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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (ZOOLOGIA)

DIVERSIFICAÇÃO DE ANUROS NO CHACO SUL-AMERICANO

FRANCISCO ADOLFO BRUSQUETTI ESTRADA

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Zoologia).

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RESUMO

Os processos de diversificação biológica na América do Sul têm sido foco de vários estudos nos últimos tempos; porém, nota-se um marcado viés para os biomas florestados como a Amazônia e a Mata Atlântica. Nesse contexto, o Chaco é um dos biomas que tem recebido menor atenção. Nesta tese apresentamos o primeiro esforço por entender quais eventos e processos têm sido responsáveis pela diversificação de anuros endêmicos deste bioma, incluindo *Lepidobatrachus asper*, *Lepi. laevis*, *Lepi. llanensis* e *Leptodactylus bufonius*. Para atingir esse objetivo utilizamos sequências de DNA mitocondrial e nuclear de uma ampla amostragem de cada uma das espécies. Nos primeiros dois capítulos investigamos a diversificação do gênero *Lepidobatrachus*, sendo que no primeiro deles nossos objetivos foram analisar a estrutura genética e delimitar um período de tempo de diversificação que nos permita associá-la com eventos históricos e assim gerar as primeiras hipóteses sobre diversificação de anuros no Chaco. A estrutura genética identificada corresponde à distribuição geográfica dos espécimes analisados, assim como também revela algumas quebras ao longo da paisagem. A principal dessas barreiras corresponde à área central do Chaco, que por sua extrema aridez poderia estar atuando como uma barreira climática para a dispersão desses animais. Por outro lado, a dinâmica histórica dos rios da região poderia ter influenciado na estrutura genética das espécies do gênero *Lepidobatrachus*. Sugerimos uma antiga e rápida radiação para este gênero, com introgressões marinhas e períodos de alta temperatura e aridez como os principais responsáveis por sua diversificação. No segundo capítulo, aprofundamos no estudo da estrutura genética usando frequência alélica. Investigamos a história demográfica e usamos modelos de isolamento com migração para testar as hipóteses propostas no primeiro capítulo e entender os processos que resultaram na diversidade que hoje conhecemos dentro do gênero *Lepidobatrachus*. Nossos resultados sugerem que as antigas formações continentais estáveis, como os arcos estruturais e os crátons, tiveram um importante papel na diversificação do gênero *Lepidobatrachus*, atuando como refúgios durante as introgressões marinhas. A barreira climática do Chaco central é confirmada para uma das espécies do gênero. Adicionalmente, encontramos que os rios exerceram diferentes efeitos nas diferentes espécies do gênero, o que pode estar relacionado ao tempo de persistência de cada uma das espécies na área de influência desses rios. A maior persistência de *Lept. llanensis* resultou num padrão

genético modelado pela dinâmica desses rios alóctones. No terceiro e último capítulo analisamos a estrutura genética e demografia histórica de *Lept. bufonius*, uma espécie de ampla distribuição, para ter acesso a história evolutiva mais recente do Chaco. Os resultados suportam eventos de expansão recente e constante fluxo gênico entre as populações. As expansões estão relacionadas aos períodos interglaciais, enquanto o fluxo gênico é mantido por dispersões curtas que seguem um modelo "stepping-stone", permitindo assim uma alta conectividade, inclusive entre as populações mais distantes. Esse mesmo padrão foi registrado em anuros de outras regiões semiáridas pelo mundo.

ABSTRACT

Diversification processes in South America have been the focus of several studies in recent times; however, with a marked bias towards the forested biomes such as the Amazon and the Atlantic Forest. In this context, the Chaco has been one of the biomes that have received less attention. In this thesis we present the first effort to understand the events and processes that have been responsible for the diversification of endemic frogs in this biome, including *Lepidobatrachus asper*, *Lepi. laevis*, *Lepi.llanensis*, and *Leptodactylus bufonius*. For this purpose we used DNA sequences, both mitochondrial and nuclear, of a large sample of each species. In the first two chapters we investigate the diversification of the genus *Lepidobatrachus*. In the first one, our goals were to assess genetic structure and to delimit a diversification timeframe, which allow us to do associations with historical events and, thereby, generate the first hypotheses about diversification within the Chaco. The identified genetic structure is concordant with the geographic distribution of sampled specimens as well as shows some breaks along the landscape. The most important of these breaks matches with the central area of the Chaco, which due to the extreme aridity may act as a climatic barrier for the dispersal of these animals. On the other hand, the historical dynamics of the rivers of this region has influenced the genetic structure of these species. We suggest an old and rapid radiation for *Lepidobatrachus*, with marine introgressions and periods of high temperature and aridity as the main responsible in the genus diversification. In the second chapter, we deepen the genetic structure using allele frequency, we investigated the demographic history and used isolation-with-migration models to test hypotheses proposed in the first chapter and to

understand the processes that resulted in the current diversity of the genus *Lepidobatrachus*. Our results support that old and stable continental formations, such as structural arches and cratons, have played an important role in the genus diversification, acting as refuges during marine introgressions. The central Chaco climatic barrier is confirmed for one species of the genus. Additionally, we find different effects of the rivers on the species of the genus, related to the time of persistence of each one in the area of influence of these rivers. The longer persistence of *Lepi. llanensis* resulted in a genetic pattern shaped by dynamics of allochthonous rivers. In the third and last chapter we analyzed the genetic structure and historical demography of the widely distributed species *Lept. bufonius* in order to access the recent evolutionary history of this biome. Our results support recent expansion events and current gene flow among populations. The expansions are related to interglacial periods. Gene flow is maintained by short dispersions that follow a "stepping-stone" model enabling high connectivity, even among the most remote populations. The same pattern was registered in frogs in other semiarid regions of the world.

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INTRODUÇÃO GERAL

A diversificação de espécies nos Neotrópicos não está associada a apenas um espaço de tempo ou evento (RULL, 2011), mas sim a diversos eventos e processos que vêm acontecendo desde o Eoceno superior / Oligoceno inferior até o Pleistoceno (RULL, 2008). A orogênese dos Andes no Mioceno, o fechamento do Istmo do Panamá, e as introgressões marinhas, juntamente com as mudanças climáticas do Quaternário, são os principais eventos deste período de tempo (NORES, 2004; GARDA & CANATELLA, 2007). Na América do Sul, eventos associados à diversificação têm sido muito mais estudados em florestas tropicais como a Amazônia e a Mata Atlântica (TURCHETTO-ZOLET *et al.,* 2013), deixando um pouco de lado as formações mais abertas.

Os biomas abertos da América do Sul ocupam atualmente uma grande superfície de áreas subúmidas a semiáridas que atingem em conjunto uma extensão de aproximadamente 4.6 milhões de km^2 (DUELLMAN, 1999). Essas formações se estendem desde o centro da Argentina até o norte do Brasil, incluindo grande parte do Paraguai e o sudeste da Bolívia, correspondendo aos biomas do Chaco, Cerrado e Caatinga. Este conjunto de biomas já foi denominado de diversas maneiras: "corredor de savanas", "cadeia de savanas desde o Chaco paraguaio e Mato Grosso até Ceará e Pernambuco" (SCHMIDT & INGER, 1951) e, por fim, como "diagonal de formações abertas" (VANZOLINI, 1963). Historicamente, estas formações têm sido associadas erroneamente à baixa diversidade de espécies e poucos endemismos. Caatinga, Cerrado e Chaco foram muitas vezes considerados parte de uma única formação, mas análises fitogeográficas suportam a existência de três biomas floristicamente distintos e bastante peculiares (CABRERA & WILLINK, 1973; RIZZINI, 1979).

Entre estes biomas, o Chaco é o mais negligenciado em termos de conhecimento sobre sua história evolutiva (WERNECK, 2011), uma vez que carece de estudos sobre possíveis promotores da diversificação de espécies, como barreiras de dispersão ou eventos históricos. O Chaco ocorre desde o sudeste da Bolívia até o centro da Argentina, ocupando grande parte do Paraguai e uma pequena porção no sul do Brasil, no estado do Mato Grosso do Sul. Tem uma superfície aproximada de 1 milhão de km^2, estando principalmente representado na Argentina (62% da sua

extensão) e no Paraguai (25%). O Chaco encontra-se limitado pelas formações pré-Andinas a oeste (Yungas e Montes), pelo Espinal ao sudeste, pelos rios Paraguai e Paraná assim como pela Mata Atlântica Interior (Floresta Estacional Semidecidual) ao leste, e pela Chiquitanía Boliviana e o Cerrado ao norte. O Chaco é considerado como uma unidade biogeográfica distinta, mas sua composição vegetal suporta o reconhecimento de duas formações: o Chaco Seco e o Chaco Úmido (ADAMOLI *et al*., 1990).

Devido à sua situação geográfica, o bioma Chaco está associado a diversos eventos históricos, como introgressões marinhas, glaciações e as mudanças climáticas associadas (HERNANDEZ *et al*., 2005; ORTIZ-JAUGUERIZAR & CLADERA, 2006). As introgressões marinhas do Mioceno têm sido apontadas como possíveis promotores da diversificação para alguns vertebrados no sul da América do Sul (CANDELA *et al*., 2012; MORANDO *et al*., 2014). Pelo menos três importantes introgressões marinhas têm sido registradas para essa região (OTTONE, *et al*., 2013). A mais importante em extensão, conhecida como mar Paranense, ocorreu entre 15 e 13 milhões de anos atrás, cobrindo quase toda a superfície do atual Chaco (HERNANDEZ, *et al*., 2005; CANDELA *et al*., 2012; OTTONE, *et al*., 2013). Este mar interno se estendeu através de uma grande superfície de terra, inundando todas as terras baixas compreendidas entre as antigas formações continentais estáveis, como os arcos estruturais e os crátons (HERNANDEZ *et al*., 2005). Outros importantes eventos que fazem parte da história do bioma Chaco são as glaciações do Quaternário. Mesmo que as geleiras não tenham avançado mais que as latitudes mais austrais do sul da América do Sul e que tenham persistido apenas nos Andes, as mudanças climáticas e ambientais associadas a estes eventos resultaram em deslocamentos cíclicos dos biomas do sul, assim como dos mais próximos a estes, como o Chaco (COSACOV *et al*., 2010; ORTIZ-JAUREGUIZAR & CLADERA, 2006).

O Chaco é uma grande planície aluvial (PENNINGTON *et al*., 2000), historicamente considerada como um continuum, carente de barreiras geográficas para a dispersão de organismos (BUCHER, 1982). Porém, a influência de possíveis barreiras como os rios que atravessam a região nunca foi formalmente testada. Os principais rios que irrigam o Chaco são os rios Pilcomayo, Bermejo, Salado, Dulce e Paraguay. Com a exceção do Paraguay, esses rios são todos alóctones com suas

cabeceiras nos Andes. Esses rios atravessam o Chaco na direção oeste-leste até confluírem com os rios Paraguay e Paraná. Estes rios mantêm águas correntes apenas na temporada chuvosa. No resto do ano os canais perdem suas águas por infiltração (IRIONDO, 1993), principalmente nas regiões mais afastadas das cabeceiras, além disso, esses rios carregam grandes quantidades de sedimento que eventualmente enchem os canais fazendo com que os rios mudem seus cursos, formando grandes sistemas de leques aluviais.

Historicamente o Chaco tem sido associado com baixa diversidade e endemismos (VANZOLINI, 1963). Porém, até agora ao redor de 100 espécies de anuros foram registradas no Chaco, sendo 21 espécies e dois gêneros endêmicos (TNC *et al.,* 2005). Entre estes habitantes do Chaco o gênero *Lepidobatrachus* é um interessante modelo para o estudo e o teste de hipóteses sobre possíveis promotores de diversificação neste bioma. Este gênero de ceratophryídeos contém três espécies (*Lepi. asper*, *Lepi. laevis*, and *Lepi. llanensis*) com distribuição geográfica restrita ao Chaco (FAIVOVICH *et al*., 2014), que apresentam certas características que poderiam ser associadas à baixa vagilidade. Estas espécies, quando ativas, são quase totalmente aquáticas, habitando poças temporárias. Quando as poças estão secando, estes sapos se enterram no solo úmido e produzem um casulo de pele morta que os ajuda na proteção contra a dessecação durante o período de estivação (FAIVOVICH *et al*., 2014). A baixa vagilidade faz deste gênero um candidato ideal para o estudo da diversificação no Chaco. Os ceratophryídeos têm diversificado em ambientes semiáridos (FAIVOVICH *et al*., 2014) e *Lepidobatrachus* especificamente no Chaco, tendo assim uma longa história dentro deste bioma. Isso nos permite ter acesso à história antiga do Chaco. Outro habitante do Chaco, porém com uma historia mais recente dentro do bioma e diferente história de vida é *Lept. bufonius*. Esta espécie é menos dependente de corpos d'água, utilizando-os apenas para a reprodução, e não se enterra durante o período seco do ano. O fato de ter uma historia mais curta dentro do Chaco faz desta espécie um modelo interessante para estudar a história evolutiva mais recente do bioma, permitindo também comparar se espécies com biologias distintas são diferentemente influenciadas pelos fatores e eventos históricos que fazem parte da historia do Chaco.

Esta tese está organizada em três capítulos que investigam desde os eventos e processos mais antigos até os mais recentes que têm atuado como promotores da diversificação das espécies do gênero *Lepidobatrachus* e da espécie *Lepto. bufonius*, assim propondo hipóteses sobre a diversificação no bioma Chaco. Os dois primeiros capítulos tratam sobre a diversificação do gênero *Lepidobatrachus*. No primeiro deles investigamos a estrutura genética e delimitamos o período de tempo de diversificação dentro do gênero, com o objetivo de correlacionar com eventos históricos que poderiam ter influenciado na história evolutiva do gênero e gerar assim hipóteses sobre a diversificação no Chaco. No segundo capítulo aprofundamos nas hipóteses geradas no primeiro capítulo com o propósito de esclarecer os mecanismos que têm gerado a diversidade atual dentro de *Lepidobatrachus*, assim como também testamos com maior robustez as possíveis barreiras de dispersão e a influência dos rios na estrutura genética das diferentes espécies do gênero. No último capítulo focamos na história mais recente do Chaco, investigando a estrutura genética e a demografia histórica da espécie de ampla distribuição *Lepto. bufonius*.

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CAPITULO 1

What happened in the Chaco? Miocene events as main drivers of diversification of the endemic frog genus *Lepidobatrachus* (Ceratophryidae: Anura)

ABSTRACT

Aim. To investigate genetic structure and estimate the diversification time frame for the genus *Lepidobatrachus* to correlate them with historical events and generate hypotheses about Chaco diversification.

Location. Chaco biome.

Methods. We sampled 175 individuals from 53 localities for the genus *Lepidobatrachus*. We sequenced one mitochondrial and six nuclear genes. To infer genetic structure we inferred gene trees and to estimate a time frame of diversification within the genus we used a multi-locus coalescent model implemented in *Beast.

Results. The mitochondrial gene tree recovered the three species and some structure within them. In most nuclear gene trees, the three species were recovered although without interspecific resolution. According to *Beast analysis the diversification of the genus occurred in the Miocene.

Main conclusions. The genetic structure detected within species corresponds to specimens geographical distribution and reveals some breaks in landscape. The main break corresponds to an area of central Chaco that may act as a climatic barrier to dispersal. The dynamic of allochthonous rivers during the Quaternary shaped a complex genetic pattern in *Lepidobatrachus*. We suggest an old and rapid radiation in *Lepidobatrachus* during the Miocene, with marine introgressions and periods of high temperature and arid conditions as the main drivers of *Lepidobatrachus* diversification.

1. INTRODUCTION

The South American dry biomes currently occupy a large area of strongly seasonal sub-humid to semiarid formations of about 4,600,000 km^2 (DUELLMAN, 1999). These formations correspond to Chaco, Cerrado, and Caatinga biomes and extend from central Argentina to northeast Brazil, including large portions of Paraguay and Bolivia. Collectively, this biome group has been named as "chain of savannas" by Schmidt & Inger (1951) and "diagonal of open formations" by Vanzolini (1963). However, Caatinga, Cerrado, and Chaco are different biomes and each one has its own floristic identity (CABRERA & WILLINK, 1973; RIZZINI, 1979). In terms of evolutionary history knowledge, among these biomes the Chaco is the most neglected (WERNECK, 2011), since this peculiar biome lacks scientific studies addressing putative drivers of species diversification, like dispersal barriers or historical events.

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). It is characterized by xerophytic vegetation, formed by a mosaic of grassland, savannas, open woodlands, and xeric thorn forest (WILLIG *et al*., 2000). It is distributed from southeast Bolivia to center Argentina, occupying more than 60% of Paraguay and a little portion of southern Brazil, in Mato Grosso do Sul state. The approximate extension of the Chaco is 1.000.000 km^2 (BUCHER, 1982), being the second most extensive continuous forested area in South America, after the Amazon forest (SANDOVAL & BARQUEZ, 2013). Plant composition supports two macro units within the Chaco: the Dry Chaco and the Humid Chaco (ADAMOLI *et al*., 1990). The Chaco, as currently known, is the result of the Andean uplift (GREGORY-WODZICKI, 2000), marine introgressions (HERNANDEZ *et al*., 2005), and several alluvial fan systems (IRIONDO, 1993) that continuously influence its distribution and climatic conditions, since the Paleogene until today.

