to find genetic signatures of these events. These signatures should help in the identification of stable areas where populations have persisted for longer periods of time, as well as the direction of subsequent expansions, when habitats became suitable again. The same type of pattern is expected for refugia during glaciations, for which we expect higher genetic diversity at stable areas and genetic signatures of expansion in unstable or recently colonized areas (Hewitt, 1996).

Here we used a multilocus dataset with samples of the three species of *Lepidobatrachus* from throughout the entire distribution of the genus to address two main questions: (1) are any of the historical events suggested as drivers of diversification of vertebrates of southern South America (marine introgressions and glaciations) also related to *Lepidobatrachus* diversification; and (2) is the Chaco indeed a barrier-free biome as has been suggested? To answer these questions we: estimated the diversification time frame for the genus to correlate it with historical events; estimated genetic diversity indices and tested for demographic expansions in order to identify areas where each lineage may have been isolated; and investigate the genetic structure and genetic differentiation among populations, with the specific objective of identifying putative dispersal barriers across the Chaco.

2. Material and methods

2.1. Sampling

We included 24 samples from six localities of *L. asper*, 79 samples from 26 localities of *L. laevis*, and 89 samples from 27 localities of *L. llanensis* (Fig. 1; Appendix A). Voucher specimens are housed in the Herpetological Collection of the Instituto de Investigación Biológica del Paraguay (IIBP-H), Asunción, Paraguay; Laboratorio de Genética Evolutiva (LGE, JNL, and LL), Posadas, Argentina; Museo Argentino de Ciencias Naturales (MACN and BB), Buenos Aires, Argentina; and in the Coleção Zoológica de Referência da Universidade Federal do Mato Grosso do Sul (ZUFMS), Corumbá, Brazil.

2.2. Laboratory procedures and molecular methods

We extracted total genomic DNA from samples preserved in 95–100% ethanol (muscle or liver), using the DNeasy extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We amplified one mitochondrial fragment and six nuclear introns via polymerase chain reaction (PCR) using published primers (Table 1) and a commercial kit (Master Mix, Fermentas). In cases where a locus was not previously referred with an abbreviation, we used the pair of primers to name it throughout the text. For the amplification of the mitochondrial gene we used a step-up reaction (UP) following Lyra et al. (2016). The UP reaction consisted of an initial denaturation step with 3 min at 95 °C, 10 cycles of denaturation for 20 s at 95 °C, annealing for 20 s at 50 °C (increasing + 0.5 °C in each subsequent cycle) and extension for 50 s at 60 °C; followed by 25 cycles of denaturation for 15 s at 95 °C, annealing for 20 s at 50 °C, extension for 50 s at 60 °C, and final extension for 5 min at 60 °C. For the amplification of the nuclear genes we used an initial denaturation step of 3 min at 94 °C, followed by 35 cycles (45 cycles for difficult samples) (30 s of denaturation at 95 °C, 30 s of annealing at 50–64.3 °C, and 45 s of extension at 72 °C), and a final extension step of 7 min at 72 °C (see Table 1 for details). We purified PCR products using ExoSAP (Fermentas) and sent them to Macrogen Inc. (Seoul, South Korea) for sequencing. We checked chromatograms and edited sequences in CodonCode Aligner v. 3.5.4 (Codon Code Corporation). GenBank Accession Numbers are listed in Appendix B, sequences with less than 200 base pairs are deposited in the Dryad Repository (<https://doi.org/10.5061/dryad.mn183qg>). In the

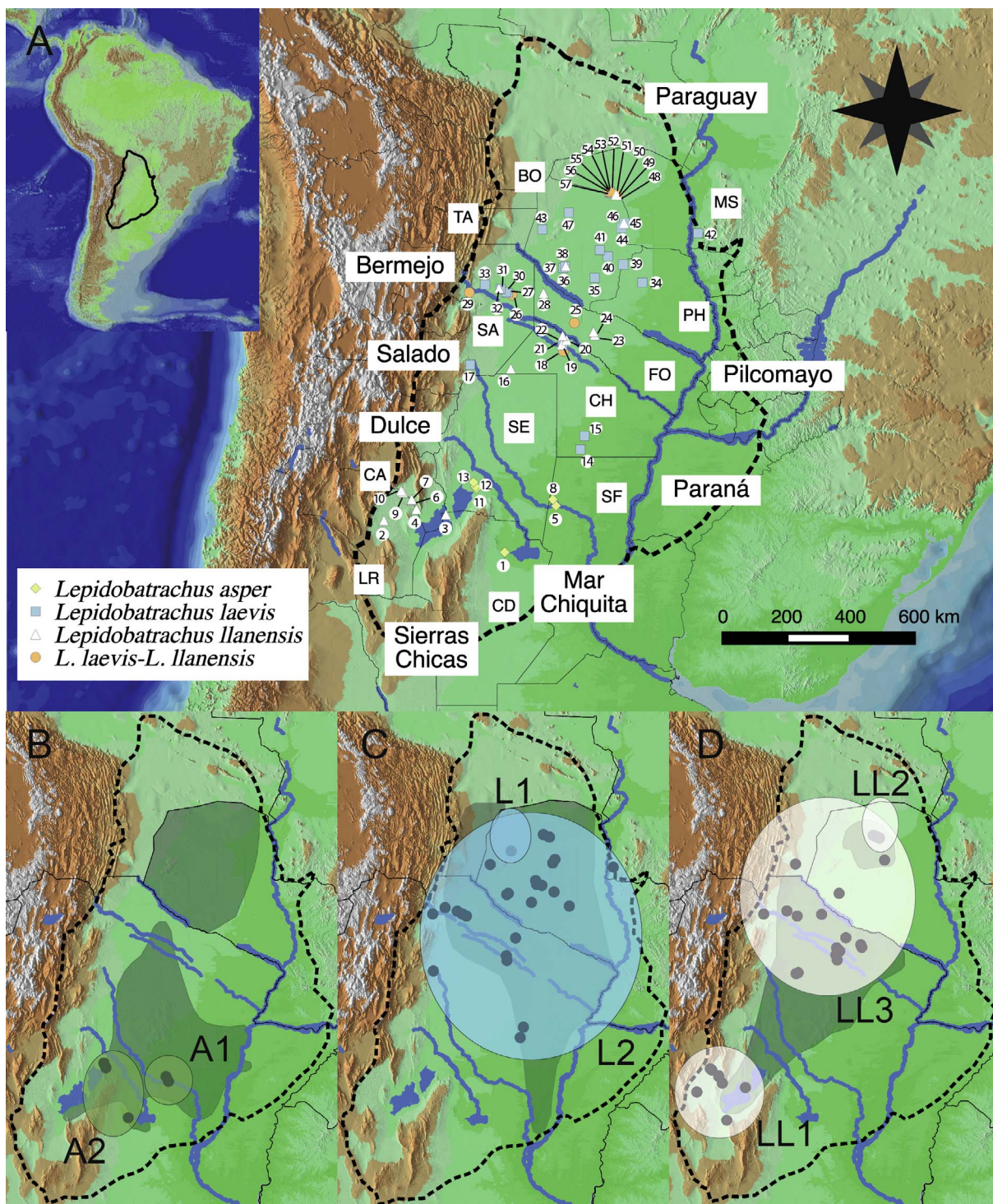


Fig. 1. (a) Sampling localities of *Lepidobatrachus* species. Area enclosed in black in map of South America and dashed in the enlarged map denotes the limits of the Chaco biome according to Morrone (2001). Putative dispersal barriers discussed in the text are highlighted with their respective names. Detailed locality information represented by numbers is shown in Appendix A. Department/province/state abbreviations: CA, Catamarca; CD, Córdoba; CH, Chaco; FO, Formosa; LR, La Rioja; SA, Salta; SF, Santa Fe; SE, Santiago del Estero (Argentina); MS, Mato Grosso do Sul (Brazil); BO, Boquerón; PH, Presidente Hayes (Paraguay). (b), (c), and (d) correspond to sampling localities, distribution of haplogroups (based on mitochondrial results), and approximate geographic distribution (in darker green), based on IUCN (2017), of *L. asper*, *L. laevis*, and *L. llanensis*, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

subsequent analyses we used one to five samples per locality for the mitochondrial gene and one or two samples per locality for the nuclear genes (Fig. 1; Appendix B).

We aligned sequences from each fragment separately with MUSCLE (Edgar, 2004) in MEGA 6 (Tamura et al., 2013) and checked by eye. We separated sequences of individuals with heterozygous indels with the

algorithm Process Heterozygous Indels in CodonCode Aligner v. 3.5.4 and used Phase 2.1 (Stephens et al., 2001) implemented in DnaSP 5.1 (Librado and Rozas, 2009) to resolve haplotypes of heterozygous individuals, discarding those resolved with less than 0.90 posterior probability. We generated haplotypes in DnaSP. Within nuclear loci, we tested for recombination with PhiTest implemented in Splitstree v4.2

Table 1

Primers and annealing temperature (°C) used for amplification of each locus. In the cases where a locus was not previously referred with acronyms, we use the pair of primers to name it throughout the text.

