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

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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 savannas 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 km\(^2\) (Bucher, 1982). The Chaco is an extensive sedimentary alluvial plain with soils derived from the accumulation of fine loess and alluvial sediments.
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 allochthonous, 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 intrusions (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 intrusions 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 intrusions have been recorded for this region (Ottone et al., 2013). The most important in extension, the Paranenae 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 intrusions, Pleistocene glaciations with associated refugia have also been proposed as diversification mechanisms for southern South America (Núñez 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*, *L. laevis*, and *L. 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 intrusions 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 intrusions 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 intrusions 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 intrusions 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 Colección Zoológica de Referencia da Universidade Federal do Mato Grosso do Sul (ZUPMS), 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.mn183gq). In the
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

<table>
<thead>
<tr>
<th>Locus ID (base pair number)</th>
<th>Primer sequence 5′-3′</th>
<th>Annealing</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO1 (600 bp)</td>
<td>ANF1 ACHAAYCAYAAGAYATYGG</td>
<td>45/50</td>
<td>Jungler et al. (2013)</td>
</tr>
<tr>
<td>GTPDH (297 bp)</td>
<td>ANR1 CCGGCTAAGTCAAGTCCTG</td>
<td>54.7</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>Glyceraldehyde-3 Phosphate Dehydrogenase (intron 4)</td>
<td>MZV26 AAATGTAAGCTAAGATGACAC</td>
<td>54.7</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>MVZ 27–28 (352 bp)</td>
<td>MZV27 ATTATCCGTAACAGGAATCT</td>
<td>54.7</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>Lactase Dehydrogenase Chain Beta (Intron 3)</td>
<td>MZV28 GTAACTCTGGAAGTCTGTAAG</td>
<td>57</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>MVZ 29–30 (198 bp)</td>
<td>MZV29 ACCCTCATACTACTAAGGAGAC</td>
<td>57</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>V Box Binding (Intron 1)</td>
<td>MZV30 CTGAGGCTCTACATGTTT</td>
<td>57</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>MVZ 39–40 (180 bp)</td>
<td>MZV39 GATGCTGAGGACACTGCTTCC</td>
<td>57</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>X. laevis MGC82783 protein (Intron 2)</td>
<td>MZV40 AGACGACTTTCAAAACCCAGAATAC</td>
<td>54.7/56.7/59</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>MVZ 47–48 (340 bp)</td>
<td>MZV47 AGTTGAAATGACAGTCAGGTCTAGG</td>
<td>54.7/56.7/59</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>Fibrinogen, A alpha polypeptide (Intron 1)</td>
<td>MZV48 GAGGATTATCGACGACTGCTAAAAG</td>
<td>57</td>
<td>Bell et al. (2011)</td>
</tr>
<tr>
<td>RPL3 (413 bp)</td>
<td>RPL35F AAGAAGTCYCACCTCATGGAGAT</td>
<td>50/53/64.3</td>
<td>Pinho et al. (2009)</td>
</tr>
<tr>
<td>Ribosomal Protein L3 (Intron 5)</td>
<td>RPL36RA AGTTTCTTGGTGTCGCACGGCTAG</td>
<td>50/53/64.3</td>
<td>Pinho et al. (2009)</td>
</tr>
</tbody>
</table>

(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), NDH dehydrogenase subunit 1 (ND1) (961 bp), exon 2 of chemokine receptor 4 (CKR4) (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 (SIAH) homolog 1 (SIAH1) (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. lantenis, and two individuals of each species used as outgroup. Sequences were not combined across loci and individuals. Nucleotide substitution model, range of the rate 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 best empirical fits.