Historically, this formation has been associated with low diversity and few endemic species (VANZOLINI, 1963). However, at least 100 anuran species were recorded in the Chaco until now, including 21 endemic species and two endemic genera (TNC *et al.,* 2005). Within these Chacoan inhabitants the genus

Lepidobatrachus is an interesting model to study and test hypotheses about diversification drivers in this biome. This ceratophryid genus contains three species (*L. asper*, *L. laevis*, and *L. llanensis*) with geographical distribution restricted to the Chaco (FAIVOVICH *et al*., 2014). These species are mostly aquatic, inhabit temporary ponds, and exhibit several characteristics associated with the survival in semiarid environments, such as cocoon formation and short duration of larval development (FAIVOVICH *et al*., 2014). When ponds are drying these frogs burrow into the humid soil and produce a dead skin cocoon, which helps to protect against desiccation during the dry period of estivation (FAIVOVICH *et al*., 2014). In the same way, the short larval development is also related with the ephemeral nature of the ponds, helping to prevent larval desiccation. These set of characteristics, closely related with ephemeral water regime, would lead us to think of *Lepidobatrachus* as organisms with low dispersal ability, which contributes to make this genus a great candidate for phylogeographic studies and for understanding patterns of diversification in the Chaco.

One of the first steps to study diversification of a group of species is the delimitation of a temporal framework, and time-calibrated trees are a great tool for this purpose (DRUMMOND *et al*., 2012). 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). According to Faivovich *et al.* (2014), the taxonomic position of the fossils historically assigned to Ceratophryidae; *Beelzebufo ampinga* (EVANS *et al*., 2008), *Baurubatrachus pricei* (BAEZ & PERÍ, 1989), and *Wawelia geroldhi* (CASAMIQUELA, 1963), is not clear and their use as calibration points is controversial. Thus, the older Ceratophryidae fossils are currently represented by *Lepidobatrachus australis* (NICOLI, 2015) and *Ceratophrys ameghinorum* (FERNICOLA, 2001). Both fossil species are from the late Miocene-early Pliocene from Farola Monte Hermoso, Buenos Aires, Argentina (TOMASSINI *et al*., 2011, 2013; FERNICOLA, 2001, NICOLI, 2015). However, the use of these two fossils also presents some problems; none of them was included in any phylogenetic analysis and the chronoestratigraphy from? Farola Monte Hermoso presents some uncertainty (see FAIVOVICH *et al*., 2014).

Besides fossil calibrations, there are some other available strategies in combination with molecular phylogenies to estimate divergence times, such as the use of known divergence times based on other fossil calibration or on dated biogeographical events. Some authors have estimated divergence time between *Ceratophrys* and *Lepidobatrachus* (ROELANTS *et al*., 2007; HEINICKE *et al*., 2009; RUANE *et al*., 2011). However, due to the uncertainty in phylogenetic placement and to the temporal assignment of calibration points used by these authors, it is necessary to be very careful with the use of this data.

Here we used multi locus phylogeography with samples of the three species of *Lepidobatrachus* along the total distribution of the genus to (1) investigate the genetic structure and genetic differentiation between populations, with the specific objective of identifying putative dispersal barriers across the Chaco and (2) estimate a diversification time frame for the genus *Lepidobatrachus,* aiming to correlate these results with historical events and generate diversification hypotheses in the Chaco.

2. MATERIAL AND METHODS

2.1 Sampling

We included 17 samples from four localities of *L. asper*, 80 samples from 29 localities of *L. laevis*, and 78 samples from 27 localities of *L. llanensis*, all of this cover almost totally the genus distribution and the Chaco biome (Fig. 1; see Appendix 1).

Voucher specimens are housed in the Herpetological Collection of the Instituto de Investigación Biológica del Paraguay (IIBPH), Asunción, Paraguay; Laboratorio de Ecología y Evolución (LGE, DB, JNL, and LL), Posadas, Argentina; Museo Argentino de Ciencias Naturales (BB), Buenos Aires, Argentina; and in Coleção Zoológica de Referência da Universidade Federal de Mato Grosso do Sul (ZUFMS), Corumbá, Brazil.

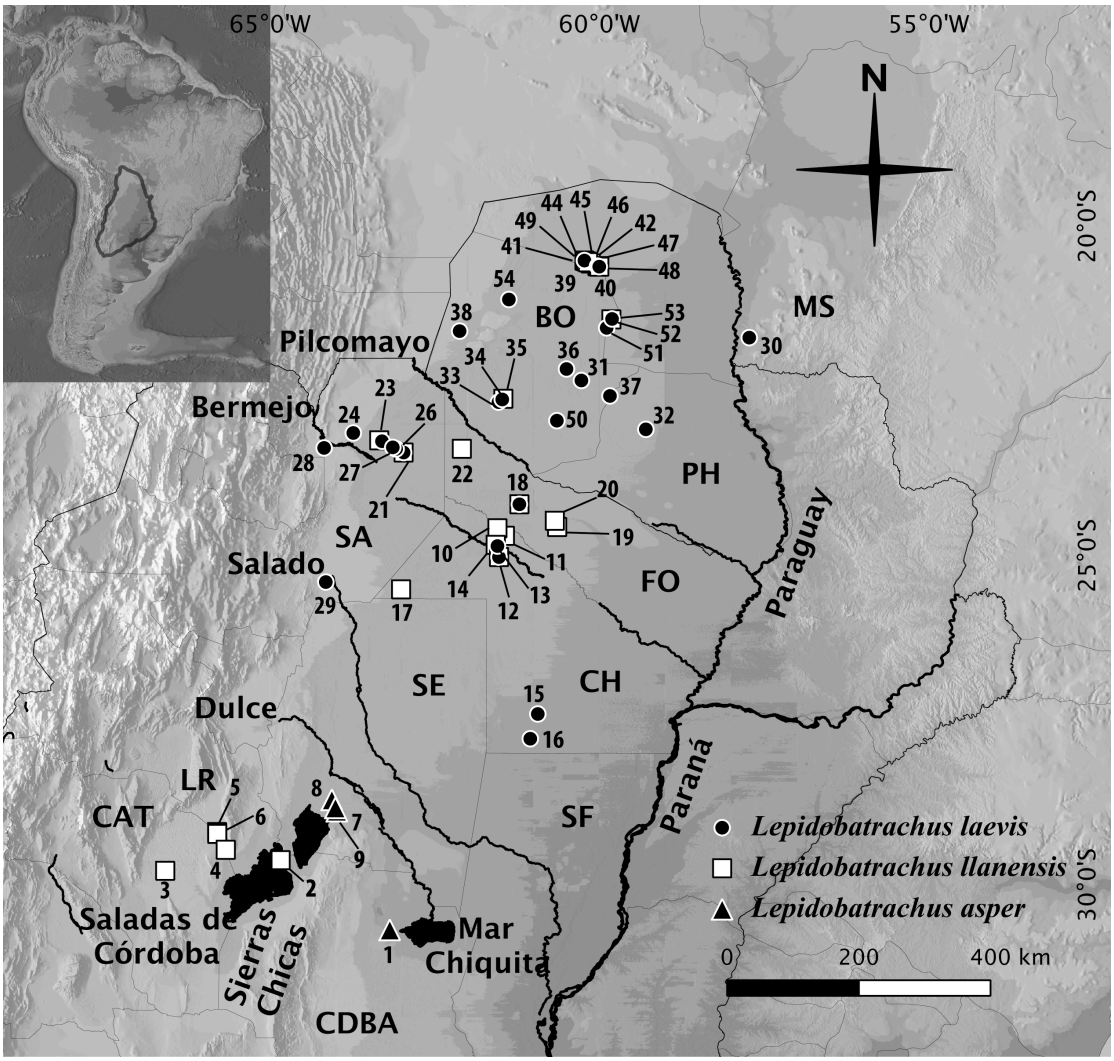

Figure 1: Sampling localities of *Lepidobatrachus* species. Area enclosed in black in South America map denotes the limits of Chaco biome follow Morrone (2001). Putative dispersal barriers discussed in the text are highlighted with their respective names. Detailed locality information represented by numbers is shown in Appendix 1. Department/province/state abbreviations: CAT, Catamarca; CDBA, Córdoba; CH, Chaco; FO, Formosa; LR, La Rioja; SA, Salta; SF, Santa Fé; SE, Santiago del Estero (Argentina); MS, Mato Grosso do Sul (Brazil); BO, Boquerón; PH, Presidente Hayes (Paraguay).

2.2 Molecular methods

We extracted total genomic DNA from samples conserved in 95-100% ethanol (muscle or liver) using the DNeasy extraction kit (Qiagen, Valencia, CA, USA) following manufacturer 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). For the mitochondrial gene amplification we used an initial denaturation step of 3 min at 94 °C, followed by 10

cycles (15 s of denaturation at 95 °C, 20 s of annealing at 45 °C, and 50 s of extension at 60 °C), followed by 26 cycles (15 s of denaturation at 95 °C, 20 s of annealing at 50 °C, and 50 s of extension at 60 °C), and a final extension of 5 min at 60 °C. For nuclear genes amplification 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, Koreia) for sequencing. We checked chromatograms and edited sequences in CodonCode Aligner v. 3.5.4 (Codon Code Corporation). In the subsequent analyses we used one to five samples per locality for mitochondrial gene and one or two samples per locality for the nuclear genes (Fig. 1; see Appendix 2).

2.3 Gene trees

We aligned sequences from each fragment separately with MUSCLE (EDGAR, 2004) in MEGA 6 (TAMURA *et al*., 2013) and checked by eye. Within nuclear loci, we tested for recombination with PhiTest implemented in Splitstree v4.2 (HUSON & BRYANT, 2006); and GARD (Genetic Algorithm Recombination Detection) (KOSAKOVSKY POND *et al*., 2006), MAXCHI (SMITH, 1992; POSADA & CRANDALL, 2001), and CHIMAERA (POSADA & CRANDALL, 2001) implemented in the program RDP3 (HEATH *et al*., 2006). We separated sequences of individuals with heterozygous indels with CodonCode Aligner v. 3.5.4 (Codon Code Corporation) and used Phase 2.1 (STEPHENS *et al*., 2001) implemented in DnaSP 5.1 (LIBRADO & ROZAS, 2009) to resolve haplotypes of heterozygous individuals, discarding those resolved with less than 0.90 of posterior probability. We generated unique haplotypes datasets in DnaSP 5.1 (LIBRADO & ROZAS, 2009). We estimated evolutionary models that best fit each nuclear intron with the program JModeltest 0.1.1 (POSADA, 2008) under the Akaike information criterion (AKAIKE, 1973) (Table 2).

We estimated gene trees for each locus under Bayesian Inference (BI) with MrBayes (RONQUIST & HUELSENBECK, 2003). We applied two independent runs, with four chains each for 30 million generations, sampling every 1000, with

priors under default settings. We verified convergence in TRACER 1.5 (RAMBAUT *et al*., 2013) and by examining standard deviation of split frequencies between independent runs (< 0.01). The first 7500 trees were discarded as burn-in. The CO1 dataset was partitioned by codon position. To polarize the gene trees we used *Ceratophrys aurita* as outgroup.

Table 1: Primers and annealing temperature (°C) used on amplification of each locus.

Locus ID (length on base pair number)	**Primer sequence 5'- 3'**	**Annealing**	**Reference**
CO1 (637 bp)	ANF1 ACHAAYCAYAAAGAYATYGG	45/50	**Jungfer** *et al.*, 2013
Cytochrome c oxidase subunit 1	ANR1 CCGGTCTGAACTCAGATCACGT		**Jungfer** *et al.*, 2013
MVZ 15-16 (299 bp)	MVZ15 ACACCCACTCCTCTATCTTTGATG	54.7	**Bell** *et al.*, 2011
Glyceraldehyde-3 Phosphate Dehydrogenase (intron 4)	MVZ16 AAATGTAAGCTAAGAGATCCACAAC		**Bell** *et al.*, 2011
MVZ 27-28 (356 bp)	MVZ27 ATTATTCCGTAACAGCAAACTC	54.7	**Bell** *et al.*, 2011
Lactose Dehydrogenase Chain Beta (Intron 3)	MVZ28 GTAACCATGGCAACTGGTAG		**Bell** *et al.*, 2011
MVZ 29-30 (221 bp)	MVZ29 ATCCTCCATACTACTTAAGGAGACC	57	**Bell** *et al.*, 2011
Y Box Binding (Intron 1)	MVZ30 CTGAAGCCTCTGTACATGTTTTG		**Bell** *et al.*, 2011
MVZ 39-40 (188 bp)	MVZ39 GGATCTGCTAGAGACCTGTCACTTC	57	**Bell** *et al.*, 2011
X. laevis MGC82783 protein (Intron 2)	MVZ40 ACAGAGTCTTCAAACCCAGCAATAC		**Bell** *et al.*, 2011
MVZ 47-48 (349 bp)	MVZ47 AGTGAAAGATACAGTCACAGTGCTAGG	54.7/56.7/59	**Bell** *et al.*, 2011
X. laevis Fibrinogen, A alpha polypeptide (Intron 1)	MVZ48 GGAGGATATCAGCACAGTCTAAAAAG		**Bell** *et al.*, 2011
RPL3 (418 bp)	RPL35F AAGAAGTCYCACCTCATGGAGAT	50/53/64.3	**Pinho** *et al.*, 2009
Ribosomal Protein L3 (Intron 5)	RPL36RA AGTTTCTTTGTGTGCCAACGGCTAG		**Pinho** *et al.*, 2009

2.4 Isolation by distance and genetic differentiation between populations

We tested isolation by distance [Mantel test in Alleles in Space 1.0 (MILLER, 2005)], gene flow (*Fst* in DnaSP 5.10) and net mean distances [*Da* in DnaSP (TAMURA & NEI, 1993)] between mitochondrial clades within species in order to identify genetic breaks.

2.5 Species tree and diversification time estimate

To infer species trees and estimate a timeframe of diversification for the genus *Lepidobatrachus* we used the multi-locus coalescent model implemented in *Beast (HELED & DRUMMOND, 2010) on Beast 2.1.3 (BOUCKAERT *et al*., 2014), considering the seven loci (CO1 and the six nuclear introns). For this analysis, we included only samples with sequences for at least four loci (see Appendix 2). Because *Beast does not offer all evolutionary models, for nuclear genes we used the closest ones estimated by jModeltest (Table 2).

Table 2: Site and clock models used in gene trees and species tree analyses. Models for CO1 used in gene tree correspond to each codon position.

locus ID	**Gene trees**	**Species tree**	
	site model	**site model**	**clock model**
CO1	TIM3/F81/TIM2+G	TrN+I	relaxed clock log normal
MVZ 15-16	HKY+I+G	TrN+G	relaxed clock log normal
MVZ 27-28	TIM2+I	GTR+G	strict clock
MVZ 29-30	HKY+G	HKY+G	strict clock
MVZ 39-40	TIM3+G	TrN+I+G	strict clock
MVZ 47-48	TVM+G	GTR+G	relaxed clock log normal
RPL3	TIM2+I+G	GTR+I	strict clock

To assess clock models that better 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 good fit. CV is an indicative of how much variation among rates are implied by the data; values below 0.1 are considered a strong evidence for the use of the strict clock (DRUMOND & BOUCKAERT, 2014). See Table 2 for clock and site models used for each locus.

We used secondary calibration dates and gene mutation rates to delimit a time frame for diversification within the genus *Lepidobatrachus*. As commented above, several issues about fossils historically related with ceratophryids were discussed by Faivovich *et al*. (2014). According to them, the older confirmed fossils of Ceratophryidae are the fossil species *L. australis* and *C. ameghinorum*, both with ~5 MYr (TOMASSINI *et al*., 2011, 2013; NICOLI, 2015). However, we chose to not include this information in our analysis due to the uncertainty in relation to the phylogenetic position of the fossils and the chronology of the geologic units where they were found. Several authors have estimated divergence times for *Ceratophrys* and *Lepidobatrachus* with different methods and calibration points (ROELANTS *et al*., 2007; RUANE *et al* 2011; HEINICKE *et al*., 2009). All these authors used a combination of fossil records and paleogeographical events as calibration points. In *Beast analysis we used the most conservative divergence time obtained by these authors, proposed by Ruane *et al*. (2011), as root constraint with normal distribution (mean = 12, sigma = 1.9, in order to cover 7 to 17 Ma).

Among the fragments used here, only CO1 mutation rate is available; however, published estimations were made indirectly. Freilich *et al*. (2014) estimated that CO1 mutation rate is about 25% slower than ND2 mutation rate corresponding to 0.78% per lineage per million years. Meng *et al*. (2014), based on general mitochondrial rates, proposed 0.65% per linage per million years. Based on available rates we used 0.78% per linage per million years as mean in a normal distribution (sigma = 0.0005) under an uncorrelated lognormal relaxed clock model in order to cover both estimations. We ran 200 millions generations sampling every 20,000 with Yule model tree prior and constant population. Convergence and effective sample size (ESS>200) was assessed with Tracer 1.5. 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.