Locus ID (base pair number)	Primer sequence 5'–3'	Annealing	Reference
CO1 (600 bp)	ANF1 ACHAAYCAYAAAGAYATYGG	45/50	Jungfer et al. (2013)
Cytochrome c oxidase subunit 1	ANR1 CCGGTCTGAACCTCAGATCACGT		Jungfer et al. (2013)
GAPDH (297 bp)	MVZ15 ACACCCACTCCTCTATCTTTGATG	54.7	Bell et al. (2011)
Glyceraldehyde-3 Phosphate Dehydrogenase (intron 4)	MVZ16 AAATGTAAGCTAAGAGATCCACAAC		Bell et al. (2011)
MVZ 27–28 (352 bp)	MVZ27 ATTATTCGTAACAGCAAACCTC	54.7	Bell et al. (2011)
Lactose Dehydrogenase Chain Beta (Intron 3)	MVZ28 GTAACCATGGCAACTGGTAG		Bell et al. (2011)
MVZ 29–30 (198 bp)	MVZ29 ATCCTCCATACTACTTAAGGAGACC	57	Bell et al. (2011)
Y Box Binding (Intron 1)	MVZ30 CTGAAGCCTCTGTACATGTTTTG		Bell et al. (2011)
MVZ 39–40 (180 bp)	MVZ39 GGATCTGTAGAGACCTGTCACTTC	57	Bell et al. (2011)
<i>X. laevis</i> MGC82783 protein (Intron 2)	MVZ40 ACAGAGTCTTCAAACCCAGCAATAC		Bell et al. (2011)
MVZ 47–48 (340 bp)	MVZ47 AGTGAAGATACAGTCACAGTGTAGG	54.7/56.7/59	Bell et al. (2011)
Fibrinogen, A alpha polypeptide (Intron 1)	MVZ48 GGAGGATATCAGCACAGTCTAAAAAG		Bell et al. (2011)
RPL3 (413 bp)	RPL35F AAGAAGTCYCACCTCATGGAGAT	50/53/64.3	Pinho et al. (2009)
Ribosomal Protein L3 (Intron 5)	RPL36RA AGTTTCTTTGTGTGCCAACGGCTAG		Pinho et al. (2009)

(Huson and Bryant, 2006).

2.3. Species-tree and diversification-time estimation

To infer the species tree and to estimate a timeframe of diversification for the genus *Lepidobatrachus*, we used the multilocus coalescent model implemented in *Beast* (Heled and Drummond, 2010) on *Beast* 2.4.3 (Bouckaert et al., 2014), considering the seven loci (*CO1* and the six nuclear introns) and available sequences for *Lepidobatrachus* species in GenBank for the genes *12S* (926 bp), *16S* (1428 bp), cytochrome *b* (*Cytb*) (1003 bp), NADH dehydrogenase subunit 1 (*ND1*) (961 bp), exon 2 of chemokine receptor 4 (*CXCR4*) (676 bp), proopiomelanocortin A gene (*POMC*) (556 bp), recombination-activating gene 1 (*RAG1*) (428 bp), exon 1 of rhodopsin (*RHOD*) (316 bp), seven in absentia homolog 1 (*SIAH*) (397 bp), and tyrosinase (*TYR*) (532 bp), for a total of five mitochondrial and 12 nuclear fragments, and 9603 bp. For this analysis, we included only samples with sequences for at least four loci including *CO1* sequence to diminish missing data and to ensure some amount of common sequence (see Appendix B). We included a total of 11 individuals of *L. asper*, 38 individuals of *L. laevis*, 29 individuals of *L. llanensis*, and two individuals of each species used as outgroup. Sequences were not combined across loci and individuals. Nucleotide substitution model, range of the rate of heterogeneity, and proportion of invariant positions were inferred during the MCMC analysis with *bModelTest* package (Bouckaert, 2015) implemented in *Beast*, with transition-transversion split option and empirical frequencies.

To assess clock models that best fit our dataset we ran exploratory analyses of each locus in standard *Beast* with uncorrelated lognormal relaxed clock using Coefficient of Variation (CV) as indicator of goodness of fit. CV is indicative of how much variation among rates is implied by the data; values below 0.1 are considered strong evidence for the use of the strict clock (Drummond and Bouckaert, 2015). The Strict Clock model was used only for the nuclear loci MVZ 27–28, MVZ 29–30, MVZ 39–40, and RPL3; the Relaxed Clock Log Normal model was used for all the other loci.

Fossil calibration is largely the best practice to estimate divergence time; however, justifying the use of a fossil record is not trivial (Parham et al., 2012). Several authors have recently questioned the taxonomic position of the fossils historically assigned to Ceratophryidae, *Beelzebubo ampinga* (Evans et al., 2008), *Baurubatrachus pricei* (Báez and Perí, 1989), and *Wawelia geroldi* (Casamiquela, 1963) (Agnolín, 2012; Báez et al., 2005; Faivovich et al., 2014; Nicoli et al., 2016), and their use as calibration points (Faivovich et al., 2014; Nicoli et al., 2017). On the other hand, in the last few years fossil data for Ceratophryidae significantly increased, with several new fossil records for the genus *Ceratophrys* and one for the genus *Lepidobatrachus* (Fericola, 2001; Tomassini et al., 2011; Nicoli, 2014; Nicoli et al., 2017). The only

record of *Lepidobatrachus* corresponds to the fossil species *Lepidobatrachus australis* (Nicoli, 2015), which is from the late Miocene-early Pliocene from Farola Monte Hermoso, Buenos Aires, Argentina (Tomassini et al., 2011, 2013; Fericola, 2001; Nicoli, 2015), dated between 5 and 6.8 Ma (Cione et al., 2007). In *Beast* analysis we used a log-normal distribution with $M = 1.0$, $S = 1.25$, offset = 5 based on the minimum possible age of the *L. australis* fossil in order to constrain the minimum bound for the node of the clade that includes all species of the genus *Lepidobatrachus*. The maximum bound, although it is a soft limit, is based on the combination of the *Ceratophrys-Lepidobatrachus* divergence suggested by Roelants et al. (2007), Heinicke et al. (2009), and Ruane et al. (2011) (without *B. ampinga* as a calibration point) and on the *Ceratophrys-Chacophrys + Lepidobatrachus* divergence suggested by Frazão et al. (2015). With these parameters the 5–95% prior distribution lies between 5.23 and 26.2 Ma.

Among the markers used, a mutation rate is available for only *CO1*. Freilich et al. (2014) estimated that the *CO1* mutation rate is about 25% slower than the *ND2* mutation rate, and corresponds to 0.78% per lineage per million years. Meng et al. (2014), based on general mitochondrial rates, proposed 0.65% per lineage per million years. Based on both available rates we used 0.7% per lineage per million years as the mean in a normal distribution ($\sigma = 0.0005$) under an uncorrelated lognormal relaxed clock model in order to cover both estimations (5–95% prior distribution between 0.618 and 0.782%). We ran 400 million generations sampling every 40,000 with Yule model tree prior and constant population size. All mitochondrial genes were set up to use the same tree model. We used the software Tracer 1.5 (Rambaut et al., 2013) to check stationarity of the Markov chains by examining the effective sample size (ESS) values ($ESS > 200$ was expected at stationarity). Analyses were repeated three times to assess consistency among results. The species tree was inferred with TREEANNOTATOR as a maximum clade credibility tree and median heights as node ages; the first 1000 trees were discarded as burn-in.

2.4. Population structure: network haplotype genealogies and Bayesian population assignment analyses

We generated haplotype genealogies for each locus using Haploviewer (Salzburger et al., 2011). Haploviewer turns phylogenetic trees into haplotype networks. We used DNAML available in PHYLIP v.3.695 package (Felsenstein, 2005) to generate a maximum-likelihood tree. To visualize the genetic structure within species and the influence of the main rivers on genetic structure we generated each genealogy two times, identifying haplotypes (1) by species and (2) by populations separated by rivers.

To assess the genetic assignment of individuals to genetic clusters we used a model-based clustering method implemented in STRUCTURE

2.3.4 (Pritchard et al., 2000). Using multilocus genotypic data, STRUCTURE divides individuals into a number of genetic clusters (K) (irrespective of locality information), to minimize deviations from Hardy-Weinberg and linkage equilibrium within each cluster, and also calculates the fractional membership of each individual to each cluster (Q). For STRUCTURE analysis, we included only nuclear loci and only samples with sequences for at least four loci (see Appendix B). We used the program xmf2struct (available at: <http://www.xavierdidelot.xtreemhost.com/clonalframe.htm>) to convert sequences to STRUCTURE input; this program encodes each variable site as an allele. We performed ten independent runs for each K with admixture model and independent allele frequencies inferring lambda. For each K ranging from 1 to 15, we performed 5×10^5 iterations as burn-in and 5×10^5 additional iterations. The most likely K was based on the highest mean value of the likelihood distribution via the on-line program STRUCTURE HARVESTER v.0.6.93 (Earl, 2012). We assembled the multiple runs for each K in CLUMPP v.1.1.2 (Jakobsson and Rosenberg, 2007) and visualized with DISTRUCT v.1.1 (Rosenberg, 2004).