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, Beelzebufo ampinga (Evans et al., 2008), Baurubatrachus pricei (Báez and Perí, 1989), and Wawelia geroldhi (Casamiquela, 1963) (Agolini, 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 (Fernicola, 2001; Tomassi et al., 2011; Nicoli, 2014; Nicoli, 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 (Tomassi et al., 2011, 2013; Fernicola, 2001; Nicoli, 2015), dated between 5 and 6.8 Ma (Clione 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 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 haploviever (Salzburger et al., 2011). Haploviever 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) (irrespectively of locality information), to minimize deviations from Hardy-Weinberg and linkage equilibria 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 xma2struc (available at: http://www.xavierdidiot. xtremehost.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 (Hd: the probability that two randomly chosen haplotypes are different; Nei, 1987) and per-site nucleotide diversity (Pt: the average number of nucleotide differences per site between two randomly chosen DNA sequences; Nei, 1987) in DnaSP software. We estimated Fst in DnaSP and net mean distances (Da in DnaSP (Tamura and Nei, 1993)) between mitochondrial haplogroups within species in order to identify genetic breaks. Significance of Fst and Da 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 Fs (Fu, 1997) and Ramos-Önssin and Rozas’s R2 tests (Ramos-Ønssin and Rozas, 2002). Significance levels of Fs, R2, 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), 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 (theta) 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 Paivovich 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 samples 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 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

---

**Table 2**

<table>
<thead>
<tr>
<th>haplogroups</th>
<th>N</th>
<th>H</th>
<th>Hd</th>
<th>Pi</th>
</tr>
</thead>
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<tr>
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<td>8</td>
<td>2</td>
<td>0.429</td>
<td>0.00143</td>
</tr>
<tr>
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<td>15</td>
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<td>0.419</td>
<td>0.00140</td>
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<td>77</td>
<td>23</td>
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<td>0.00384</td>
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<tr>
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<td>5</td>
<td>0.602</td>
<td>0.00147</td>
</tr>
<tr>
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<td>3</td>
<td>0.318</td>
<td>0.00056</td>
</tr>
<tr>
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<td>31</td>
<td>23</td>
<td>0.981</td>
<td>0.00780</td>
</tr>
</tbody>
</table>

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.
the species distribution (LL1). In contrast, all tests supported a recent population expansion scenario for the northern populations. 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 of the observation window. A sharp increase in population size was inferred for L. laevis (Fig. 5c) in the last 0.25 Ma after a long time of 1.875 Ma). The southern population groups separated by an extensive gap in the province of Santiago del Estero, Argentina (Fig. 1d). Southern populations (LL1) exhibit genetic diversity in both haplogroups (Table 2), and the short persistence of the observation window. A sharp increase in population size was inferred for L. laevis (Fig. 5c) in the last 0.25 Ma after a long time of 1.875 Ma). The southern L. llanensis haplogroup, LL1 (Fig. 5d), shows a long (“observation window” of 5 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.

3.4. 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 e
to isolation of populations on emergent lands that surrounded the current Chaco during the Paranense Sea. 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 northwest 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 *Hamonota* 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 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 arches are extensions of the Brazilian shield, a large sub-surface 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.

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3.5. Isolation-with-migration model

According to 1Ma2 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).

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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
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. EBSP 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, EBSP 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 (Salft, 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 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 the Chaco distribution, at areas protected by Michicola Arch, are also described in (Salft, 1982).

**Fig. 5.** Extended Bayesian skyline plots (EBSP) 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.)
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 introgressions is the Asunción Arch. This formation protected a large land surface during the middle-Miocene marine introgressions and covers parts of southern Paraguay southward along Misiones, Corrientes, and Entre Ríos provinces in Argentina (Hernández et al., 2005). Fossil records from the Hermanderlas 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 (Ortíz...
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 intrusions 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 (Fst = 0.911) but low genetic differentiation (Du = 0.014), which suggest a recent divergence. EBSP results support a Pleistocene divergence with low persistence 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 (Iriondo, 1993; Iriondo 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 (Iriondo, 1993; Iriondo 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 (Fst range from 0.63 to 0.94) and genetic distance (Du range from 0.008 to 0.017) (values for Fst and Du 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 (Rubial, 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% = 0.190–0.071 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, GTmax = 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 Paivovich, 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...
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 Coordenación 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 11220110/00875), 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.

References