3. RESULTS

3.1 Gene trees

Mitochondrial gene tree recovered the three species well supported; however, internal relationships were not resolved (Fig. 2). With only three haplotypes, one with all samples from Córdoba province, Argentina, no structure was found within *L. asper*. *Lepidobatrachus llanensis* was the most structured among the three species. Haplotypes of northern Chaco, on Boquerón department, Paraguay (Parque Nacional Defensores del Chaco), split in two non-sister groups. Haplotypes of central distribution species (Formosa, Salta, and Chaco provinces, Argentina) group together as well as haplotypes of southern distribution (Córdoba, La Rioja, and Catamarca provinces, Argentina). On the other hand, the less structured is *L. laevis*, with a small group of two samples from northern Chaco (Prieto Farm, Boquerón department, Paraguay) as sister of a large clade grouping all other sampled individuals. Within this large clade only two well-supported and geographically coincident groups were formed, occupying Salta and Chaco provinces, in Argentina, and Boquerón department in Paraguay. In most nuclear gene trees (Fig. 3), the three species were recovered although without interspecific resolution. In MVZ 15-16 gene tree (Fig. 3A) haplotypes of *L. asper* were nested inside *L. laevis*.

3.2 Isolation by distance and genetic differentiation between mitochondrial clades

Mitochondrial test showed positive correlation between genetic distance and geographic distance for *L. asper* (r = 0.25792, p = 0.000) and *L. llanensis* (r = 0.17713, p = 0.000), which corresponds to isolation-by-distance pattern. No correlation was found for *L. laevis* (r = -0.80707, p = 0.902). We found relative high genetic differentiation between some clades within *L. llanensis* (*Fst* range from 0.044 to 0.911, Table 3). The genetic distance was very similar and relatively low between all the clades within the species (*Da* range from 0.003 to 0.019, Table 3).

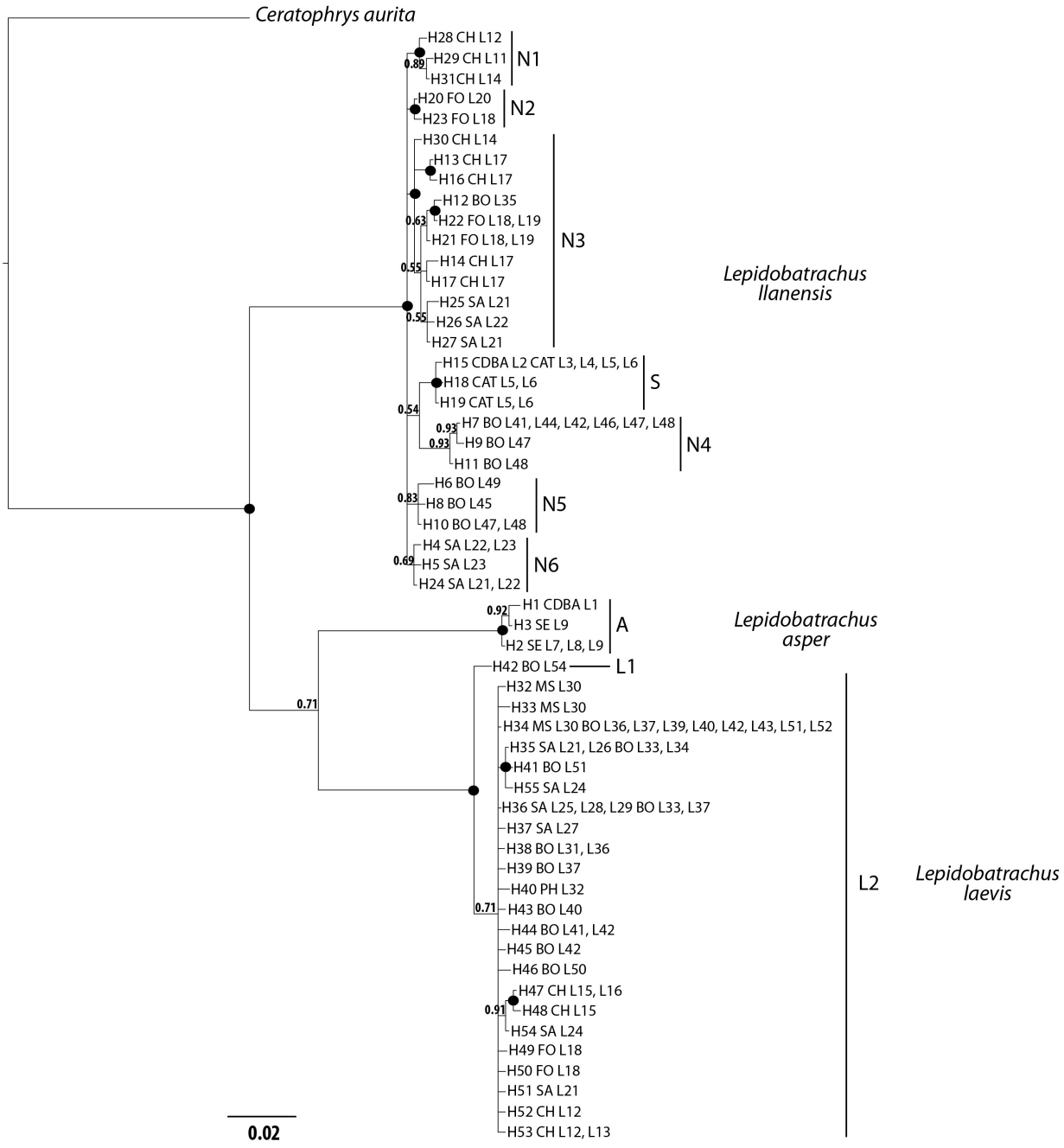

Figure 2: Fifty percent majority rule consensus tree from Bayesian inference analysis of mitochondrial (CO1) fragment (637 bp). Black dots indicate nodes supported by Bayesian posterior probabilities ≥ 0.95. Terminal names correspond to haplotype number followed province, department or state code and locality code (see Fig. 1 and Appendix 1). *Lepidobatrachus llanensis* clades: N1, N2, N3, N4, N5, N6 correspond to sampled sites of north Santiago del Estero gap; S to sampled sites of south of the gap. A to *Lepidobatrachus asper*. L1 and L2 correspond to *Lepidobatrachus laevis*. Scale bar corresponds to substitution/site.

Figure 3: Fifty percent majority rule consensus tree from Bayesian inference analysis of nuclear gene trees. A) MVZ 15-16, B) MVZ 27-28, C) MVZ 29-30, D) MVZ 39-40, E) MVZ 47-48, F) RPL3. Black dots indicate nodes supported by Bayesian posterior probabilities ≥ 0.95. Black triangle corresponds to *Lepidobatrachus asper*, black circle to *Lepidobatrachus laevis*, and white square to *Lepidobatrachus llanensis*. Outgroup is not shown. Scale bar corresponds to substitution/site.

Table 3: Pairwise *Fst* (below diagonal) and *Da* (above diagonal) values for mitochondrial fragment (CO1) between clades of *Lepidobatrachus* species (see Fig. 2): A (n = 15) corresponds to *Lepidobatrachus asper*; L1 (n = 2) and L2 (n = 78) to *Lepidobatrachus laevis* clades; N1 (n = 3), N2 (n = 2), N3 (n = 14), N4 (n = 13), N5 (n = 4), N6 (n = 6), and S (n = 30) to *Lepidobatrachus llanensis*. (L1* corresponds to a unique haplotype on mitochondrial tree).

	A	**L1***	**L2**	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**S**
A		0.107	0.108	0.111	0.109	0.110	0.112	0.106	0.108	0.112
L1*	0.962		0.011	0.111	0.111	0.109	0.104	0.105	0.108	0.109
L2	0.908	0.514		0.112	0.113	0.111	0.109	0.106	0.109	0.111
N1	0.886	0.946	0.895		0.005	0.006	0.018	0.006	0.005	0.010
N2	0.915	0.974	0.924	0.471		0.003	0.019	0.004	0.003	0.010
N3	0.836	0.901	0.857	0.369	0.470		0.019	0.005	0.004	0.010
N4	0.945	0.990	0.931	0.825	0.911	0.762		0.019	0.019	0.017
N5	0.875	0.944	0.893	0.077	0.400	0.320	0.806		0.005	0.010
N6	0.920	0.977	0.923	0.218	0.533	0.379	0.908	0.044		0.009
S	0.938	0.980	0.923	0.722	0.851	0.690	0.901	0.712	0.829	

3.3 Species tree

As our main goal with the species tree analysis was to delimit a timeframe of diversification within the genus *Lepidobatrachus* we assigned samples to the three species of the genus. The analysis recovered *L. llanensis* as sister taxa of *L. asper* + *L. laevis* with moderate support (posterior probability = 0.88). According to our data, the first split has occurred in the Miocene [6–11.6 Ma; 95% highest posterior density (HPD) interval] and the second in the late Miocene-early Pliocene (4.2–9.6 Ma; 95% HPD interval) (Fig. 4).

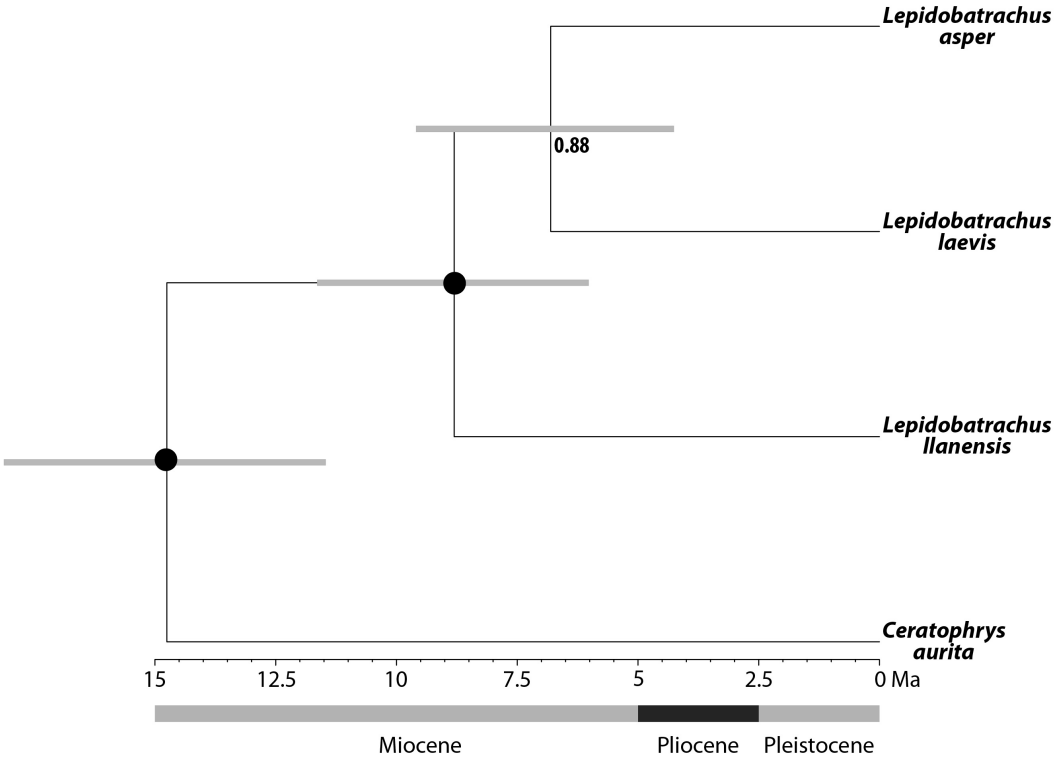

Figure 4: *Beast species tree analysis. Black dots indicate nodes supported by Bayesian posterior probabilities ≥ 0.95. Node bars correspond to 95% highest posterior density intervals of divergence times expressed in Ma.

4. DISCUSSION

4.1 Gene trees: genetic structure and putative dispersal barriers within the Chaco

The nuclear genes used here cannot resolve relationships among or within species. The low phylogenetic resolution of nuclear genes is expected because of the larger effective population size (BIRKY *et al*., 1989; BALLARD & WHITLOCK, 2004), the close relationship among the species (FAIVOVICH *et al*., 2014), and additionally, in our case, because of the low variation due to short sequence lengths (MIRARAB *et al*., 2014).

Mitochondrial gene tree (Fig. 2) recovered all the species but failed to recover the relationships within species. We detected some structure within species, which is concordant to geographical distribution of the specimens and suggests some breaks in

the landscape. The main break within *L. llanensis* corresponds to a gap of about 400 km in central Santiago del Estero province between southern *L. llanensis* clade (south of Santiago del Estero sampled sites) and all other samples (see Fig. 1). The geographic distance is correlated with genetic distances within *L. llanensis* ($r = 0.177$) and therefore the genetic structure could be explained by isolation by distance. However, between south and each north clade we found a relative high differentiation (*Fst* range from 0.69 to 0.9) and genetic distance (*Da* range from 0.009 to 0.017) (values for *Fst* and *Da* in Table 3). This distribution gap may not simply correspond to a sampling gap, once it matches with southern and/or western distribution limit of other Chacoan species (e.g. *L. laevis*, *Scinax acuminatus*, *Melanophryniscus klappenbachi*, *Rhinella major*, *Dermatonotus muelleri*). Also, other endemic species with similar distribution range of *L. llanensis*, such as *Chacophrys pierottii* and *Leptodactylus bufonius*, show a comparable spatial distribution pattern. In fact, this gap corresponds to an area of high temperature known as the "South American heat pole" (PROHASKA, 1959) with summer temperatures of about 47 ºC. Furthermore, low precipitation regime (BOLETTA *et al*., 1989) and high salinity (RUIBAL, 1962) are important environmental characteristics of this area that may act as a climatic barrier to *L. llanensis* dispersal and also maybe to other anuran species.

This disjunct distribution can also be associated with recent local extinctions caused by landscape changes due to severe logging and ranching since early last century at this region of Argentina (BUCHER & HUSZAR, 1999). However, based on genetic distance between south and north sampled sites of *L. llanensis* (*Da* between 0.009 and 0.017) and CO1 mutation rate (0.78%), this divergence seems to be much older than the recent Santiago del Estero landscape transformation, corresponding to late Pleistocene (between 0.57 and 1 Ma).

South American Late Pleistocene (approximately the last 1 Ma) is marked by increments on temperature and dryer conditions (ORTIZ-JAUREGUIZAR & CLADERA, 2006), enhancing seasonality and extreme conditions of central Santiago del Estero. Only species with high salinity tolerance as *Pleurodema guayapae*, *P. tucumanum*, and *L. asper* (RUIBAL, 1962) were recorded from central Santiago del Estero (FERRARO & CASAGRANDA, 2009; FAIVOVICH, 1994). Furthermore, Carroll (1996) found low tadpoles survival rates at short time thermal shocks of 45 ºC

on the congeneric *L. laevis*. These results suggest that the combination of high salinity and high temperature should be responsible to make this area an impermeable barrier to *L. llanensis* dispersal and thus a putative vicariant agent for north and south distribution of this species.

Our sampling of *L. asper* covers only partially the known distribution of the species. Following Faivovich (1994), besides Córdoba, and southern Santiago del Estero the species has been recorded in Corrientes, Chaco, and Santa Fé provinces, and in the Paraguayan departments of Presidente Hayes and Alto Paraguay. Nevertheless, the last of these records are from the 80's and the species has never been found again in these latter localities. We sampled basically two localities of *L. asper* from southern Chaco distribution. Even taking into account that genetic structure within *L. asper* can be also related with isolation by distance (r = 0.258), between localities of Saladas de Córdoba (south of Mar Chiquita lake) and Santiago del Estero we found no evidence of gene flow (*Fst* = 0.941) that can be related to one of the last foothills of the Sierras Chicas formation (Fig. 1). However, without a better sampling we cannot test the influence of this formation on genetic structure within *L. asper*.

Lepidobatrachus laevis is the less genetically structured and was the only species without evidence of isolation by distance (r = -0.0807). The only haplotype from north Chaco (Prieto Farm, Boquerón) is sister of all other haplotypes but without support (posterior probability of 0.71) and no evidence of genetic differentiation (*Da* = 0.01 and *Fst* = 0.513).

Both samplings of *L. laevis* and *L. llanensis* allow us to test the influence of rivers on genetic structure, since we have samples of both sides of the main Chacoan rivers: Pilcomayo, Bermejo, Salado, Dulce, and Paraguay. With the exception of the Paraguay River, these rivers are allochthonous and have their headwaters in the Andes. These rivers cross the Chaco in west-east direction until the confluence with the Paraguay and Paraná rivers. They have flowing waters only in rainy season, and in the rest of the year their channels lose water by infiltration (IRIONDO, 1993), especially in regions away from the headwaters. Furthermore, these rivers carries a

large amount of sediments that eventually fill the channels and make the rivers change their courses, forming large alluvial fan systems.

The Dulce and Salado rivers cross the Chaco between the south and north distribution of *L. llanensis*. However, divergence time estimation between these two groups of localities (between 0.57 and 1 Ma) is markedly much older than expected for any influence of these rivers. During the Quaternary, the Chaco experienced climatic changes interspersing between dry and humid periods (IRIONDO, 1993). In dry periods these allochthonous rivers were ephemeral with highly seasonal channels. Only between the last glacial maximum (21,000 years ago) and the late Holocene two dry periods were identified (IRIONDO, 1993). Nevertheless, this constant climatic change during the Quaternary has had an impact in central distribution of this species, where the dynamic of the Pilcomayo and Bermejo rivers shaped a complex genetic pattern. Besides of water volume differences between dry and humid periods, these rivers change their course due to sedimentation of their channels. Some mitochondrial haplotypes of south and north Bermejo were grouped in different clades, however other haplotypes of the same localities grouped together in other clade. The same pattern was observed for localities of both side of Pilcomayo River. This pattern probably is the response to recurrent connections between localities of both sides of these rivers, which has promoted relatively short and intermittent vicariant events.