2.5. Genetic diversity, genetic differentiation between populations and neutrality tests

For each mitochondrial haplogroup we estimated haplotype diversity (H_d : the probability that two randomly chosen haplotypes are different; Nei, 1987) and per-site nucleotide diversity (P_i : the average number of nucleotide differences per site between two randomly chosen DNA sequences; Nei, 1987) in DnaSP software. We estimated F_{st} in DnaSP and net mean distances [D_a in DnaSP (Tamura and Nei, 1993)] between mitochondrial haplogroups within species in order to identify genetic breaks. Significance of F_{st} and D_a was assessed with the Permutation Test by 5000 replications. To detect significant deviations from the null hypothesis of neutral evolution and constant population size we performed Tajima's D (Tajima, 1989), Fu's F_s (Fu, 1997) and Ramos-Onsins and Rozas's R_2 tests (Ramos-Onsins and Rozas, 2002). Significance levels of F_s , R_2 , and r were estimated with 10,000 coalescent simulation replicates. Statistics and significance analyses were made in DnaSP.

2.6. Demographic history

We used the multilocus coalescent-based extended Bayesian Skyline plot (EBSP; Heled and Drummond, 2008) implemented in Beast 2.4.3 to estimate changes in effective population size through time. We analyzed each mitochondrial haplogroup separately using all loci, mitochondrial and nuclear (LL2 was not analyzed due to low number of sequences in nuclear loci). For each analyzed group we used the *COI* substitution rate as reference, with 0.007 substitution/site/year based on Freilich et al. (2014) and Meng et al. (2014) estimations, with a strict molecular clock and uniform priors [except for RPL3 marker of LL3, which was gamma distributed (Alpha 0.0010, Beta 1000, Offset 0.0)]. Nucleotide substitution model, range of the rate of heterogeneity, and proportion of invariant positions were inferred during the MCMC analysis with bModelTest package implemented in Beast with transition-transversion split option and empirical frequencies. We ran 400 million Markov chain Monte Carlo simulations sampled every 40,000 chains. Chain convergence was assessed in Tracer 1.5 by effective sample size (ESS) examination (> 200).

2.7. Isolation-with-migration model

We estimated time of divergence, gene flow after split with its direction, and population size of ancestral and current populations between populations of *L. llanensis* from north and south of the Santiago del Estero gap with an isolation-with-migration model (Hey and Nielsen, 2007) implemented in IMA2 (Hey, 2010). Analyses were made using LL1 and LL3 as sampling populations because of their closer

relationship (see Fig. 3a). Following the authors of IMA2, we first tested the HKY model (Hasegawa et al., 1985) for mitochondrial data and the IS model (Kimura, 1969) for nuclear data. However, HKY was used for all loci because IS was not compatible with our nuclear data. We used 0.7% per lineage per million years as the *COI* mutation rate based on Freilich et al. (2014) and Meng et al. (2014), transformed to a mutation rate per locus per year. Upper bounds for population size, migration, and divergence time ($-q$, $-m$, and $-t$, respectively) were based on the population mutation rate (θ) estimated in DnaSP following the authors' recommendations. After pilot-run adjustment we used uniform distribution for population-size and divergence-time priors with upper bounds set as $-q18.95 -m0.53 -t7.58$. We ran in M mode (MCMC) three times with different seed numbers with 20 Markov chains with the following heating terms: $-hfg -hn40 -ha0.975 -hb0.75$. Each simulation corresponds to 10×10^5 of burn-in and 20,000 saved genealogies. We assumed a generation time of one year based on the rapid larval development (including metamorphosis) and fast postmetamorphic growth of *Lepidobatrachus* species (Fabrezi and Quinzio, 2008; also see Faivovich et al., 2014). MCMC mixing was assessed through Effective Sample Sizes (ESSs) and trend-line plots; both denote levels of autocorrelation among samples and swapping rates between chains over the run.

3. Results

3.1. Species-tree

Since our main goal with the species-tree analysis was to delimit a timeframe of diversification within *Lepidobatrachus*, we assigned samples to the three species of the genus. All runs resulted in the same tree and effective sample sizes (ESS) were > 200 for all parameters denoting a high confidence of the estimates. The analysis recovered *L. asper* as the sister taxon of *L. laevis* + *L. llanensis*. According to our data, the first and second split were relatively close and with a high level of overlap [8.73–16.82 and 6.48–13.26 Ma of 95% highest posterior density (HPD) interval, respectively], corresponding to the second half of the Miocene (Fig. 2).

3.2. Population structure: network haplotype genealogies and Bayesian population assignment analyses

The *COI* haplotype network (Fig. 3a) showed three main haplogroups separated by ~ 60 mutations, which correspond to each *Lepidobatrachus* species. The four haplotypes of *L. asper* were grouped in pairs separated by nine mutations. One pair corresponds to all haplotypes from Santa Fe (A1) and the other corresponds to haplotypes from Córdoba and Santiago del Estero (A2) (Fig. 1b). In *L. laevis*, one haplotype shared by the only two individuals from Teniente Prieto (Boquerón) (L1) (Fig. 1c) was separated by seven mutations from all other haplotypes (L2). In this major haplogroup (L2), four most frequent haplotypes were evident, each one surrounded by several rare haplotypes. Within *L. llanensis*, three haplogroups were evident. The first one, with few and common haplotypes, corresponded to individuals from Córdoba, La Rioja, and Catamarca provinces, in the southern distribution of the species (LL1) (Fig. 1d). Populations from the northern distribution of the species were clustered in two main haplogroups, one corresponding to a highly frequent haplotype shared by a large number of individuals, and two more of only one individual each. All individuals that share this haplogroup are from Defensores del Chaco (Boquerón, Paraguay) (LL2) (Fig. 1d), which was separated from all other *L. llanensis* by 11 (LL1) and 10 (LL3) mutation steps. The other haplogroup (LL3) shows a high number of rare haplotypes connected to each other by one to three mutations.

The only nuclear marker (Appendix C) that does not clearly separate the three *Lepidobatrachus* species, is GADPH. In this genealogy, *L. asper* shows three haplotypes; one shared by Córdoba and Santiago del Estero

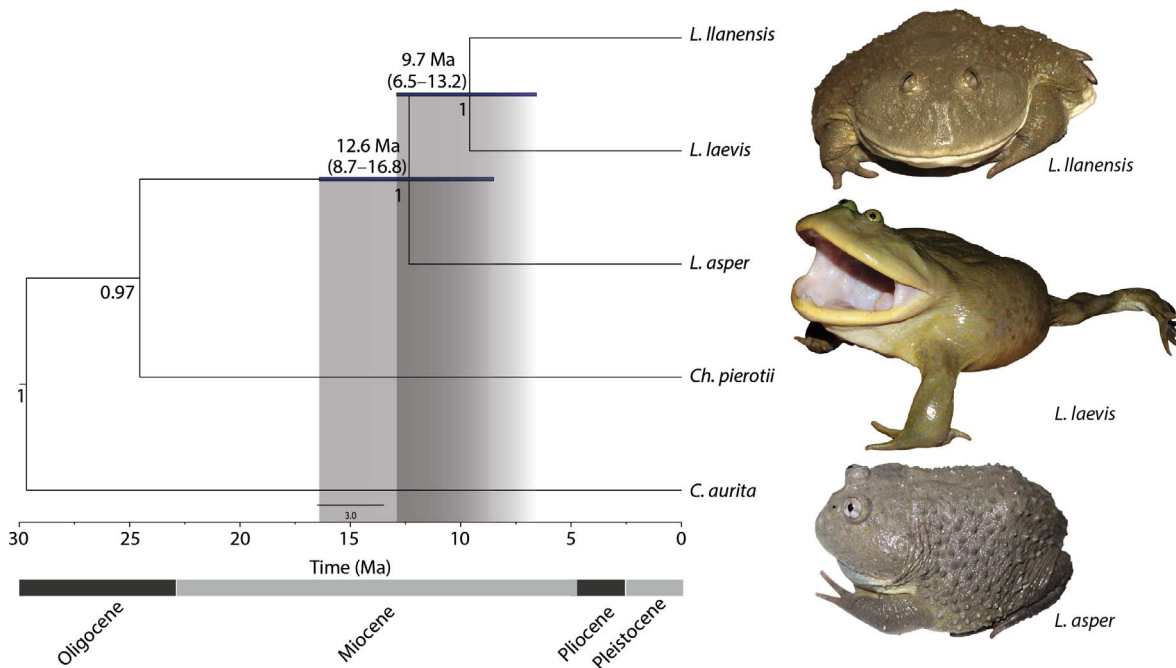


Fig. 2. [†]Beast species tree analysis. Numbers below nodes correspond to Bayesian posterior probabilities. Numbers above nodes correspond to time of divergence with the 95% highest posterior density intervals (HPD) in parenthesis and also represented by bars. Divergence times expressed in million years. *Ch.* corresponds to *Chacophrys* and *C.* to *Ceratophrys*.

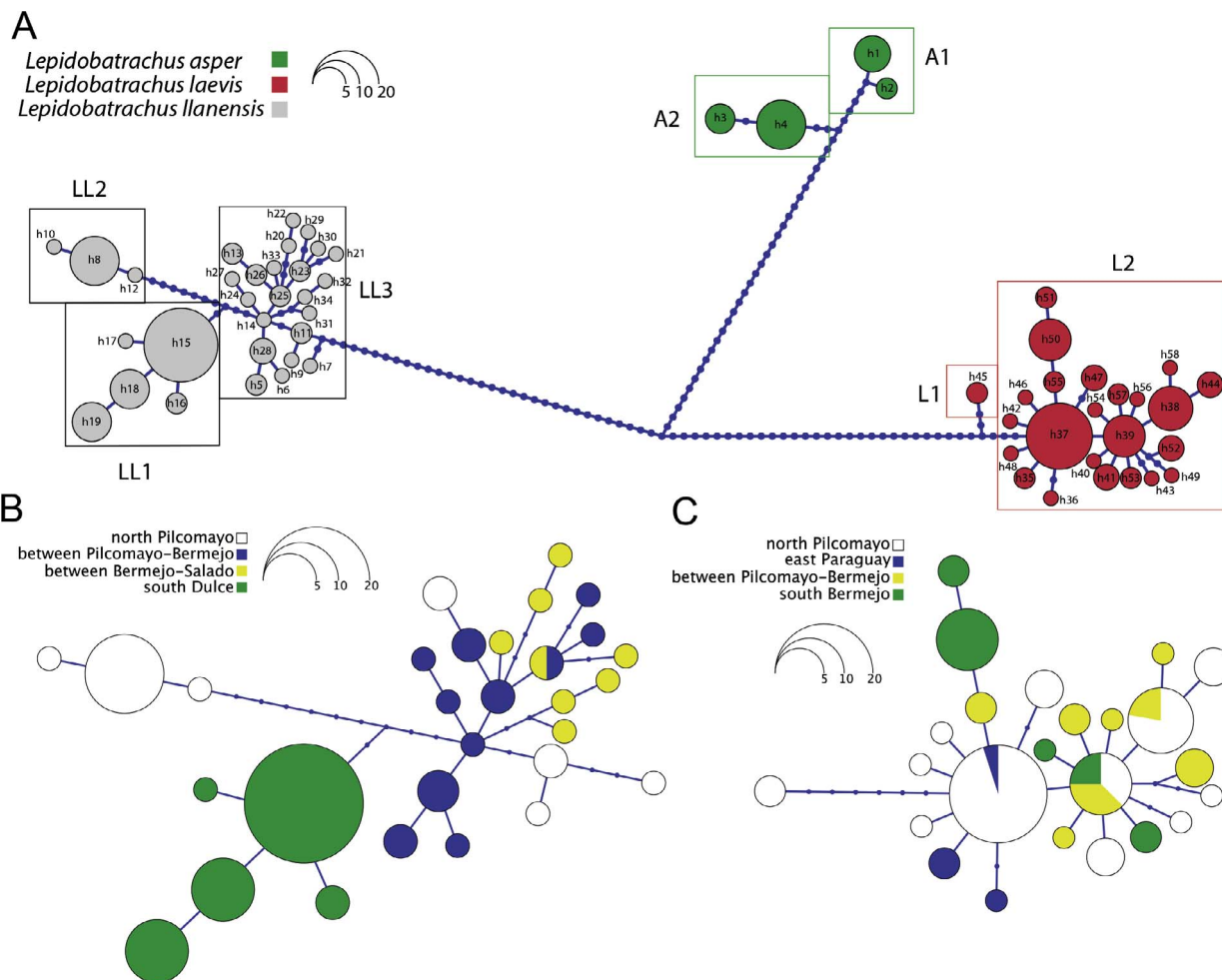


Fig. 3. (a) Mitochondrial haplotype network of *Lepidobatrachus* species. Blue dots indicate unsampled mutations. Circle size corresponds to number of copies of the haplotype in our sample. (b) *L. llanensis* and (c) *L. laevis* haplotypes, colors represent populations separated by rivers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

individuals and the other two shared by Santa Fe individuals. The Córdoba + Santiago del Estero haplotype was closer to *L. laevis* haplotypes, and the Santa Fe haplotypes to *L. llanensis* haplotypes. In MVZ 27–28 and RPL3, *L. asper* haplotypes were shared by individuals from Córdoba and Santa Fe, but in MVZ 27–28 an individual from Córdoba shared a haplotype with Santiago del Estero individuals as well. In MVZ 29–30, MVZ 39–40, and MVZ 47–48, each locality exhibited exclusive haplotypes.

The most common pattern in *L. laevis* is many rare haplotypes without any geographic pattern (GADPH, MVZ 29–30, MVZ 39–40). In MVZ 47–48, the most frequent haplotype is surrounded by some rare haplotypes; however, the rest of the haplotypes do not show any pattern. In MVZ 27–28, the two most frequent haplotypes, and in RPL3, the one most frequent haplotype, were surrounded by many rare haplotypes (Appendix C).

In *L. llanensis*, GADPH and RPL3 exhibit a predominance of rare haplotypes without any geographic pattern. In MVZ 39–40 the most frequent haplotype is surrounded by some rare haplotypes, but the others did not show any pattern. In MVZ 29–30, the four most frequent haplotypes exhibit few associated rare haplotypes. The pattern for MVZ 27–28 and MVZ 47–48 is that of only one most frequent haplotype surrounded by some rare haplotypes (Appendix C).

For *L. llanensis*, the *CO1* genealogy shows exclusive haplotypes for all between-rivers comparisons, except for one haplotype shared by two individuals separated by the Bermejo River (Fig. 3b). Populations south of the Dulce River were clearly separated; however, a long geographic distance (about 400 km) and the Santiago del Estero gap are between these populations and the remaining populations of *L. llanensis*. Haplotypes north of the Pilcomayo River constitute two haplogroups. One of these haplogroups, which contains haplotypes from almost all the localities, was separated by several mutational steps. The remaining haplotypes from north of the Pilcomayo, and those from both sides of Bermejo River, exhibited mixed relationships. For *L. laevis*, the *CO1* genealogy shows high levels of admixture between populations separated by rivers, and shared haplotypes between individuals from both sides of all rivers (Fig. 3c).

In STRUCTURE analyses, the mean likelihood reached a plateau at

Table 2

Genetic diversity of *Lepidobatrachus* main mitochondrial haplogroups. Higher genetic diversity is expected for populations with longer persistence time on the referred area.

haplogroups	N	H	Hd	Pi
<i>Lepidobatrachus asper</i> A1	8	2	0.429	0.00143
<i>Lepidobatrachus asper</i> A2	15	2	0.419	0.00140
<i>Lepidobatrachus laevis</i> L2	77	23	0.898	0.00384
<i>Lepidobatrachus llanensis</i> LL1	42	5	0.602	0.00147
<i>Lepidobatrachus llanensis</i> LL2	12	3	0.318	0.00056
<i>Lepidobatrachus llanensis</i> LL3	31	23	0.981	0.00780

Note: Columns correspond to: number of individuals (N), number of haplotypes (H), haplotype diversity (Hd: the probability that two randomly chosen haplotypes are different), and nucleotide diversity (Pi: the average number of nucleotide differences per site between two randomly chosen DNA sequences). Haplogroup names follow Fig. 3. *Lepidobatrachus laevis* L1 not included because it corresponds to a unique haplotype in the mitochondrial genealogy.

K = 3, continuing with little variation up to K = 15 (Fig. 4a). In K = 3 each deme corresponds to one *Lepidobatrachus* species. Starting from K = 4 an ephemeral admixture in some individuals is evident but keeping the same main structure that of K = 3.

3.3. Genetic diversity, genetic differentiation between mitochondrial haplogroups, and neutrality tests

Haplotype diversity (Hd) and nucleotide diversity (Pi) were relatively low in both *L. asper* haplogroups (A1 and A2). In *L. laevis* (only for L2 because haplogroup L1 had only two individuals) Hd is very high and Pi relatively low. In *L. llanensis*, the southern haplogroup (LL1) shows medium to high Hd values and very low Pi. Both genetic diversity indices (Hd and Pi) were very low for LL2 and very high for LL3 (Table 2). We found relatively high genetic differentiation and low genetic distance among all haplogroups within the species (Fst range from 0.63 to 0.94 and Da range from 0.007 to 0.017; Table 3).