The Paraguay River is the largest river analyzed here. Unlike the other rivers, this river does not cross the Chaco in west-east direction and, until recently, all the species of *Lepidobatrachus* have been recorded from its oriental margin. Sugai *et al*. (2013) cited *L. laevis* (as *L. asper*) from Patolá Farm, Porto Murtinho, a Chacoan fragment of southern Brazil at the other river margin . We included four individuals of this locality in our analysis that resulted in three haplotypes, two exclusives and one shared with individuals of Paraguayan Central Chaco (Paraguay River occidental margin). This fact suggests a recent colonization of the region. Frutos & Van Des Busche (2002) found similar results in armadillo populations from both sides of this river. Based on water level records analyses of upper Paraguay River, Collischonn *et al*. (2001) found that between 1960 and 1972 an abrupt water level decrease occurred, which may have permitted the dispersion of *L. laevis* to the other side of the river.

4.2 Species tree: diversification time frame within *Lepidobatrachus*

Our species tree analysis recovered a different topology than from Faivovich *et al*. (2014) with *L. llanensis* as sister of *L. asper* + *L. laevis* (Fig. 4) instead of *L. asper* as sister of *L. llanensis* + *L. laevis*, but with only moderate support (posterior probability of 0.88). This incongruence can be explained in different ways, like sampling efforts, selected loci, or may have been caused by methodological differences. It is known that concatenation-based methods may result in different topology that of coalescent-based analysis in presence of gene trees incongruence (KNOWLES, 2009). This gene-tree discordance is attributable to many biological phenomena like incomplete lineage sorting, horizontal gene transfer, or hybridization (PAMILO & NEI, 1987; AVISE, 1989; MADDISON, 1997).

The *Beast species tree analysis suggests an old and rapid radiation of *Lepidobatrachus* genus with divergence times between the species with high degree of overlap (see Fig. 4). Diversification within *Lepidobatrachus* began with *L. llanensis* split in middle Miocene about 9 Ma (6–11.6 Ma 95% HPD interval), followed by *L. asper*-*L. laevis* late Miocene divergence about 6 Ma (4.2–9.5 Ma 95% HPD interval). The main events occurred within this timeframe are the middle Miocene marine introgression in Chaco and Paraná basins, known as Paranense Sea (HERNANDEZ *et al*., 2005) and the late Miocene climatic change (CERLING *et al*., 1997; BEERLING & OSBORNE, 2006; OSBORNE, 2008).

Three marine introgression events were proposed in the literature for the studied region (HERNANDEZ *et al*., 2005; OTTONE *et al*., 2013). The more ancient one was from late Oligocene/early Miocene, between 25 and 20 Ma, which occupied a great portion of Chaco and Pampas. The earliest introgression was estimated from late Miocene, between 10 and 5 Ma, with no evidence at Chaco (OTTONE *et al*., 2013). The middle Miocene introgression, 15 and 13 Ma, called Paranense Sea, was the most important in size (HERNANDEZ *et al*., 2005) with final phases of regression dated in 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 the Argentinan Chaco covered Santa Fé, 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 identified at southern Boquerón (OTTONE *et al*., 2013). North and northeastern marine distribution reached the Michicola and Asunción highs, respectively (HERNANDEZ *et al*., 2005). The Michicola and Asunción highs are extensions of the Brazilian shield, a large subsurface that act as a barrier of drainage systems (LUNDBERG *et al*., 1998). This distribution suggests a strong influence of the Paranense Sea on the *Lepidobatrachus* diversification as a vicariant agent on a Chacoan widely distributed ancestral taxa. Furthermore, this distribution suggests the existence of refuges on north, east and south of current Chaco during the Paranense Sea.

Another event that may have had an influence on *Lepidobatrachus* diversification, mainly on the divergence between *L. asper* and *L. laevis* was the late Miocene climatic change that favored the grassland habitats expansion. It is likely that this expansion in late Miocene, at least in South America, was due to higher temperature, arid conditions (CERLING *et al*., 1997), fire regime, and low atmospheric CO_2 availability (BEERLING & OSBORNE, 2006; OSBORNE, 2008). Currently, *Lepidobatrachus* species are found more associated to woodland formations than to open areas like grasslands. Late Miocene grassland expansion could have fragmented forested areas and isolated populations, triggering the divergence between *L. asper* and *L. laevis*.

As a first attempt to understand the diversification of the genus *Lepidobatrachus* we proposed some historical scenarios based on divergence times and genetic structure. However, fine-scale analyses, as allele frequencies methods and historical demography, could test this hypotheses or result on other equally acceptable diversification hypotheses. Finally, to avoid generalizations about diversification on Chacoan frogs, hypotheses proposed here have to be tested with other species models, especially with species with different life histories.

5. CONCLUSIONS

We found some genetic structure within *Lepidobatrachus* species which can be associated with some breaks on the landscape. Main break corresponds to a high aridity area in central Chaco that may acts as a dispersal barrier for *L. llanensis*. We also found some influences of main rivers (Bermejo and Pilcomayo), which due to its historical dynamics might have promoted short and intermittent vicariant events allowing recurrent connections among populations of both side of these rivers. Population of *L. laevis* from oriental margin of Paraguay river may correspond to a recent colonization, probably related with recent abrupt decrease of water level of the river. Our results also suggest and old an rapid radiation in *Lepidobatrachus*, which began with *L. llanensis* divergence on middle Miocene followed by *L. asper* and *L. laevis* separation on late Miocene. Main events that match this time frame are marine introgressions and climatic changes. Marine introgressions would have acted as a vicariant agent on a widely ancestral taxa and isolated populations that originated *L. llanensis* and *L. asper* + *L. laevis* ancestrals. Likewise, late Miocene climatic changes could have favored grassland-like habitat expansions that fragmented forested areas isolating ancestral populations of *L. asper* and *L. laevis*.

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Appendix 1: Voucher information. Abbreviations: National Route (NR), Reserva Natural (RN), Parque Nacional (PN).

Samples ID	Country	Prov/Department	Locality	Lat	Long	Locality code	Mitochondrial Haplotype	Clade
Lepidobatrachus asper								
LGE 101	Argentina	Santiago del Estero	Saladillo river	-28.88	-63.98	L9	H2	A
LGE 102	Argentina	Santiago del Estero	Saladillo river	-28.88	-63.98	L9	H2	A
LGE 103	Argentina	Santiago del Estero	Saladillo river	-28.88	-63.98	L9	H3	A
LGE 104	Argentina	Santiago del Estero	Saladillo river	-28.88	-63.98	L9	H3	A
LGE 105	Argentina	Santiago del Estero	Saladillo river	-28.88	-63.98	L9	H2	A
LGE 329	Argentina	Santiago del Estero	NR 9, km 1028	-28.71	-64.06	L8	H2	A
LGE 1347	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64	L7	H2	A
LGE 1348	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64	L7	H2	A
LGE 1349	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64	L7	H2	A
LGE 1350	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64	L7	H2	A
LGE 1351	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64	L7	H2	A
JNL 253	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	H1	A
JNL 254	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	H1	A
JNL 255	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	H1	A
JNL 257	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	H1	A
Lepidobatrachus laevis								
DB 4933	Argentina	Salta	PR81, 21 km SE from NR34	-23.14	-63.73	L24	H54	L2
DB 4943	Argentina	Salta	PR81, 21 km SE from NR34	-23.14	-63.73	L24	H55	L2
DB 4942	Argentina	Salta	Pichanal	-23.36	-64.18	L28	H36	L2
DB 5003	Argentina	Salta	Pichanal	-25.4	-64.15	L29	H36	L2
DB 8562	Argentina	Chaco	Nueva Pompeya	-24.85	-61.54	L13	H53	L2
LGE 5275	Argentina	Chaco	Mesón de Hierro	-27.4	-60.93	L15	H47	L2
LGE 5277	Argentina	Chaco	Mesón de Hierro	-27.4	-60.93	L15	H47	L2
LGE 5289	Argentina	Chaco	Mesón de Hierro	-27.4	-60.93	L15	H47	L2
LGE 5292	Argentina	Chaco	Mesón de Hierro	-27.4	-60.93	L15	H48	L2

Samples ID	Country	Prov/Department	Locality	Lat	Long	Locality code	Mitochondrial Haplotype	Clade
LGE 5295	Argentina	Chaco	Mesón de Hierro	-27.4	-60.93	L15	H47	L2
LGE 5315	Argentina	Chaco	Mesón de Hierro	-27.4	-60.93	L15	H48	L2
LGE 5425	Argentina	Chaco	Santa Sylvina	-27.78	-61.05	L16	H47	L2
LGE 5426	Argentina	Chaco	Santa Sylvina	-27.78	-61.05	L16	H47	L2
LGE 5427	Argentina	Chaco	Santa Sylvina	-27.78	-61.05	L16	H47	L2
LGE 5428	Argentina	Chaco	Santa Sylvina	-27.78	-61.05	L16	H47	L2
IIBPH 1457	Paraguay	Boquerón	20 km E from Filadelfia	-22.34	-60.27	L31	H38	L2
IIBPH 1749	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	H34	L2
IIBPH 1750	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	H36	L2
IIBPH 1751	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	H34	L2
IIBPH 1752	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	H34	L2
IIBPH 1753	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	H39	L2
IIBPH 1811	Paraguay	Boquerón	Chaco Boef Farm	-22.17	-60.5	L36	H38	L2
IIBPH 1812	Paraguay	Boquerón	Chaco Boef Farm	-22.17	-60.5	L36	H38	L2
IIBPH 1813	Paraguay	Boquerón	Chaco Boef Farm	-22.17	-60.5	L36	H34	L2
IIBPH 1884	Paraguay	Presidente Hayes	RN Fortín Salazar	-23.08	-59.29	L32	H40	L2
IIBPH 1906	Paraguay	Boquerón	Toro Reta Farm	-22.67	-61.53	L33	H35	L2
IIBPH 2749	Paraguay	Boquerón	Toro Reta Farm	-22.67	-61.53	L33	H36	L2
IIBPH 2286	Paraguay	Boquerón	Infante Rivarola	-21.59	-62.12	L38	H41	L2
IIBPH 2287	Paraguay	Boquerón	Infante Rivarola	-21.59	-62.12	L38	H41	L2
IIBPH 2288	Paraguay	Boquerón	Infante Rivarola	-21.59	-62.12	L38	H41	L2
IIBPH 2349	Paraguay	Boquerón	Teniente Prieto Farm	-21.11	-61.37	L54	H42	L1
IIBPH 2350	Paraguay	Boquerón	Teniente Prieto Farm	-21.11	-61.37	L54	H42	L1
IIBPH 2360	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L39	H34	L2
IIBPH 2361	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L39	H34	L2
IIBPH 2362	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L39	H34	L2
IIBPH 2365	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L40	H34	L2
IIBPH 2366	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L40	H43	L2
IIBPH 2367	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L40	H34	L2

Samples ID	Country	Prov/Department	Locality	Lat	Long	Locality code	Mitochondrial Haplotype	Mitochondrial Clade
IIBPH 2378	Paraguay	Boquerón	PN Defensores del Chaco	-20.52	-60.22	L41	H44	L2
IIBPH 2379	Paraguay	Boquerón	PN Defensores del Chaco	-20.52	-60.22	L41	H44	L2
IIBPH 2391	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	H34	L2
IIBPH 2392	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	H34	L2
IIBPH 2393	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	H44	L2
IIBPH 2395	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	H34	L2
IIBPH 2396	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	H34	L2
IIBPH 2397	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	H45	L2
IIBPH 2398	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	H34	L2
IIBPH 2407	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L43	H34	L2
ZUFMS 2638	Brazil	Mato Grosso do Sul	Porto Murtinho	-21.69	-57.72	L30	H32	L2
ZUFMS 2639	Brazil	Mato Grosso do Sul	Porto Murtinho	-21.69	-57.72	L30	H32	L2
ZUFMS 2640	Brazil	Mato Grosso do Sul	Porto Murtinho	-21.69	-57.72	L30	H33	L2
ZUFMS 2641	Brazil	Mato Grosso do Sul	Porto Murtinho	-21.69	-57.72	L30	H34	L2
IIBPH 2819	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	H35	L2
IIBPH 2820	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	H35	L2
IIBPH 2821	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	H36	L2
IIBPH 2822	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	H36	L2
IIBPH 2862	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	H35	L2
IIBPH 2863	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	H35	L2
IIBPH 2864	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	H35	L2
IIBPH 2865	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	H35	L2
IIBPH 2866	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	H35	L2
IIBPH 2884	Paraguay	Boquerón	road Montania-Madrejón	-21.54	-59.89	L51	H34	L2
IIBPH 2885	Paraguay	Boquerón	road Montania-Madrejón	-21.54	-59.89	L51	H34	L2
IIBPH 2886	Paraguay	Boquerón	road Montania-Madrejón	-21.54	-59.89	L51	H34	L2
IIBPH 2887	Paraguay	Boquerón	road Montania-Madrejón	-21.54	-59.89	L51	H34	L2
IIBPH 2888	Paraguay	Boquerón	road Montania-Madrejón	-21.54	-59.89	L51	H34	L2
IIBPH 2906	Paraguay	Boquerón	Pitiantuta Farm detour	-21.41	-59.8	L52	H34	L2

Samples ID	Country	Prov/Department	Locality	Lat	Long	Locality code	Mitochondrial Haplotype	Clade
IIBPH 2930	Paraguay	Boquerón	Pirizal, Linea 1	-22.95	-60.64	L50	H46	L2
LGE 8180	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	H49	L2
LGE 8197	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	H50	L2
LGE 8201	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	H50	L2
LGE 8202	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	H49	L2
LGE 8203	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	H49	L2
LGE 8232	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	H35	L2
LGE 8247	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	H51	L2
BB 1953	Argentina	Salta	Pluma de Pato	-23.38	-63.08	L26	H35	L2
BB 1996	Argentina	Salta	Pluma de Pato	-23.26	-63.3	L25	H36	L2
BB 2009	Argentina	Salta	Morillo	-23.36	-63.13	L27	H37	L2
LL 1	Argentina	Chaco	Nueva Pompeya	-25.02	-61.52	L12	H52	L2
LL 2	Argentina	Chaco	Nueva Pompeya	-25.02	-61.52	L12	H53	L2
Lepidobatrachus llanensis								
DB 7700	Argentina	Chaco	Wichi	-24.7	-61.43	L11	H29	N1
DB 8702	Argentina	Chaco	Nueva Pompeya	-24.84	-61.57	L14	H30	N3
DB 8703	Argentina	Chaco	Nueva Pompeya	-24.84	-61.57	L14	H31	N1
LGE 1360	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	H13	N3
LGE 1362	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	H14	N3
LGE 1660	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	H16	N3
LGE 1758	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	H17	N3
LGE 2324	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 2325	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 2326	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 2331	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 2332	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 2333	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 5536	Argentina	Catamarca	San Martín	-29.19	-65.8	L5	H18	S
LGE 5537	Argentina	Catamarca	San Martín	-29.19	-65.8	L5	H15	S

Samples ID	Country	Prov/Department	Locality	Lat	Long	Locality code	Mitochondrial Haplotype	Clade
LGE 5539	Argentina	Catamarca	San Martín	-29.19	-65.8	L5	H18	S
LGE 5540	Argentina	Catamarca	San Martín	-29.19	-65.8	L5	H19	S
LGE 5541	Argentina	Catamarca	San Martín	-29.19	-65.8	L5	H15	S
LGE 5546	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	H15	S
LGE 5547	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	H15	S
LGE 5548	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	H15	S
LGE 5550	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	H15	S
LGE 5551	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	H15	S
LGE 5587A	Argentina	Catamarca	San Martín	-29.22	-65.8	L6	H18	S
LGE 5587B	Argentina	Catamarca	San Martín	-29.22	-65.8	L6	H18	S
LGE 5587C	Argentina	Catamarca	San Martín	-29.22	-65.8	L6	H18	S
LGE 5587D	Argentina	Catamarca	San Martín	-29.22	-65.8	L6	H19	S
LGE 5593A	Argentina	Catamarca	Telarito	-29.46	-65.67	L4	H15	S
LGE 5593B	Argentina	Catamarca	Telarito	-29.46	-65.67	L4	H15	S
IIBPH 2380	Paraguay	Boquerón	PN Defensores del Chaco	-20.52	-60.22	L41	H7	N4
IIBPH 2381	Paraguay	Boquerón	PN Defensores del Chaco	-20.55	-60.17	L44	H7	N4
IIBPH 2384	Paraguay	Boquerón	PN Defensores del Chaco	-20.56	-60.13	L45	H8	N5
IIBPH 2394	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	H7	N4
IIBPH 2400	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.03	L46	H7	N4
IIBPH 2401	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.03	L46	H7	N4
IIBPH 2402	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L47	H7	N4
IIBPH 2403	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L47	H9	N4
IIBPH 2404	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L47	H10	N5
IIBPH 2405	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L48	H7	N4
IIBPH 2406	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L48	H7	N4
IIBPH 2408	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L48	H7	N4
IIBPH 2409	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L48	H7	N4
IIBPH 2410	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L48	H11	N4
IIBPH 2412	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60	L48	H10	N5