According to neutrality tests, there is no statistical support to accept a recent history of population expansions for both *L. asper* haplogroups (A1 and A2) and the *L. llanensis* haplogroup from the southern part of

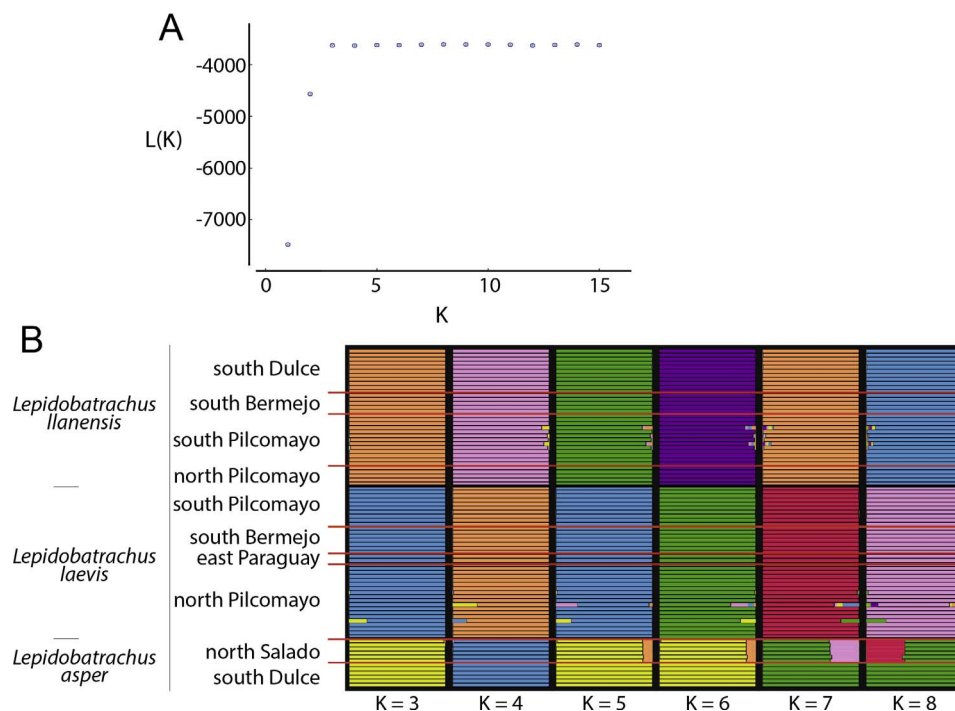


Fig. 4. Bayesian clustering and specimen assignment to the three *Lepidobatrachus* species using STRUCTURE. (a) Mean likelihood distributions as a function of K = 1–15. (b) Bars correspond to each specimen and their membership coefficient (q). Clusters are represented by color. Sampling sites are plotted by species (Appendix A).

Table 3

Pairwise *Fst* and *Da* values for mitochondrial fragment (*COI*) among haplogroups within *Lepidobatrachus* species (see Fig. 3). *Fst* is inversely proportional to gene flow; lower values denote high levels of gene flow. *Da* is a genetic distance calculation based on nucleotide substitutions per site between populations.

	<i>Fst</i>	<i>Da</i>
<i>Lepidobatrachus asper</i> A1-A2	0.911	0.014
<i>Lepidobatrachus laevis</i> L1 ⁺ -L2	0.862	0.012
<i>Lepidobatrachus llanensis</i> LL1-LL2	0.947	0.017
<i>Lepidobatrachus llanensis</i> LL2-LL3	0.828	0.017
<i>Lepidobatrachus llanensis</i> LL1-LL3	0.664	0.008

Note: A1 (n = 8), A2 (n = 15), L1 (n = 2), L2 (n = 77), LL1 (n = 42), LL2 (n = 12), LL3 (n = 31). L1⁺ corresponds to a unique haplotype in the mitochondrial genealogy.

Table 4

Neutrality tests (Tajima's *D*, Fu's *Fs*, *R2* test) within *Lepidobatrachus* haplogroups. Significant values (highlighted in bold) denote deviations from the null hypothesis of neutral evolution, whose rejection suggest an increase in population size.

haplogroups	<i>D</i>	<i>Fs</i>	<i>R2</i>
<i>Lepidobatrachus asper</i> A1	0.41421	1.653	0.2143
<i>Lepidobatrachus asper</i> A2	0.95399	2.222	0.2095
<i>Lepidobatrachus laevis</i> L2	−1.80184	−14.438	0.0404
<i>Lepidobatrachus llanensis</i> LL1	−0.12498	−0.625	0.1109
<i>Lepidobatrachus llanensis</i> LL2	−1.45138	−1.325	0.1863
<i>Lepidobatrachus llanensis</i> LL3	−1.69113	−15.484	0.0662

Note: Significant values ($p < 0.05$) in bold. Haplogroup names follow Fig. 3. *Lepidobatrachus laevis* L1 not included because it corresponds to a unique haplotype in the mitochondrial genealogy.

the species distribution (LL1). In contrast, all tests supported a recent population expansion scenario for *L. laevis* (L2) and *L. llanensis* haplogroups from the northern part of its distribution (LL2 and LL3, except for *R2* for LL2) (Table 4).

3.4. Demographic history

In EBSP analyses the “observation window” is delimited by the last coalescent event; some events, such as evolutionary bottlenecks, yield short coalescent times, which reduce the “observation window.” Although with a recent sudden increases (about 1500 years), EBSP shows a mostly constant effective population sizes in both *L. asper* haplogroups, A1 (Fig. 5a) and A2 (Fig. 5b), with “observation windows” of 0.020 and 0.025 Ma, respectively. A sharp increase in population size was inferred for *L. laevis* L2 (Fig. 5c) in the last 0.25 Ma after a long time of stability (“observation window” of 1.875 Ma). The southern *L. llanensis* haplogroup, LL1 (Fig. 5d), shows a short (“observation window” of 0.07 Ma) and constant demographic history, unlike LL2 (Fig. 5e), which shows a long (“observation window” of 5 Ma) and constant demographic history with a marked population-size increase 0.8 Ma ago.

3.5. Isolation-with-migration model

According to IMA2 analyses, lineages of northern and southern *L. llanensis* diverged about 0.038 Ma [95% highest posterior density interval (HPD) = 0.019–0.071 Ma]. After divergence, no evidence of migration was detected in any direction between the two populations. The estimate of effective population size was clearly higher for the northern populations.

4. Discussion

4.1. Species tree: diversification time frame within *Lepidobatrachus*

The results of the ^{*}Beast species tree suggests a Mid Miocene diversification in *Lepidobatrachus* with divergence times between species

with high degree of overlap (Fig. 2). Diversification within *Lepidobatrachus* began with the *L. asper* split about 12.6 Ma (8.7–16.8 Ma 95% HPD interval), followed by *L. laevis*-*L. llanensis* divergence about 9.7 Ma (6.5–13.2 Ma 95% HPD interval), both within the second half of the Miocene. The main event that occurred within this timeframe is the middle Miocene marine introgression into the Chaco and Paraná basins, called the Paranense Sea (Hernández et al., 2005). This marine introgression was the most important in size (see Hernández et al., 2005; Ottone et al., 2013) and occurred between 15 and 13 Ma (Hernández et al., 2005) with its final phases of regression estimated at about 8.7 Ma (Candela et al., 2012).

The Paranense Sea occupied a great portion of the current Chacoan distribution during the middle Miocene incursion. The extension of the sea in Argentinean Chaco covered Santa Fe, Formosa, and Chaco provinces, a great portion of Santiago del Estero, western and eastern borders of Córdoba, eastern border of Salta, and the east corner between Catamarca and La Rioja; in Paraguay only a small incursion was suggested, in southern Boquerón (Ottone et al., 2013). The northern and northeastern marine distribution reached the Michicola and Asunción archs, respectively (Hernández et al., 2005). The Michicola and Asunción archs are extensions of the Brazilian shield, a large subsurface that acts as a barrier of drainage systems (Lundberg et al., 1998). The distribution of the Paranense Sea suggests a strong influence on *Lepidobatrachus* diversification as a vicariant agent on widely distributed ancestral Chacoan populations. Furthermore, this distribution suggests the existence of possible refuges to the north, east, and south of the current Chaco during the Paranense Sea.

The Paranense Sea has previously been suggested as an important driver of diversification for other groups. Based on fossil faunas, Candela et al. (2012) suggested that the Paranense Sea acted as an important geographic barrier promoting vicariance between Mesopotamic and northwestern Patagonic faunas. Delsuc et al. (2012) dated the divergence between the two species of fairy armadillos (*pichiciegos*) at about 17 Ma and suggested that the diversification was promoted by the disruption of the ancestral range, isolating populations to the south and north of the current Chaco. Finally, Morando et al. (2014) suggested that the three species groups of the gecko genus *Homonota* split by isolation of populations on emergent lands that surrounded the Paranense Sea. For species of *Lepidobatrachus*, we suggest that marine introgressions may have forced populations of a widely distributed ancestral species to become isolated in areas protected from flooding, which probably acted as refugia during marine introgressions; this condition might have favored opportunities for speciation by geographical isolation.

4.2. Refugia during marine introgressions

To suggest a refugium for *L. asper* is very difficult due to the poor sampling. Our sampling of *L. asper* covers only the southern portion of the known distribution of the species (Fig. 1b). Following Faivovich (1994), besides Córdoba, Santa Fe, and southern Santiago del Estero, the species has been recorded in Corrientes and Chaco provinces, and in the Paraguayan departments of Presidente Hayes and Alto Paraguay. Nevertheless, the last of these records is from 1980, and the species has never been found again in these latter localities. However, the low genetic diversity in both haplogroups (Table 2), and the short persistence time resulting from EBSP (Fig. 5A and B), may correspond to an evolutionary bottleneck, which suggests a Upper Pleistocene colonization of the southern Chaco, but as stated before, a better sampling is necessary to study the evolutionary history of this species.

Currently, *L. llanensis* shows a disjunct distribution with two population groups separated by an extensive gap in the province of Santiago del Estero, Argentina (Fig. 1d). Southern populations (LL1) exhibit lower genetic diversity and population structure than the northern populations (LL3) (Table 2; Fig. 3), which suggests a later colonization of the southern distribution. Paradoxically, the other northern

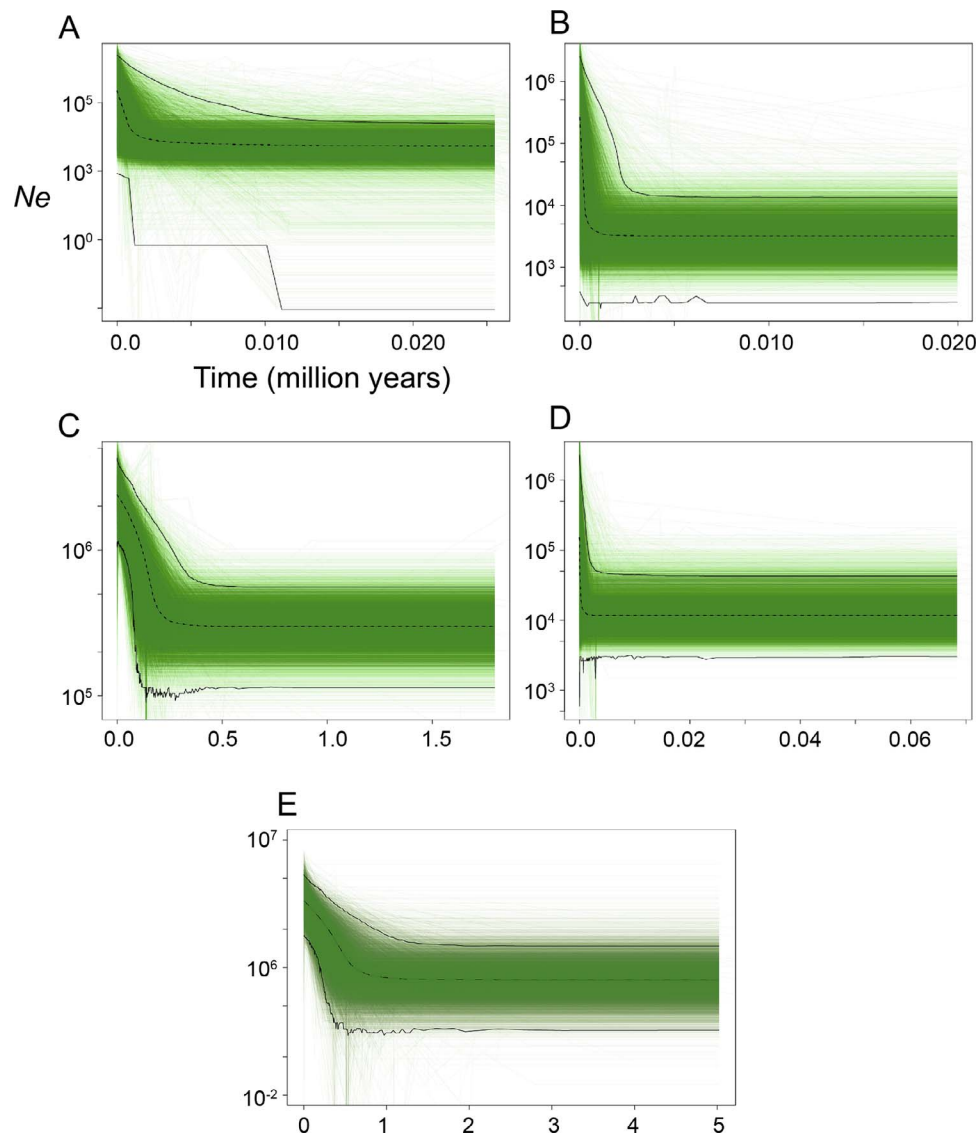


Fig. 5. Extended Bayesian skyline plots (EBS) of *Lepidobatrachus* haplogroups. (a) *L. asper* A1, (b) *L. asper* A2, (c) *L. laevis* L1, (d) *L. llanensis* LL1, and (e) *L. llanensis* LL3 (LL2 was not analyzed due to low number of sequences in nuclear loci). Horizontal continuous line and surrounded green area corresponds to mean and 95% highest posterior density limits for the effective population size (represented in Y axis in logarithmic scale), respectively. X axis is time in million years. Calculation of effective population size uses a generation time of one year. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

population (LL2) shows the lowest indices of genetic diversity (Table 2), and neutrality tests support a recent increase in population size (Table 4). This pattern suggests a later colonization by this lineage in the area, but with our current sampling the source of this colonization is not clear. EBS analyses support a constant population history for the southern populations (LL1) and an expansion event for the northern LL3 (also supported by neutrality test, Table 4), although this expansion would not be very recent (0.8 Ma; Fig. 5e). Furthermore, EBS analyses show a clearly longer time of persistence for northern LL3, with 5 Ma, than southern LL1 with only 0.07 Ma (Fig. 5e and d, respectively), which supports the isolation of the *L. llanensis* ancestor in northern Chaco distribution, at areas protected by Michicola Arch (part of Boquerón in Paraguay and Salta in Argentina, and the Bolivian Chaco), an old structural arch that is present at least since the end of the Devonian (Salfity, 1982).

The sympatry between *L. laevis* and *L. llanensis* in the northern part of the Chaco distribution (Fig. 1) could have been the result of expansions of *L. laevis* from the east or the south. Haplotype genealogies with a repetitive star-like pattern (Fig. 3; Appendix C) support demographic expansions, following a population bottleneck, due to recent

colonization (Slatkin and Hudson, 1991). The combination of high haplotype diversity (Hd) and low nucleotide diversity (Pi) (Table 2) also corresponds to population expansion after bottleneck events (Eizirik et al., 2001; Althoff and Pellmyr, 2002; Joseph et al., 2002; Stamatis et al., 2004). High values of haplotype diversity and low nucleotide diversity result from an accumulation of mutations, which consequently results in a high number of closely related haplotypes (i.e. many recently evolved haplotypes). Neutrality tests also support expansion events in *L. laevis* (Table 4) with a sudden increase of the population size in the last 0.25 Ma (Fig. 5c), which reinforce the idea of the absence of a northern refugium for the *L. laevis* ancestor.