Samples ID	Country	Prov/Department	Locality	Lat	Long	Locality code	Mitochondrial Haplotype	Clade
IIBPH 2368	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.2	L49	H6	N5
IIBPH 2382	Paraguay	Boquerón	PN Defensores del Chaco	-20.55	-60.17	L44	H7	N4
IIBPH 2855	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.62	-61.45	L35	H12	N3
IIBPH 2858	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.62	-61.45	L35	H12	N3
LGE 7416	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 7417	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 7418	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 7419	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	H15	S
LGE 8121	Argentina	Formosa	Las Lomitas	-24.47	-60.67	L20	H20	N2
LGE 8148	Argentina	Formosa	Laguna Yema	-24.56	-60.64	L19	H21	N3
LGE 8159	Argentina	Formosa	Laguna Yema	-24.56	-60.64	L19	H22	N3
LGE 8164	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	H23	N2
LGE 8165	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	H22	N3
LGE 8166	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	H21	N3
LGE 8207	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	H24	N6
LGE 8209	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	H25	N3
LGE 8215	Argentina	Salta	Fortín Dragones	-23.38	-62.08	L22	H4	N6
LGE 8216	Argentina	Salta	Fortín Dragones	-23.38	-62.08	L22	H26	N3
LGE 8217	Argentina	Salta	Fortín Dragones	-23.38	-62.08	L22	H24	N6
LGE 8224	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	H24	N6
LGE 8225	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	H27	N3
BB 1891	Argentina	Salta	Fortín Dragones	-23.25	-63.34	L23	H4	N6
BB 1892	Argentina	Salta	Fortín Dragones	-23.25	-63.34	L23	H5	N6
LLL 2	Argentina	Chaco	Nueva Pompeya	-25.02	-61.52	L12	H28	N1
LGE 5854	Argentina	Catamarca	Telarito	-29.46	-65.67	L4	H15	S
LGE 5858	Argentina	Catamarca	Telarito	-29.46	-65.67	L4	H15	S
LGE 5856	Argentina	Catamarca	San Martín	-29.22	-65.8	L6	H15	S
LGE 5857	Argentina	Catamarca	San Martín	-29.22	-65.8	L6	H19	S

Appendix 2: GenBank accession of sequences used in this article. (upon acceptance)

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
Lepidobatrachus asper							
LGE 101	x	x	x	x	x		x
LGE 102	x	x	x	x	x		x
LGE 103	x						
LGE 104	x						
LGE 105	x						
LGE 329	x	x	x	x	x		x
LGE 1347	x	x	x	x	x		x
LGE 1348	x	x	x	x	x		x
LGE 1349	x						
LGE 1350	x						
LGE 1351	x						
LGE 4961		x	x	x			x
LGE 4962		x	x		x		
JNL 253	x						
JNL 254	x						
JNL 255	x						
JNL 257	x						
Lepidobatrachus laevis							
DB 4933	x		x	x		x	
DB 4943	x	x	x	x	x	x	x
DB 4942	x						
DB 5003	x						
DB 8562	x		x	x	x	x	x
LGE 5275	x		x	x			x
LGE 5277	x		x	x		x	x
LGE 5289	x						
LGE 5292	x						
LGE 5295	x						
LGE 5315	x						
LGE 5425	x		x	x			x
LGE 5426	x		x	x			x
LGE 5427	x						
LGE 5428	x						
IIBPH 1457	x			x			
IIBPH 1749	x		x	x	x	x	
IIBPH 1750	x		x	x			x
IIBPH 1751	x						
IIBPH 1752	x						
IIBPH 1753	x						
IIBPH 1811	x		x		x		x
IIBPH 1812	x	x	x	x		x	
IIBPH 1813	x						

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
IIBPH 1884	x	x	x	x	x		x
IIBPH 1906	x						
IIBPH 2749	x						
IIBPH 2286	x		x	x			x
IIBPH 2287	x		x				x
IIBPH 2288	x						
IIBPH 2349	x		x	x	x	x	
IIBPH 2350	x	x	x	x		x	x
IIBPH 2360	x						
IIBPH 2361	x						
IIBPH 2362	x						
IIBPH 2365	x	x	x	x	x		
IIBPH 2366	x						
IIBPH 2367	x						
IIBPH 2378	x						
IIBPH 2379	x		x	x	x		
IIBPH 2391	x						
IIBPH 2392	x						
IIBPH 2393	x		x	x			
IIBPH 2395	x						
IIBPH 2396	x	x	x		x		x
IIBPH 2397	x						
IIBPH 2398	x						
IIBPH 2407	x						
ZUFMS 2638	x	x	x	x		x	x
ZUFMS 2639	x	x	x	x	x	x	x
ZUFMS 2640	x						
ZUFMS 2641	x						
IIBPH 2819	x		x		x		x
IIBPH 2820	x			x	x	x	x
IIBPH 2821	x						
IIBPH 2822	x						
IIBPH 2862	x		x		x		x
IIBPH 2863	x		x		x	x	x
IIBPH 2864	x						
IIBPH 2865	x						
IIBPH 2866	x						
IIBPH 2884	x		x	x	x	x	x
IIBPH 2885	x	x	x				x
IIBPH 2886	x						
IIBPH 2887	x						
IIBPH 2888	x						
IIBPH 2906	x	x	x	x	x	x	x
IIBPH 2930	x		x	x	x	x	x

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
LGE 8180	x		x	x	x	x	x
LGE 8197	x	x	x	x	x	x	x
LGE 8201	x						
LGE 8202	x						
LGE 8203	x						
LGE 8232	x	x	x	x		x	x
LGE 8247	x	x	x	x	x	x	x
BB 1953	x		x	x	x	x	x
BB 1996	x	x	x	x		x	
BB 2009	x						
LL 1	x		x	x	x	x	
LL 2	x		x	x	x	x	
Lepidobatrachus llanensis							
DB 8402		x	x	x		x	
DB 7700	x		x	x			x
DB 8702	x	x	x	x	x		x
DB 8703	x	x	x	x	x	x	
LGE 220			x	x			
LGE 1360	x			x			x
LGE 1362	x						
LGE 1660	x						
LGE 1758	x						
LGE 2324	x		x	x	x	x	
LGE 2325	x		x	x	x		x
LGE 2326	x						
LGE 2331	x						
LGE 2332	x						
LGE 2333	x						
LGE 5536	x						
LGE 5537	x			x			
LGE 5538		x	x	x	x		x
LGE 5539	x						
LGE 5540	x						
LGE 5541	x						
LGE 5546	x	x	x				
LGE 5547	x	x	x	x	x	x	x
LGE 5548	x						
LGE 5550	x						
LGE 5551	x						
LGE 5587A	x		x	x			
LGE 5587B	x	x		x	x	x	
LGE 5587C	x						
LGE 5587D	x						
LGE 5593A	x	x	x	x	x		x

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
LGE 5593B	x	x	x	x		x	x
IIBPH 2380	x	x		x	x	x	x
IIBPH 2381	x						
IIBPH 2384	x	x	x	x	x	x	
IIBPH 2394	x						
IIBPH 2400	x	x	x	x	x	x	
IIBPH 2401	x						
IIBPH 2402	x						
IIBPH 2403	x						
IIBPH 2404	x			x			
IIBPH 2405	x						
IIBPH 2406	x						
IIBPH 2408	x						
IIBPH 2409	x						
IIBPH 2410	x						
IIBPH 2412	x						
IIBPH 2368	x						
IIBPH 2382	x						
IIBPH 2854			x	x			x
IIBPH 2855	x	x	x	x	x	x	x
IIBPH 2858	x						
IIBPH 2903		x	x	x	x		
LGE 7416	x						
LGE 7417	x						
LGE 7418	x						
LGE 7419	x						
LGE 8121	x	x	x	x	x	x	x
LGE 8148	x		x	x	x	x	
LGE 8159	x	x	x	x		x	x
LGE 8164	x	x		x	x	x	
LGE 8165	x			x	x	x	x
LGE 8166	x						
LGE 8207	x		x		x	x	
LGE 8208			x	x	x	x	
LGE 8209	x						
LGE 8215	x		x		x	x	
LGE 8216	x		x	x	x	x	x
LGE 8217	x						
LGE 8224	x						
LGE 8225	x						
BB 1891	x	x		x	x	x	x
BB 1892	x			x	x	x	x
LLL 2	x		x	x			x
LGE 5854	x						

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
LGE 5858	x						
LGE 5856	x						
LGE 5857	x						
Ceratoprhys aurita							
CFBH 2319	KP295685	x	x	x	x	x	x

CAPITULO 2

Diversification of the Chacoan endemic frog genus *Lepidobatrachus* (Anura, Ceratophryidae): the role of marine introgression, old and stable continental formations, climatic barriers, and allochthonous rivers

ABSTRACT

Some Miocene events are suggested as main diversification drivers for the Chacoan endemic frog genus *Lepidobatrachus*. However, mechanisms that have influenced these species diversification are still unclear, especially concerning the effects of Miocene marine introgressions. To address this question, and others closely related, we examined mitochondrial and nuclear DNA sequences of the three *Lepidobarachus* species through almost all geographic distribution. Our results support an important role of highs and cratons as refuges during marine introgressions. We also confirmed the presence of a climatic barrier to dispersal on central Chaco distribution, as previously suggested in the literature. Additionally, we found differential effects of main Chacoan rivers on *Lepidobatrachus* species, related to persistence time of populations. Longer persistence of *L. llanensis* resulted on a genetic pattern shaped by dynamics of these allochthonous rivers.

1. INTRODUCTION

The Chaco biome comprises almost one million km^2 (BUCHER, 1982), it is characterized by xerophytic vegetation, in a mosaic of grassland, savannas, open woodlands, and xeric thorn forests (WILLIG *et al*., 2000). The Chaco is part of the Dry Diagonal, together with Cerrado and Caatinga biomes (VANZOLINI, 1963). Due to plant composition and climatic characteristics, the Chaco was recently considered as part of the seasonally dry tropical forests (SDTFs) (NEVES, *et al*., 2015).

Miocene marine introgressions are suggested as probable diversification drivers for vertebrates in southern South America (CANDELA *et al*., 2012; MORANDO *et al*., 2014). At least three extensive marine introgressions were recorded for this region (OTTONE, *et al*., 2013), and the most important in extension, known as Paranense Sea, occurred between 15 and 13 Million years ago (Ma), covering almost all the current Chaco distribution (HERNANDEZ, *et al*., 2005; CANDELA *et al*., 2012; OTTONE, *et al*., 2013). This internal sea covered a massive land surface on southern South America, flooding all lowlands comprised between old highs and cratons (HERNANDEZ *et al*., 2005).

Recently, Brusquetti *et al*. (see Chapter 1) suggested the middle Miocene marine introgressions as one of the main diversification drivers of the endemic Chacoan frog genus *Lepidobatrachus*. According to their results, 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. If marine introgressions have isolated *Lepidobatrachus* populations in areas protected from flooding we should be able to find genetic signatures of these events. These signatures should help us to identify stable areas where populations have a longer persistence, as well the direction of posterior expansions, when habitats became suitable again. We expect higher genetic diversity at stable areas and genetic signatures of expansion at unstable or recently colonized areas (HEWITT, 1996).

Other historical event cited by Brusquetti *et al*. (see Chapter 1) as a putative promoter of *Lepidobatrachus* diversification is the late Miocene climatic change,

which corresponds to grassland habitats expansion (CERLING *et al*., 1997; BEERLING & OSBORNE, 2006; OSBORNE, 2008). Brusquetti *et al*. (see Chapter 1) hypothesized that late Miocene grassland expansion could have fragmented forested areas, and hence, isolated populations of *L. asper* + *L. laevis* ancestor (associated with woodland formations). This condition might have favored the speciation opportunities by geographical isolation. However, our sampling of *L. asper* is too restricted (see FAIVOVICH, 1994 and BRUSQUETTI *et al*., Chapter 1) to go deep on this two species divergence mechanism.

The Chaco is an extensive sedimentary alluvial plain (PENNINGTON *et al*., 2000), historically considered as a continuum, without geographical barriers to organism dispersion (BUCHER, 1982). Nevertheless, Brusquetti *et al*. (see Chapter 1) found an extensive distribution gap on *L. llanensis* that corresponds to central Santiago del Estero province, Argentina. This gap also matches with the distribution limits of other Chacoan species and corresponds to a high temperatures and soil salinity area known as "South American heat pole" (PROHASKA, 1959). This extreme area may act as climatic barrier for *L. llanensis* populations of the north and south of the gap. Brusquetti *et al*. (see Chapter 1) also found some evidence that suggest an influence of main Chacoan rivers on the genetic structure of *L. llanensis*.

To better understand the diversification process of this group of amphibians we studied genetic structure and diversity, intra-specific divergence, and demographic history based on mitochondrial and nuclear markers. We used haplotype genealogies, a multi-locus distance based network, and allele frequencies to assess the genetic structure within the species and the rivers influence. In order to identify areas where each lineage possibly has been isolated and the direction of the posterior expansions we used genetic diversity indices and tested for demographic expansions. In the case of the putative climatic barrier represented by the Santiago del Estero gap we used an Isolation-with-Migration model to estimate divergence time between *L. llanensis* populations of north and south of the gap, gene flow after split and its direction, and population size.

2. MATERIAL AND METHODS

2.1 Sampling

In order to cover the genus *Lepidobatrachus* and thereby the Chaco distribution, we included 16 samples of *L. asper* from four localities, 79 samples of *L. laevis* from 29 localities, and 78 samples of *L. llanensis* from 26 localities (Fig. 1; Appendix 1). Vouchers are housed at the Herpetological Collection of the Instituto de Investigación Biológica del Paraguay (IIBPH), Asunción, Paraguay; Laboratorio de Ecología y Evolución (LGE, DB, JNL, and LL), Posadas, Argentina; Museo Argentino de Ciencias Naturales (BB), Buenos Aires, Argentina; and in Coleção Zoológica de Referência da Universidade Federal de Mato Grosso do Sul (ZUFMS), Corumbá, Brazil.

2.2 Laboratory procedures and molecular methods

We extracted total genomic DNA from muscle or liver samples conserved in 95–100% ethanol using the DNeasy extraction kit (Qiagen, Valencia, CA, USA). We used polymerase chain reaction (PCR) to amplify one mitochondrial fragment and six nuclear introns using specific primers (Table 1) and a commercial kit (Master Mix, Fermentas). To amplify the mitochondrial fragment we used an initial denaturation step of 3 min at 94 °C, followed by 10 cycles (15 s of denaturation at 95 °C, 20 s of annealing at 45 °C, and 50 s of extension at 60 °C), followed by 26 cycles (15 s of denaturation at 95 °C, 20 s of annealing at 50 °C, and 50 s of extension at 60 °C), and a final extension of 5 min at 60 °C. To amplify nuclear fragments 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). Purified PCR products (ExoSAP, Fermentas) were sent to Macrogen Inc. (Seoul, Koreia) for sequencing.

Table 1: Primers and annealing temperature (°C) used in amplification of each locus.

Locus ID (length on base pair number)	Primer sequence 5'- 3'	Annealing	Reference
CO1 (637 bp)	ANF1 ACHAAYCAYAAAGAYATYGG	45/50	**Jungfer** *et al.*, 2013
Cytochrome c oxidase subunit 1	ANR1 CCGGTCTGAACTCAGATCACGT		**Jungfer** *et al.*, 2013
MVZ 15-16 (299 bp)	MVZ15 ACACCCACTCCTCTATCTTTGATG	54.7	**Bell** *et al.*, 2011
Glyceraldehyde-3 Phosphate Dehydrogenase (intron 4)	MVZ16 AAATGTAAGCTAAGAGATCCACAAC		**Bell** *et al.*, 2011
MVZ 27-28 (356 bp)	MVZ27 ATTATTCCGTAACAGCAAACTC	54.7	**Bell** *et al.*, 2011
Lactose Dehydrogenase Chain Beta (Intron 3)	MVZ28 GTAACCATGGCAACTGGTAG		**Bell** *et al.*, 2011
MVZ 29-30 (221 bp)	MVZ29 ATCCTCCATACTACTTAAGGAGACC	57	**Bell** *et al.*, 2011
Y Box Binding (Intron 1)	MVZ30 CTGAAGCCTCTGTACATGTTTTG		**Bell** *et al.*, 2011
MVZ 39-40 (188 bp)	MVZ39 GGATCTGCTAGAGACCTGTCACTTC	57	**Bell** *et al.*, 2011
X. laevis MGC82783 protein (Intron 2)	MVZ40 ACAGAGTCTTCAAACCCAGCAATAC		**Bell** *et al.*, 2011
MVZ 47-48 (349 bp)	MVZ47 AGTGAAAGATACAGTCACAGTGCTAGG	54.7/56.7/59	**Bell** *et al.*, 2011
X. laevis Fibrinogen, A alpha polypeptide (Intron 1)	MVZ48 GGAGGATATCAGCACAGTCTAAAAAG		**Bell** *et al.*, 2011
RPL3 (418 bp)	RPL35F AAGAAGTCYCACCTCATGGAGAT	50/53/64.3	**Pinho** *et al.*, 2009
Ribosomal Protein L3 (Intron 5)	RPL36RA AGTTTCTTTGTGTGCCAACGGCTAG		**Pinho** *et al.*, 2009

To check chromatograms and edit sequences we used CodonCode Aligner v. 3.5.4 (Codon Code Corporation). We aligned each fragment separately with MUSCLE (EDGAR, 2004) on MEGA 6 (TAMURA *et al*., 2013). To verify absence of recombination we used PhiTest implemented in Splitstree v4.2 (HUSON &

BRYANT, 2006); and GARD (Genetic Algorithm Recombination Detection) (KOSAKOVSKY POND *et al*., 2006), MAXCHI (SMITH, 1992; POSADA & CRANDALL, 2001), and CHIMAERA (POSADA & CRANDALL, 2001) implemented in the program RDP3 (HEATH *et al*., 2006). We separated sequences of individuals with heterozygous indels with CodonCode Aligner v. 3.5.4 (Codon Code Corporation), and to resolve haplotypes of heterozygous individuals, we used Phase 2.1 (STEPHENS *et al*., 2001) implemented in DnaSP 5.10 (LIBRADO & ROZAS, 2009), discarding unresolved ones (< 0.90 of posterior probability). We generated unique haplotypes datasets in DnaSP 5.1.

2.3 Network haplotype genealogies and genetic diversity

We generated haplotype genealogies for each locus using Haploviewer (SALZBURGER *et al.*, 2011). Haploviewer turns phylogenetic trees into haplotype genealogies. We used DNAPARS available in PHYLIP v.3.695 package (FELSENSTEIN, 2005) to generate a Maximum Parsimony tree with "Rearrange on one best tree" as search method, which treat indels as fifth state and infers branch lengths. To visualize the genetic structure within species and the influence of putative dispersal barriers on genetic structure we generated each genealogy three times, grouping the haplotypes 1) by species and 2) by populations separated by rivers; and to assess the influence of the gap on *L. llanensis* genetic structure 3) by populations of north and south of the gap of Santiago del Estero. We also assessed genetic diversity within mitochondrial haplogroups. We estimated haplotype diversity (*Hd*) and per site nucleotide diversity (*Pi*) in DnaSP software.

2.4 Multilocus genetic distance network and Bayesian population assignment analyses

We used a multi-locus distance based network to visually represent divergence patterns within *Lepidobatrachus* individuals. We generated genetic distance matrix for each locus in MEGA6 (TAMURA *et al*., 2013) with *p*-distances between individuals, including transitions and transversions, uniform rates, and pairwise deletion. We combined individual locus matrices into one multi-locus distance matrix (same weight across loci) with POFAD v. 1.03 (JOLY & BRUNEAU 2006) and

constructed a genetic network in SPLITSTREE v. 4.6 (HUSON & BRYANT, 2006) using the NeighborNet algorithm.

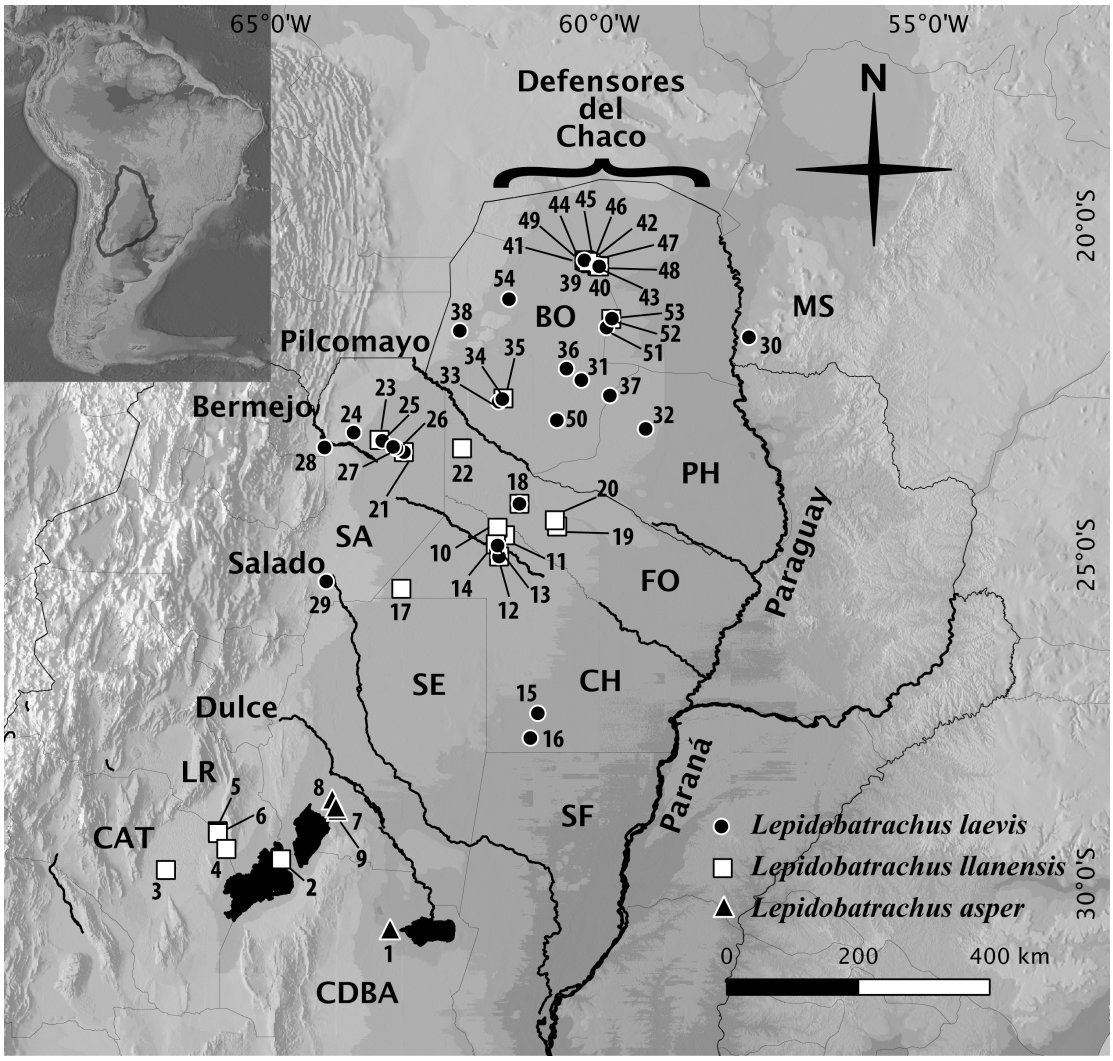

Figure 1: Sampling localities of *Lepidobatrachus* species. Area enclosed in black in South America map denotes the limits of Chaco biome follow Morrone (2001). Putative dispersal barriers discussed in the text are highlighted with their respective names. Detailed locality information represented by numbers is shown in Appendix 1. Department/province/state abbreviations: CAT, Catamarca; CDBA, Córdoba; CH, Chaco; FO, Formosa; LR, La Rioja; SA, Salta; SF, Santa Fé; SE, Santiago del Estero (Argentina); MS, Mato Grosso do Sul (Brazil); BO, Boquerón; PH, Presidente Hayes (Paraguay).

To assess genetic assignment of individuals to genetic clusters we used a model-based clustering method implement in STRUCTURE 2.3.4 (PRITCHARD *et al*., 2000). Using multi-locus genotypic data, STRUCTURE divides individuals into a number of genetic clusters (K) (irrespectively 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). In

order to assess the species boundaries and identify genetic admixture between them, we assigned a-priori population to individuals following the three *Lepidobatrachus* species. In addition, we assigned an a-priori population to individuals following species and rivers to assess the rivers influence on genetic structure of each species. For STRUCTURE analysis, we included only nuclear loci and samples with sequences for at least four loci (see Appendix 2). We used the program xmfa2struct (available at: http:// www.xavierdidelot.xtreemhost.com/clonalframe.htm) to convert sequences to STRUCTURE input; this program encodes each variable site as an allele. In both, species and rivers analyses, we performed ten independent runs for each K with admixture model and independent alleles frequencies inferring lambda. We performed for each K, ranging from 1 and 15, $5x10^5$ iterations as burn-in and $5x10^5$ additional iterations. Most likely K was based on the method of Evanno *et al*. (2005) 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 & ROSENBERG, 2007) and visualized with DISTRUCT v.1.1 (ROSENBERG, 2004).

2.5 Isolation-with-migration model

To test the Santiago del Estero climatic barrier we used an isolation-with-migration model (HEY & NIELSEN, 2004) implemented in IMa2 (HEY, 2010). We estimated divergence time and gene flow after split and its direction, between *L. llanensis* populations of north and south of the gap. Also, we estimated population size of ancestral and current populations. Analyses were made using *L. llanensis* S and *L. llanensis* N4 as sampling populations because of their sister relationship (see BRUSQUETTI *et al*., Chapter 1; Fig. 2). Following IMa2 authors, we first tested HKY model (HASEGAWA *et al*., 1985) for mitochondrial data and 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 mutation rate for CO1, estimated by (FREILICH *et al*., 2014) as 0.78% per lineage per million years 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 population mutation rate (theta) estimated in DnaSP v5.10 following author's recommendations. After pilot runs adjustment we used uniform distribution for population size and divergence time priors with upper bounds set as -q4.27 -m2.34 -t1.70. We ran in M mode (MCMC)

three times with different seed numbers with 20 Markov chains with a geometrical heating mode (-hfg -ha0.96 -hb 0.9). Each simulation corresponds to $10x10^5$ 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 & QUINZIO, 2008; also see FAIVOVICH *et al*., 2014). MCMC mixing was assessed through Effective Sample Sizes (ESSs) and trend line plots that both denote levels of samples autocorrelation, and swapping rates between chains over the run.

2.6 Demographic history and time of *Lepidobatrachus llanensis* populations of north and south of Santiago del Estero gap (based on mtDNA)

We tested for demographic expansions on *L. llanensis* populations of north and south of Santiago del Estero gap by mismatch distribution analyses (MDA) (ROGERS & HARPENDING, 1992), Tajima's *D* (TAJIMA, 1989), Fu's *Fs* (FU, 1997) and Ramos-Onsins and Rozas's *R2* tests (RAMOS-ONSINS & ROZAS, 2002). We used Harpending's raggedness statistic (*r*) (Harpending, 1994) to test the goodness of fit between expected and observed distribution on MDA. Significance levels of *Fs*, *R2*, and *r* were estimated with 10,000 coalescent simulation replicates. Statistics and significance analyses to test demographic expansions were made in DnaSP v5.10.

We used Bayesian Skyline plot (BSP) implemented in Beast 1.8.0 (DRUMMOND & RAMBAUT, 2007) to estimate changes in effective population size through time and each population relative age based on the time of the most recent common ancestor (tMRCA). For BSP analyses we estimated evolutionary models that best fit each mitochondrial haplogroup with JModeltest 0.1.1 (POSADA, 2008). For all analyzed populations we used 0.0078 as substitution rate based on the 0.78% per lineage per million years mutation rate estimated by Freilich (2014). Run settings were different for each population in order to obtain convergence. For populations south of the gap (S) we ran 400 millions of Markov chain Monte Carlo simulations sampled every 40000 chains. For populations north of the gap N1+N2+N3 and N4, we ran 100 millions sampled every 10000 and 800 millions sampled every 80000, respectively. Chain convergence was assessed in Tracer 1.5

(RAMBAUT & DRUMMOND, 2007) by effective sample size (ESS) examination (> 200).

3. RESULTS

3.1 Network haplotype genealogies and genetic diversity

The COI haplotype network (Fig. 2) showed three main haplogroups separated by ~30 mutations, which correspond to each *Lepidobatrachus* species. In *L. asper*, the two haplotypes from Santiago del Estero were separated by two mutations from the only haplotype from Córdoba. Within *L. llanensis*, the haplotypes from southern distribution (Córdoba, La Rioja, and Catamarca provinces) cluster together and correspond to few and frequent haplotypes, the most frequent among them clustered specimens from all southern localities sampled. Populations of north of Santiago del Estero gap showed high number of rare haplotypes, connected to each other by one or two mutation steps. An haplotype shared by a large number of specimens from Defensores del Chaco (Boquerón, Paraguay) was an exception of that pattern. Furthermore, this common haplotype together with two rare ones from the same geographical area, were separated from all other *L. llanensis* haplotypes by 10 mutation steps. In *L. laevis* we found four frequent haplotypes, each one surrounded by several rare haplotypes. Non-geographic overlap was evident between these frequent haplotypes; however, rare haplotypes do not follow any geographic pattern.

The only nuclear marker which does not separate in a clearly way the three *Lepidobatrachus* species is MVZ 15-16 (Fig. 3F). In this genealogy *L. asper* shows two haplotypes; one shared by the Córdoba specimens and the other shared by the Santiago del Estero specimens. *Lepidobatrachus asper* haplotypes showed more affinities with some *L. laevis* haplotypes from Boquerón. *Lepidobatrachus laevis* and *L. llanensis* showed similar patterns consisting in many rare haplotypes without any geographic pattern, and with haplotypes usually connected by one to three mutational steps, although with a few strongly divergent haplotypes, separated by more than 15 steps.

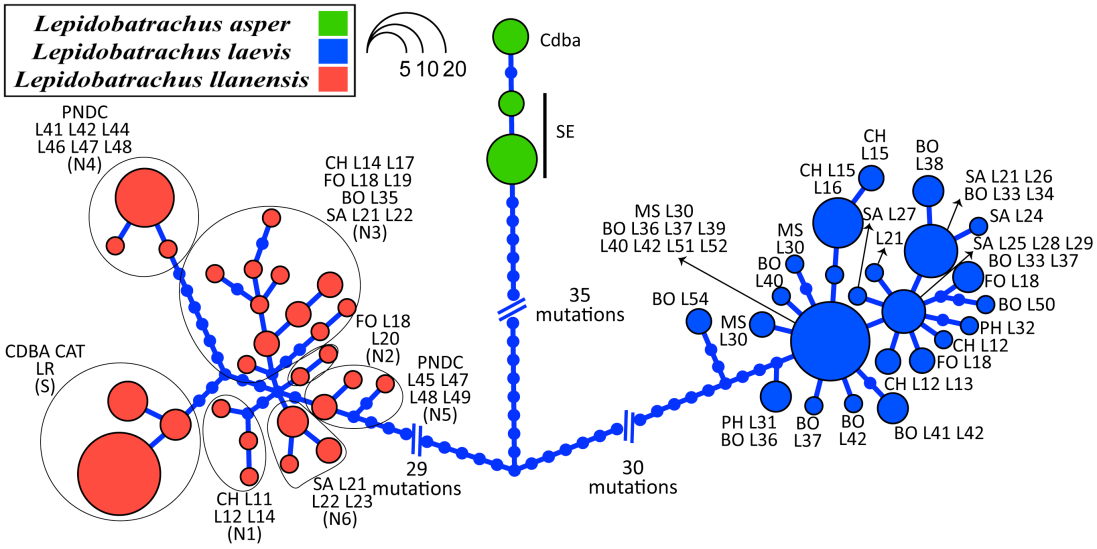

Figure 2: Mitochondrial haplotype network of *Lepidobatrachus* species. Small blue dots indicate unsampled mutations. Circle size corresponds to haplotype frequency. Provinces or departments and sampling localities (L) follow Appendix 1. *Lepidobatrachus llanensis* haplogroups in parenthesis: N1 to N6 correspond to populations of north Santiago del Estero gap; S to populations of south of the gap.

MVZ 27-28 and MVZ 29-30 genealogies (Figs 3E and 3A) showed shared haplotypes between specimens of *L. laevis* and *L. llanensis*. In MVZ 27-28 the two haplotypes were shared by specimens from the Argentinean provinces of Chaco, Salta, and Formosa, and from the Department of Boquerón in Paraguay. In MVZ 29-30, besides specimens from Chaco, Salta, Formosa, and Boquerón, specimens from Catamarca also shared one of the haplotypes. In MVZ 27-28 genealogy (Fig. 3E) common haplotypes are more frequent in the three species. *Lepidobatrachus asper* and *L. llanensis* are closer to each other, being connected by six mutational steps, whereas eight steps separate *L. asper* from *L. laevis*. *Lepidobatrachus asper* showed only two haplotypes, being one markedly more frequent and shared by specimens of all sampled localities; the other haplotype was detected in only one specimen from Córdoba. In *L. laevis* we found two haplogroups connected by four mutational steps. These two haplogroups shared haplotypes from several localities; some localities were exclusive to each one although with some geographical overlap. The most frequent haplotype of *L. llanensis* is shared by individuals from populations of south and north localities, as also happened in other less common haplotypes. However, rare haplotypes are exclusive to each group of populations. In MVZ 29-30 genealogy (Fig. 3A), *L. asper* and *L. laevis* showed large amount of mutational steps between

haplogroups and haplotypes, while two steps are the maximum observed within *L. llanensis*. Like in the other genealogies, in *L. asper*, Córdoba and Santiago del Estero populations do not share haplotypes, however in this genealogy the distance is larger, with 15 mutational steps between the two haplotypes. The pattern for *L. laevis* and *L. llanensis* was few frequent and many rare haplotypes and an uneven haplotype distribution.

In the MVZ 39-40 genealogy (Fig. 3B) *L. llanensis* and *L. laevis* showed high number of mutational steps between haplotypes, while in *L. asper* all haplotypes were connected by only one step. Furthermore, distances were larger at intra-specific than at inter-specific level. In *L. laevis* we found two haplogroups separated by 15 mutational steps but without any geographical pattern. In both haplogroups we found few frequent haplotypes surrounded by several rare haplotypes, all connected by a low number of mutational steps. In *L. llanensis* we found one haplogroup composed by one frequent haplotype surrounded by several rare haplotypes. The other haplotypes are organized in pairs, following a geographic distribution, and are separated by a relatively large number of mutational steps.