Besides areas protected by Michicola Arch, other putative refugia are located in the east and southeast of the current Chaco distribution. The Paranense Sea covered a massive land surface in southern South America, flooding all lowlands between old archs and cratons, such as Asunción and Michicola archs, and Brazilian and Rio de la Plata cratons (Fig. 6). A fossil of a recently described species of *Lepidobatrachus* (Nicoli, 2015) was found at Farola Monte Hermoso, province of Buenos Aires (Tomassini et al., 2011). This locality corresponds to Rio de la Plata Craton. Other fossils related to some species currently restricted to

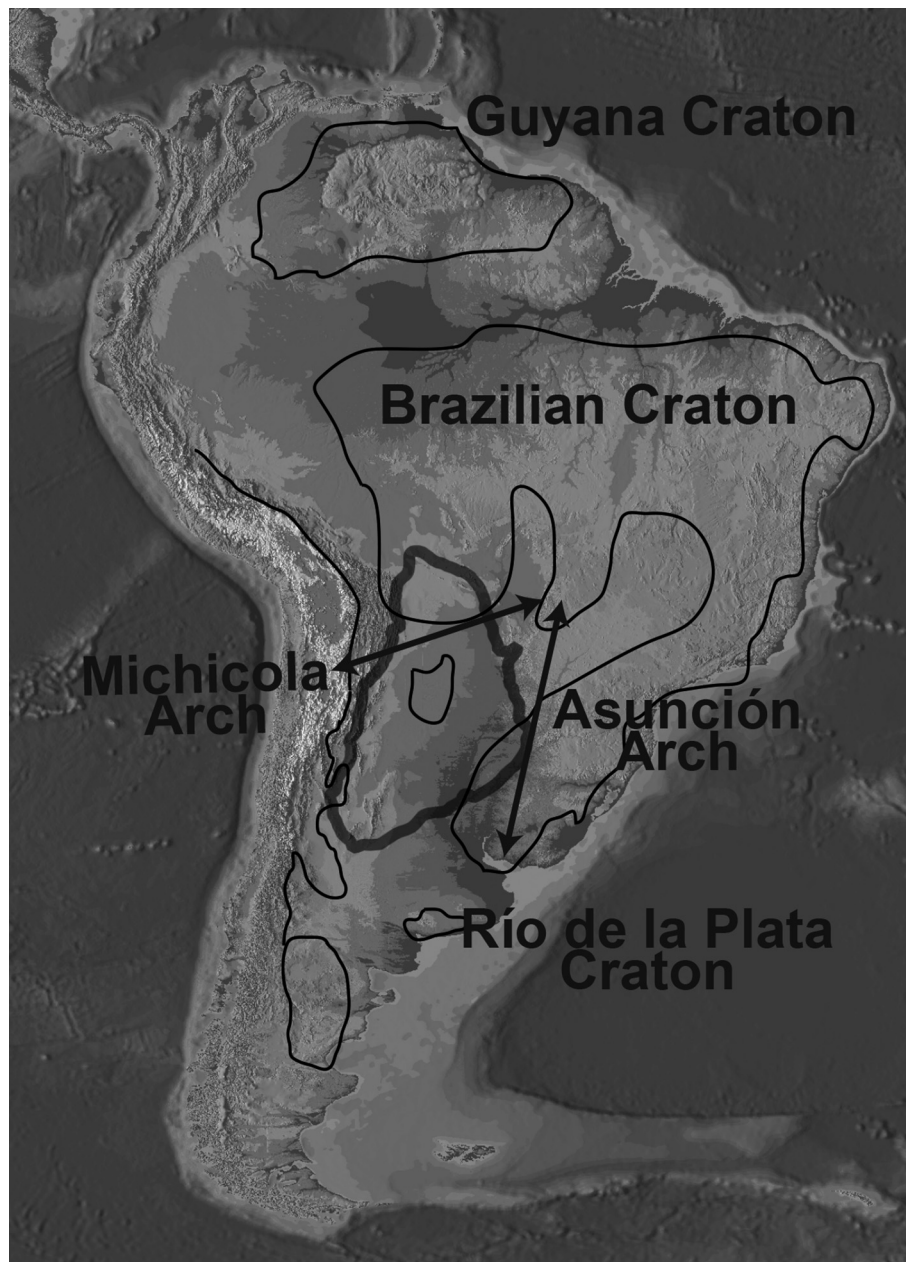


Fig. 6. Approximate distribution of main arches and cratons on South America based on Hernández et al. (2005). Double-headed arrows represent the distributions of Michicola and Asunción arches. Area enclosed in thick black denotes the limits of the Chaco biome.

Chaco wooded areas were also recovered at Farola Monte Hermoso (Tonni, 1974; Tomassini et al., 2011; Nicoli, 2015), supporting the idea that at least until the Pliocene, the climatic conditions on Farola Monte Hermoso were similar to those of the current Chaco (Tonni, 1974; Pascual, 1984; Pascual et al., 1996). Nicoli (2015) questioned some of the paleoecological inferences of these authors, especially those based on the presence of fossils of widely distributed species (extant or extinct), which occur in other regions besides the Chaco. However, the presence of fossils of species that currently are not restricted to the Gran Chaco is not ultimate evidence that Farola Monte Hermoso was not similar to the current Chaco.

Another putative refugium for Chacoan fauna during marine intrusions is the Asunción Arch. This formation protected a large land surface during the middle-Miocene marine intrusions and covers parts of southern Paraguay southward along Misiones, Corrientes, and Entre Ríos provinces in Argentina (Hernández et al., 2005). Fossil records from the Hermanderías y El Bretela deposit (Entre Ríos province)

support the domination of Chacoan-like xerophilous wood paleo-communities at least until the Pliocene (Hinojosa and Villagrán, 1997). Furthermore, several authors suggested that climatic changes generated by the final uplift of the Andes and the influence of the Humboldt Current caused expansions of xeric vegetation over an extensive area of southern South America during the middle-late Miocene and Pliocene (Solbrig et al., 1977; Axelrod, 1979; Landrum, 1981; Arroyo et al., 1995).

Isolated populations of the *Lepidobatrachus* ancestor in the southern and/or southwestern parts of the current Chaco distribution are plausible, because no evidence supports marine flooding in this part of the region, which would be southeastern La Rioja, central and southern Córdoba, and northwestern San Luis (Ottone et al., 2013). However, our data suggest a recent colonization of southwestern Chaco by *L. llanensis* from the northern part of its distribution and a short persistence of *L. asper* in the southern Chaco. The southern distribution of the Gran Chaco has been influenced by Pleistocene glaciations (Ortiz-

Jaureguizar and Cladera, 2006). Climatic and environmental changes associated with glaciation events caused cyclic distributional shifts of southern biomes, including the Chaco (Cosacov et al., 2010; Ortiz-Jaureguizar and Cladera, 2006). Furthermore, during these glaciations, a narrow area of very arid conditions, called the “Arid Diagonal,” was distributed from the Atlantic coast of Patagonia (Chubut, Argentina) to western-central Gran Chaco (Tucumán, Argentina) (Cosacov et al., 2010), reaching La Rioja, Catamarca, and southwestern Santiago del Estero (southwestern Chaco). These events may have masked any genetic signature produced by southern refugia during marine introgressions by local extinctions in the southern Chaco during the Pleistocene.

4.3. Genetic structure and putative dispersal barriers within the Chaco

STRUCTURE analyses recovered only the three species ($K = 3$; Fig. 4) without any genetic break within them. However, at $K = 5$ a shallow differentiated deme was evident, which gradually increased in differentiation to $K = 8$, where it was most evident. This deme corresponds to the mitochondrial haplogroup A1 (Fig. 3a), which groups all *L. asper* individuals from Santa Fe (Fig. 1b). The other *L. asper* haplogroup (A2; Fig. 3a) groups together all individuals from Córdoba and Santiago del Estero (Fig. 1b). Although we only partially sampled *L. asper*, this genetic structure is remarkable since the three sampled localities are almost equidistant to each other and because the last foothills of the Sierras Chicas formations are located between the localities of Córdoba and Santiago del Estero (Fig. 1a). Besides geographic distance, two rivers present between A1 and A2 may act as barriers, the Salado and the Dulce rivers (Fig. 1a). Populations corresponding to the A1 and A2 haplogroups have little or no gene flow ($F_{st} = 0.911$) but low genetic differentiation ($D_a = 0.014$), which suggest a recent divergence. EBSM results support a Pleistocene divergence with low persistence time of both A1 and A2, and also show constant population histories (Fig. 5a and b), as expected for diversification by barriers without cycles of extinctions and expansions (Amaral et al., 2013). The recent divergence between A1 and A2 may correspond to the dynamics of the Salado and Dulce rivers. During the Quaternary, the Chaco experienced climatic changes oscillating between dry and humid periods (Iriando, 1993; Iriando and García, 1993; de Vivo and Carmignotto, 2004). In dry periods these allochthonous rivers, like the other main Chacoan rivers (Pilcomayo and Bermejo), were ephemeral with highly seasonal channels. Only between the last glacial maximum (21,000 years ago) and the late Holocene were two dry periods identified (Iriando, 1993; Iriando and García, 1993). However, without better sampling we cannot confidently assess the influence of rivers or any other putative barrier on genetic structure within *L. asper*.