In MVZ 47-48 genealogy (Fig. 3D) we did not include any sample of *L. asper* because of sequencing problems. We did not find any evident geographical pattern for *L. laevis*. However, some organization is evident in northern species distribution with the northeastern and northwestern haplogroups separated by six mutational steps. Nevertheless, some geographical overlap is evident. Only one relatively more frequent haplotype was found in *L. llanensis*, which is shared by individuals from some of the north populations (Formosa, Salta, and Boquerón) and is surrounded by rare haplotypes from same localities although with one haplotype from Córdoba, southern species distribution. In both, *L. laevis* and *L. llanensis*, a pair of rare haplotypes are separated by several mutational steps.

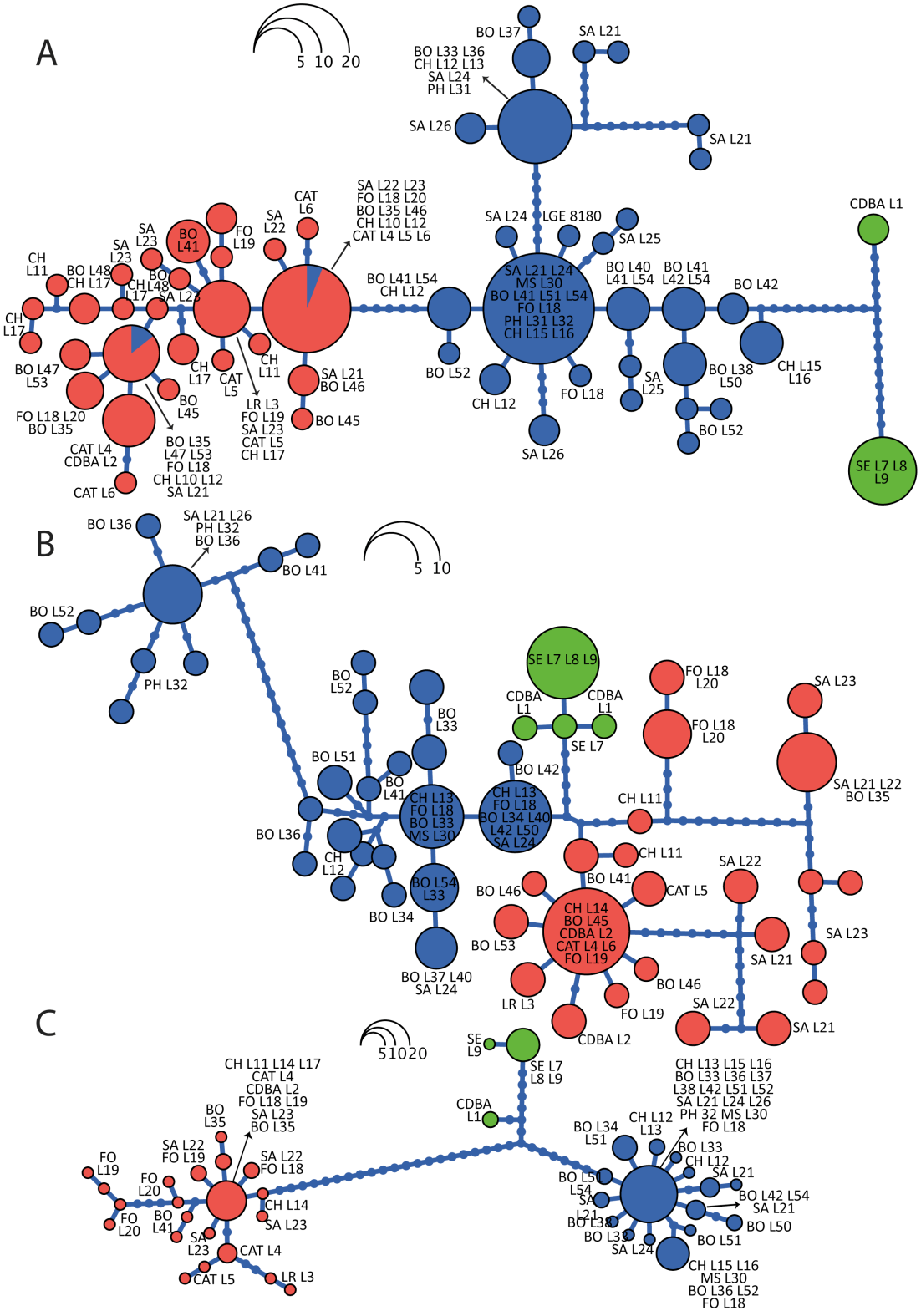

Figure 3: Network of nuclear haplotypes of *Lepidobatrachus* species. A) MVZ 29-30, B) MVZ 39-40, C) RPL3, D) MVZ 47-48, E) MVZ 27-28, and F) MVZ 15-16. Blue dots indicate unsampled mutations. Colors represent the three species: *L. asper* (green), *L. laevis* (blue), *L. llanensis* (red). Circle size corresponds to haplotype frequency. Provinces/departments and sampled localities (L) follow Appendix 1 and locus names Table 1.

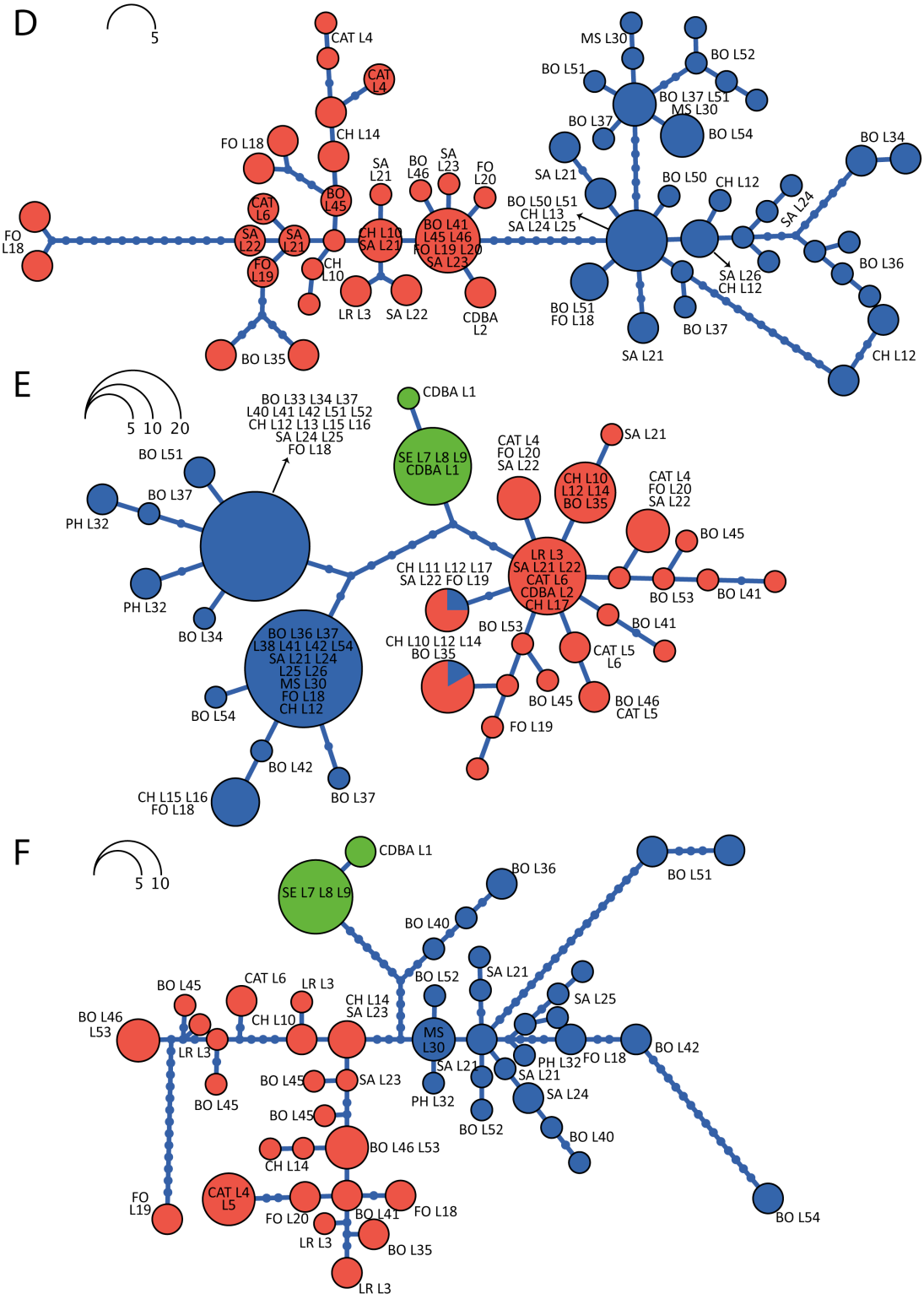

Figure 3 (continued).

In RPL3 genealogy (Fig. 3C) the three species are well separated and show more closely relationship between *L. asper* and *L. laevis*. In *L. asper* seven mutational steps separate populations from Santiago del Estero and Córdoba. All haplotypes of *L.*

laevis are basically grouped in only one haplogroup with a central and highly frequent haplotype. Specimens of almost all sampled localities from Paraguay, and from Argentinian provinces Salta and Formosa share this central haplotype. In *L. llanensis*, a similar pattern is observed with one central and highly frequent haplotype surrounded by several rare haplotypes, all connected by a low number of mutational steps.

Within *L. llanensis*, populations from north of Santiago del Estero gap (Boquerón, Salta, Formosa, and Chaco) and populations from south of the gap (Catarmarca, La Rioja, and Córdoba) show exclusive haplotypes in genealogies of CO1, MVZ 47-48, and MVZ 15-16 (Figs. 4A, 4F, and 4G). However, only in CO1 genealogy the haplotypes distribution is concordant with the geographic distribution. Shared haplotypes between north and south populations were the most common pattern in the remaining genealogies (Figs. 4B, 4C, 4D, and 4E).

Among the mitochondrial genealogies of *L. laevis* and *L. llanensis*, genetic structure is a little more concordant with rivers in *L. llanensis*, which shows exclusive haplotypes for all group of populations separated by rivers (Fig. 5). In *L. laevis* only south Bermejo River populations showed exclusive haplotypes. In *L. laevis* nuclear genealogies (Fig. S1, Supporting information) only MVZ 15-16 showed exclusive haplotypes for all group of populations separated by rivers. No correspondence between rivers and populations is the pattern in all nuclear genealogy of *L. llanensis* (Fig. S2, Supporting information).

Mitochondrial haplotype diversity (*Hd*) was relatively low in *L. asper* and in *L. llanensis* S (southwestern distribution), in *L. laevis* and in the main haplogroups of *L. llanensis* of the north distribution, except *L. llanensis* N4, *Hd* is relatively high with values from 0.733 to 1. Nucleotide diversity (*Pi*) is relatively low in all main haplogroups with values from 0.00055 to 0.00644 (Table 2).

3.2 Multilocus genetic distance network and Bayesian population assignment analyses

The multilocus genetic distance network corresponds with most genealogies, showing the three species as three divergent groups. A little more affinity was evident between *L. asper* and *L. laevis*. No structure was found within species (Fig. S3, Supporting information).

In STRUCTURE analyses by species, K = 2 was the maximum ΔK but with K = 3 with close values and a peak in K = 6 (Fig. 6A). With K = 2 STRUCTURE identified a deme including *L. asper* + *L. laevis* and other with *L. llanensis* samples. With K = 3, STRUCTURE recovered the three species; however, some mixed between *L. laevis* and *L. asper* were found in two localities of Boquerón department, Paraguay (L36 and L40). In K = 4 a genetic break is revealed within *L. llanensis* in Formosa (L19) and Salta (L21 and L22), Argentina. In both, K = 5 and K = 6, some admixture appears around *L. laevis* and *L. llanensis* (Fig. 6B). In the STRUCTURE analyses by rivers, the maximum was K = 3 with K = 2 and K = 4 with close values (Fig. 7A). These analyses with populations corresponding with main Chacoan rivers, revealed a genetic break that match with Pilcomayo and Bermejo rivers on K = 4 (Fig. 7B).

Table 2: Genetic diversity of *Lepidobatrachus* main mitochondrial haplogroups. Columns correspond to: number of individuals (*N*), number of haplotypes (*H*), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*). Haplogroups names follow Figure 2.

	N	*H*	*Hd*	*Pi*
Lepidobatrachus asper	15	3	0.59	0.00222
Lepidobatrachus laevis	80	24	0.892	0.00424
Lepidobatrachus llanensis N1	3	3	1	0.00328
Lepidobatrachus llanensis N2	2	2	1	0.00164
Lepidobatrachus llanensis N3	14	12	0.978	0.00644
Lepidobatrachus llanensis N4	12	3	0.318	0.00055
Lepidobatrachus llanensis N5	4	3	0.833	0.00328
Lepidobatrachus llanensis N6	6	3	0.733	0.00142
Lepidobatrachus llanensis S	30	3	0.439	0.00114

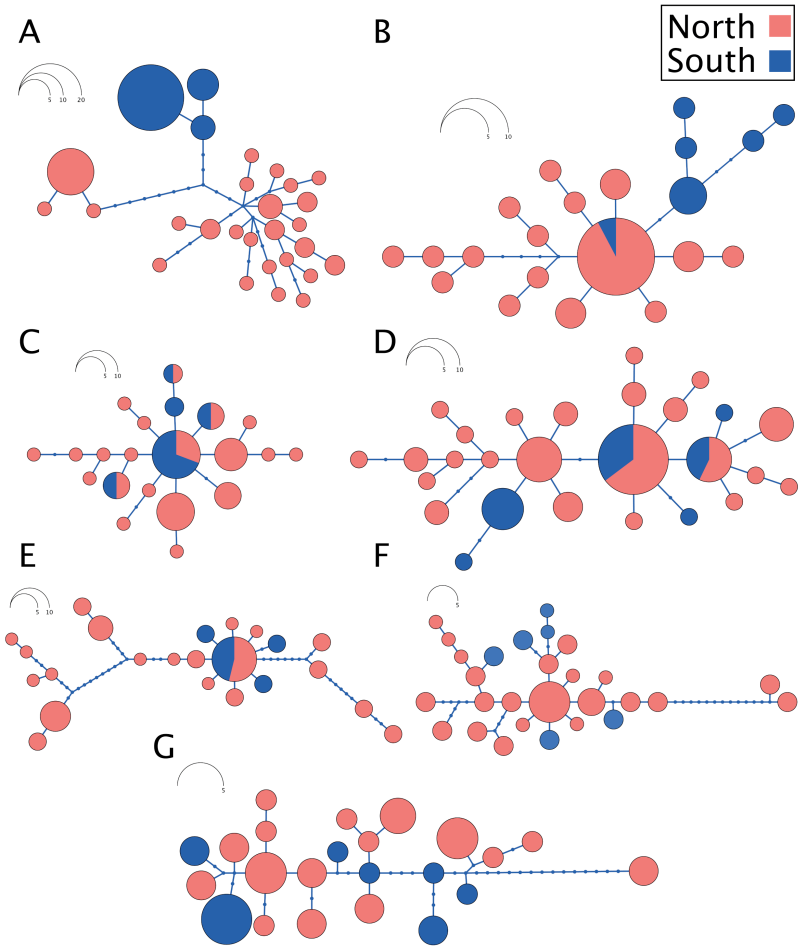

Figure 4: Haplotype network of *Lepidobatrachus llanensis*. A) CO1, B) RPL3, C) MVZ 27-28, D) MVZ 29-30, E) MVZ 39-40, F) MVZ 47-48, and G) MVZ 15-16. Small blue dots indicate unsampled mutations. Colors represent north (red) and south (blue) populations of the Santiago del Estero gap. Circle size corresponds to haplotype frequency. Locus names follow Table 1.

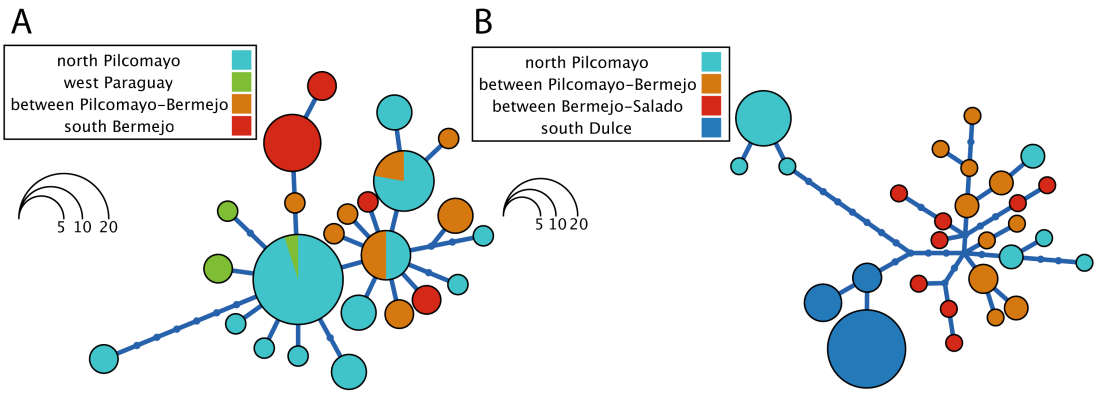

Figure 5: Mitochondrial haplotype network of A) *Lepidobatrachus laevis* and B) *Lepidobatrachus llanensis* species. Small blue dots indicate unsampled mutations. Colors represent populations separated by rivers. Circle size corresponds to frequency.