We detected some structure in the *L. llanensis* mitochondrial genealogy, which is concordant with the geographical distribution of the specimens and suggests some breaks in the landscape. The main break within this species corresponds to a gap of about 400 km in central Santiago del Estero province, Argentina, separating southern *L. llanensis* populations (LL1) from northern populations (LL2 and LL3) (Figs. 1d and 3a). Between the southern and each northern haplogroup we found a relatively high level of differentiation (F_{st} range from 0.63 to 0.94) and genetic distance (D_a range from 0.008 to 0.017) (values for F_{st} and D_a in Table 3). This distribution gap may not simply correspond to a sampling gap, since there are no historical records of the species, and it matches the southern and/or western distributional limits of other Chacoan species (e.g. *L. laevis*, *Scinax acuminatus*, *Melanophryniscus klappenbachi* and *Physalaemus cuqui*). Furthermore, another endemic species with a distribution similar to that of *L. llanensis*, *Chacophrys pierottii*, shows a comparable spatial differentiation pattern. This gap corresponds to a high-temperature area called the “South American heat pole” (Prohaska, 1959) with summer temperatures of about 47 °C. Furthermore, a low-precipitation regime (Boletta, 1989) and high salinity (Ruibal, 1962) are important environmental characteristics of this area, and may act as a climatic barrier to *L. llanensis* dispersal, and

that of other anuran species as well. This disjunct distribution could also be associated with recent local extinctions caused by landscape changes due to severe logging and ranching since early last century in this region of Argentina (Bucher and Huszar, 1999). However, IMA2 splitting time between southern and northern sampled sites of *L. llanensis* (HPD 95% = 0.019–0.071 Ma), which corresponds to the Pleistocene, seems to be much older than the Santiago del Estero anthropic landscape transformation.

South American Upper Pleistocene is marked by increasing increments of temperature and drier conditions (Ortiz-Jaureguizar and Cladera, 2006), which may have increased seasonality and extreme conditions of central Santiago del Estero. *Lepidobatrachus llanensis*, as a Chacoan pond-breeding amphibian, depends on the availability of lentic aquatic habitats, which in regions with low precipitation and high temperatures are very scarce and isolated (Lescano et al., 2015). Isolated populations have smaller population sizes with lower genetic diversity, which reduces evolutionary potential and consequently increases the risk of extinction (Reed and Frankham, 2003; Funk et al., 2005). Besides the low availability of habitats, extremely high temperatures could be lethal for frogs, both in adult and larval stages (Wells, 2007). Duarte et al. (2012), studying tadpoles of a population of Formosa (Argentina), north of the Santiago del Estero gap, found a relatively high thermal tolerance for *L. llanensis* (critical thermal limit, $CT_{max} = 44.7$ °C); however, this limit is only slightly higher than the maximum pond temperatures. Furthermore, for the congeneric *L. laevis*, Carroll (1996) found low tadpole survival rates for short-time thermal shocks of 45 °C. The thermal tolerance of *L. llanensis*, although relatively high, may not be enough for the survival of this species in the extremely high temperatures of the central region of Santiago del Estero, at least since the Upper Pleistocene. We suggest that the combination of low precipitation and high temperatures could be responsible for making this area an impermeable barrier to *L. llanensis* dispersal. The idea of a climatic barrier in central Santiago del Estero is also supported by the IMA2 results, without any evidence of migration between the two population groups after divergence.

We found the genetic structure of *L. llanensis* to be more related to rivers than that of *L. laevis* (Fig. 3B and C). In *L. laevis*, genetic evidence suggests a recent colonization of the area of influence of Pilcomayo and Bermejo rivers and, therefore, a shorter evolutionary history than *L. llanensis*, which has older populations and longer persistence in this region of the Chaco. The lower genetic structure of *L. laevis* in the Pilcomayo and Bermejo area of influence is possibly related to the late Quaternary history of these rivers, with ephemeral and highly seasonal channels that did not serve as an effective barrier. While *L. laevis* experienced recent expansions from southern regions due to lack of dispersal barriers, the Pilcomayo and Bermejo rivers have influenced the distribution of *L. llanensis* populations for a long time. The dynamics of Pilcomayo and Bermejo rivers shaped a complex genetic pattern on *L. llanensis* populations due to its long persistence in this region. Water-volume differences between dry and humid periods, and the changing watercourses, caused recurrent connections and disconnections between populations of both sides of these rivers, promoting short and intermittent vicariant events. In dynamic systems like this, a population may split into several populations or may join with others, as well as vary in size, density and location (Hey and Machado, 2003).

The Paraguay River is the largest river analyzed here and unlike the other rivers, it does not cross the Chaco in a west-east direction. Of the species of *Lepidobatrachus*, only *L. asper* was known from the oriental margin of the Paraguay River (Corrientes Province, Argentina), but without new records since early 1980s (see Faivovich, 1994). Recently, Sugai et al. (2013) cited *L. laevis* (as *L. asper*) from Patolá Farm, Porto Murtinho, a Chacoan fragment in the southwestern part of Mato Grosso do Sul state, in central Brazil, at the other margin of the river. We included four individuals from this locality yielding three haplotypes, including two exclusives but separated by only one and two mutations from individuals from the Central Paraguayan Chaco (occidental

margin of the Paraguay River) and one shared with them (Fig. 3c). This lack of genetic structure suggests constant gene flow among populations from both sides of this river. Frutos and Van Den Bussche (2002) found similar results in armadillo populations from both sides of this river. Although this river is the distributional limit for some small mammals, it is probably not acting as an impassable barrier, but different soil structures and compositions determine different habitats on each side of the river (Myers, 1982). However, the southwestern region of Mato Grosso do Sul, Brazil, where it was registered and is the only known record of *L. laevis* east of the river, is also considered part of the Chaco (see Souza et al., 2010). Based on analyses of water-level records of the upper Paraguay River, Collischonn et al. (2001) found that between 1960 and 1972 an abrupt water level decrease occurred. This kind of event may have continuously permitted connection between *L. laevis* populations from both sides of this river. However, we do not discard the contribution of vegetation rafting to the connectivity between west and east bank populations. Two records of *L. laevis* from riverine localities (west bank) are known from Paraguay (Faivovich, 1994), one of them on the bank just opposite the only known record from the east bank in Brazil. We suggest that heavy rains may carry some individuals (tadpoles or adults) to the river through small temporary streams, and that some of them are capable of crossing the river on vegetation rafts. In a similar situation, several anurans from three different families were recovered from vegetation rafts in an Amazonian river (Schiesari et al., 2003) and in the Paraná River (Achaval et al., 1979).

5. Conclusions

Based on a fossil-calibrated species tree, we suggest an important role of the middle Miocene marine incursion as a driver of diversification of *Lepidobatrachus*. This internal sea covered a great expanse of lowlands in southern South America between old archs and cratons and may have acted as a barrier to transgression. Population structure, patterns of genetic diversity and historical demography data suggest that these archs and cratons were probably suitable refugia during marine incursions, and thus favored speciation by geographic isolation. We also found some genetic structure within *Lepidobatrachus* species, which could be associated with some breaks in the landscape. The main break corresponds to an area of high aridity in central Chaco, which caused a disjunct distribution for *L. llanensis*, with two population groups. Results from the isolation-migration (IMa2) analyses, without any evidence of migration between the two population groups after divergence, also supports the idea of a barrier in this region. We also found some influences by the main rivers, which due to their historical dynamics might have promoted short and intermittent vicariant events, allowing recurrent connections among populations of both sides of the rivers.

Acknowledgements

We thank Franco L. de Souza (ZUFMS) and Julián Faivovich (MACN) for making available tissue samples under their care; to Julián Lescano and Boris Blotto for sample collections; and to Juan Boeris, Jimena Grosso, Julián Faivovich, and Tereza Thomé for their help during fieldwork. We are very grateful to the owners of the Teniente Prieto, Aguada Siete and Chaco Boef farms for allowing access to their properties, and especially to the owners of Salazar, Montana, Cotorrita and Toro Retá farms, which besides access, also provided accommodation during fieldwork. For making contact with farm owners and continually providing all kinds of support during fieldwork, we are especially thankful to Francisco Brusquetti Frutos, Alejandra Brusquetti and Luis Vargas. We thank the Secretaría del Ambiente del Paraguay (SEAM, Paraguay) for collection permits and for the access and accommodation in Teniente Enciso and Defensores del Chaco national parks. We also thank Thais Condez for comments and suggestions that helped to improve the manuscript, and Zunilda Sisa for her valuable

help with English. This research was supported by resources supplied by the Center for Scientific Computing (NCC/GridUNESP) of the São Paulo State University (UNESP) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), grant #472463/2012-8 to CFBH. FB and FN thank Programa Nacional de Incentivo a Investigadores from the Consejo Nacional de Ciencia y Tecnología (PRONII, CONACYT, Paraguay), for financial support, and FB also thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), Programa de Estudantes-Convênio de Pós-Graduação (PEC-PG), for a fellowship. DB is grateful to FONCYT (PICT 2011/1524, 2011/1895, 2012/2687, 2013/0404, 2014/1343, 2014/1930, 2014/2035, 2015/0813, 2015/0820, 2015/2381), and CONICET (PIP 112201101/00875), for financial support. CFBH thanks grant #2013/50741-7 São Paulo Research Foundation (FAPESP) and a research fellowship of CNPq.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ympev.2018.02.010>.

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