3.3 Time of divergence, population size, and gene flow between *L. llanensis* populations of south and north of Santiago del Estero gap

According to IMa2 analyses, lineages of north and south of *L. llanensis* have diverged about 0.89 Ma [95% highest posterior density interval (HPD) = 0.36–1.18 Ma] (Fig. 8). After divergence, no evidence of migration was detected in any direction between the two populations. Effective population size was not considered because HPD values for north populations did not reach low levels near either the upper or the lower limit of the prior.

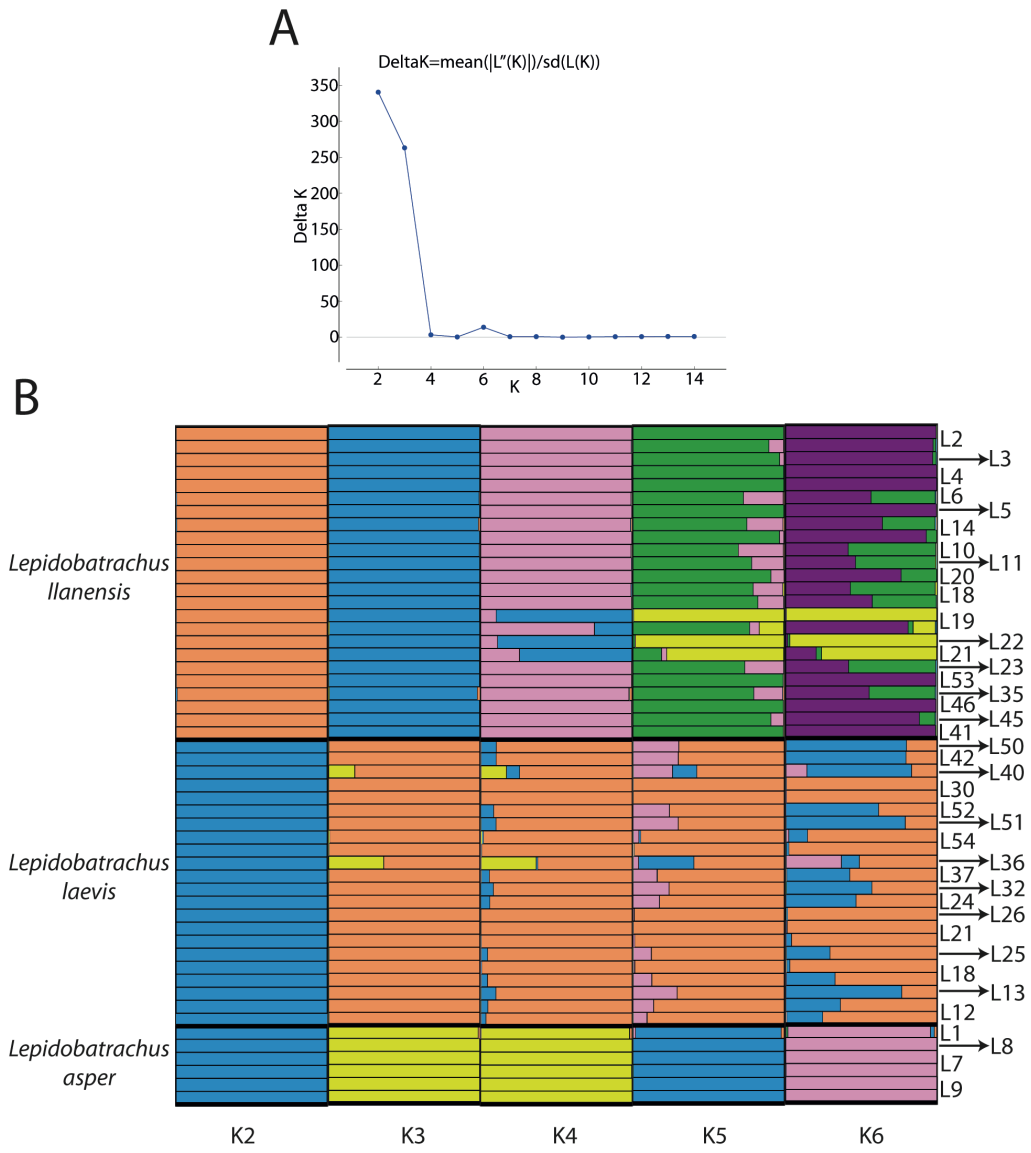

Figure 6: Bayesian clustering and specimen assignment to the three *Lepidobatrachus* species using STRUCTURE. A) Value of ΔK as a function of K = 1–15. B) Bars correspond to each specimen and their membership coefficient (q). Clusters are represented by color. Sampling sites are plotted by species (Appendix 1).

3.4 Demographic history and relative age of *Lepidobatrachus llanensis* populations of south and north of Santiago del Estero gap (based on mtDNA)

Although Mismatch Distribution Analyses (MDA) plots are not clearly unimodal (Fig. S4, Supporting information), except for *L. llanensis* N4, values of *r* were not significant for any clade (Table 3), which indicate a relatively good fit of the observed data to a model of recent population expansion.

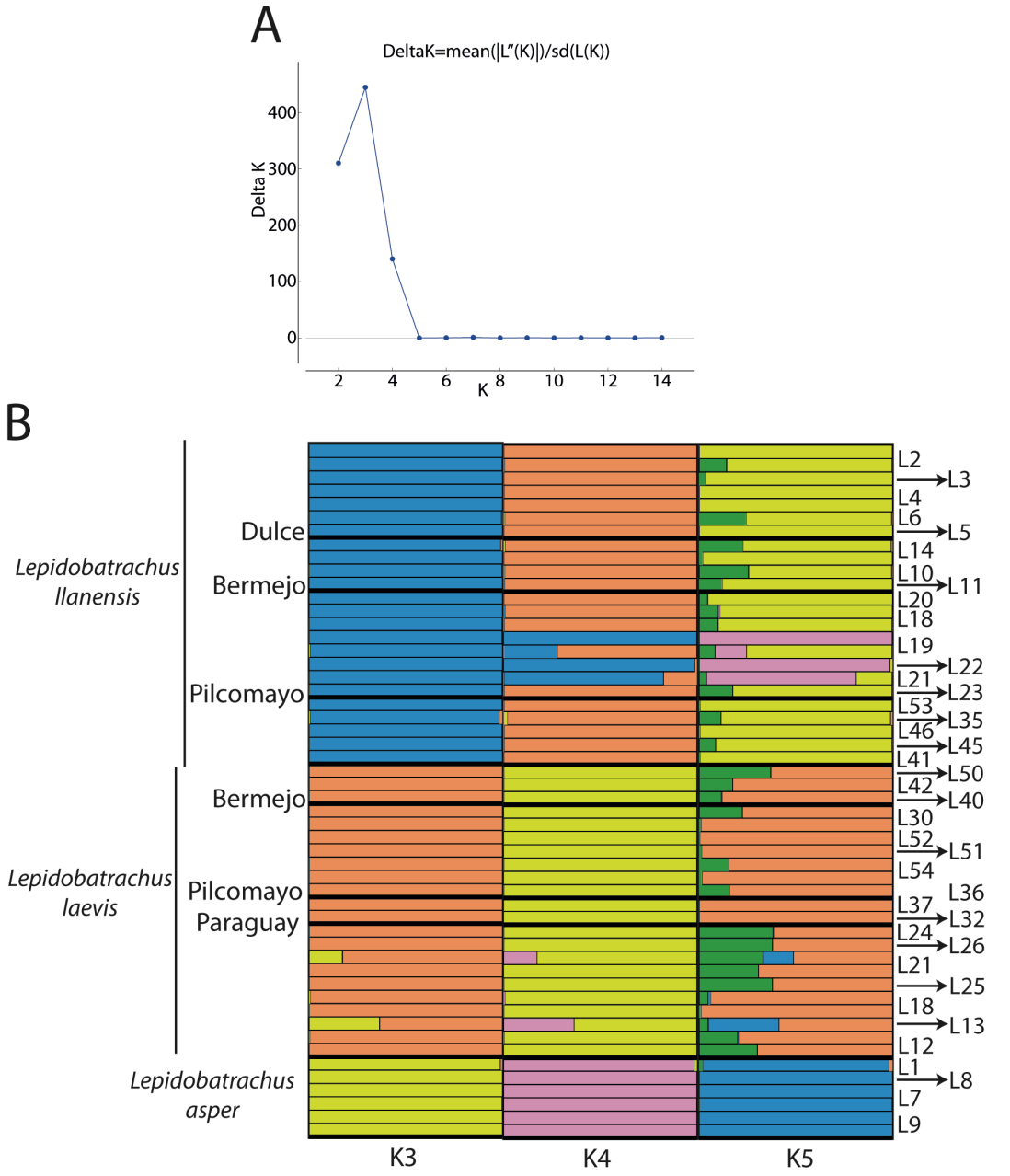

Figure 7: Bayesian clustering and specimen assignment to main Chacoan rivers using STRUCTURE. A) Value of ΔK as a function of K = 1–15. B) Bars correspond to each specimen and their membership coefficient (q). Clusters are represented by color. Sampling sites are plotted by species (Appendix 1).

According to neutrality tests, there is no statistical support to accept a recent history of population expansion for *L. asper*. In contrast, all tests supported a recent population expansion scenario for *L. laevis* (Table 3).

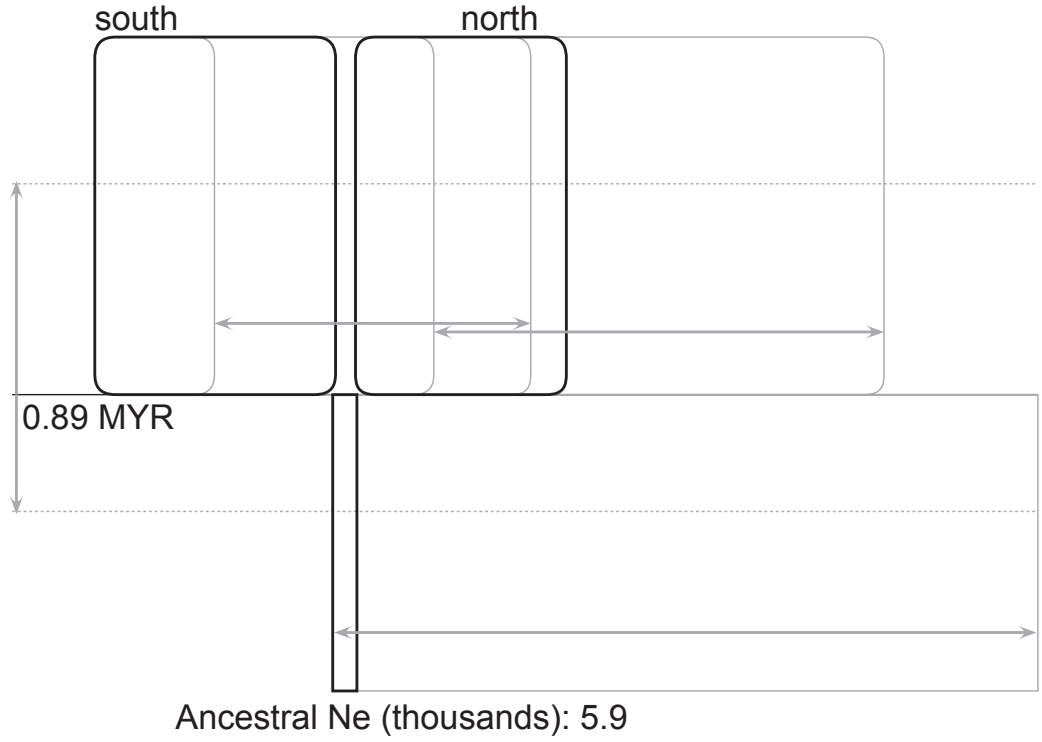

Figure 8: Divergence time and effective population size of *Lepidobatrachus llanensis* populations from south and north of Santiago del Estero gap using isolation-with-migration model. Vertical double-headed arrow corresponds to 95% HPD interval for splitting time. Horizontal double-headed arrows correspond to 95% HPD interval for populations' size.

Within *L. llanensis*, a recent expansion scenario in *L. llanensis* N1+N2+N3 was supported by significant values of *Fs* and *R2* (Table 2). In *L. llanensis* N4 only *Fs* shows significant values (*Fs* = -1.4015, *p* = 0.03968). BSP analyses show mostly constant effective population sizes in the three analyzed populations (Fig. S5). TMRCA showed *L. llanensis* N1+N2+N3 as the older clade with a mean age of about 563,000 years and shows mean ages of 94,188 years for *L. llanensis* S and 65,347 years for *L. llanensis* N4 (Fig. S5).

Table 3: Neutrality tests (Tajima's *D*, Fu's *Fs*, *R2* test) and Harpending's raggedness (*r*) tests of goodness of MDA fit within main *Lepidobatrachus* haplogroups. Significant values ($p < 0.05$) in bold. Haplogroups names follow Figure 2.

Haplogroups	*D*	*Fs*	*R2*	*r*
L. asper	1.38553	1.6261	0.2254	0.2544
L. laevis	**-1.9314**	**-14.0165**	**0.0369**	0.049
L. llanensis N1+N2+N3	-1.14657	**-11.9538**	**0.0796**	0.0228
L. llanensis N4	-1.46801	**-1.4015**	0.1804	0.2516
L. llanensis N5	-0.78012	0.1335	0.3062	0.3056
L. llanensis S	0.75921	0.7369	0.173	0.2088

4. DISCUSSION

4.1 Genetic structure in *Lepidobatrachus* species

Two nuclear genealogies were congruent in showing shared haplotypes between *L. laevis* and *L. llanensis*, MVZ 27-28 and MVZ 29-30 (Figs. 3E and A). In MVZ 27-28 two haplotypes were shared, which was restricted to north distribution of *L. llanensis* were the two species are in contact (Chaco, Salta, Formosa, and Boquerón) (Fig. 3E). However, in MVZ 29-30, one of the shared haplotypes, besides the contact zone, specimens from part of southern populations of *L. llanensis* (Catamarca province) also shared the same haplotype (Fig. 3A). This genetic signs in contact zone (sympatry or inclusively syntopy) would be a strong evidence of secondary contact introgressive hybridization (FUNK & OMLAND, 2003; WEISROCK *et al*., 2005). However, it is well known that unlinked loci have their own history with coalescent events at different times (PAMILO & NEI, 1988), which, in closely related species, may cause retention of ancestral polymorphism. Furthermore, the geographic distance among individuals that share this haplotype (Catamarca province) supports the idea of ancestral polymorphism. In STRUCTURE analyses some of these localities show admixture but almost all starting from K5, which also shows admixture of almost all samples indicating no true geographic structure (Fig. 6B). Taking into account these evidences, it is more plausible to think on incomplete lineage sorting of ancestral polymorphism than in secondary contact introgressive hybridization; however, with current data we are not able to discern between these two processes conclusively.

On the other hand, STRUCTURE detected some admixture between *L. laevis* and *L. asper* in K = 3 in two localities of Boquerón, and a genetic break in K = 4 in *L. llanensis* at some localities from Formosa and Salta (Fig. 6B). Brusquetti *et al*. (see Chapter 1) show closer relationship between *L. asper* and *L. laevis* that may result in a retention of ancestral polymorphism. The geographic distance between samples of each species reinforces this idea, because introgressions are less expected in allopatry (TOEWS & BRELSFORD, 2012). The genetic break found in *L. llanensis* in Salta and Formosa may correspond to river influence. These samples correspond to localities between Pilcomayo and Bermejo rivers (Fig. 7B). Brusquetti *et al*. (see Chapter 1) noted some influence of these rivers on genetic structure of *L. llanensis*, which resulted in a complex haplotypes arrangement due to the historical dynamics of these allochtonous rivers (see discussion about river influence on genetic structure).

4.2 Refugia during marine introgressions

Brusquetti *et al*. (see Chapter 1) proposed a direct influence of marine introgressions on *Lepidobatrachus* diversification by populations isolation in areas protected from flooding. They also suggested that *L. llanensis* ancestor was the first to diverge. Currently, *L. llanensis* shows a disjunct distribution separated by an extensive gap in Santiago del Estero province, Argentina (Fig. 1). Low genetic diversity (*Hd* and *Pi*, Table 3) evidences a posterior colonization of southern distribution, which supports the isolation of *L. llanensis* ancestor on northern Chaco distribution, at areas protected by Michicola High (part of Boquerón and Salta, and Bolivian Chaco).

The sympatry between *L. laevis* and *L. llanensis* in northern Chaco (Fig. 1) distribution could have been resulted by expansions of *L. laevis* from the south, as proposed by Brusquetti *et al*. (see Chapter 1). Haplotype genealogies with a repetitive star-like pattern on northern distribution of the species (Figs. 2 and 3) supports recent demographic expansions, following a population bottleneck, due to recent colonization (SLATKIN & HUDSON, 1991). The neutrality tests, MDA, and low genetic diversity also support recent expansion events in *L. laevis* populations (Tables 2 and 3, Fig. S4, Supporting information), which reinforce the idea of a northern refugium absence for the ancestor of *L. asper* + *L. laevis*. Isolated populations of

Lepidobatrachus ancestor in the south and/or southwestern current Chaco distribution are plausible, because no evidence supports marine flooding at southeast La Rioja, central and south of Córdoba, and northwest San Luis (OTTONE *et al*., 2013). However, our data support a recent colonization of southwestern by *L. llanensis* from the north distribution and the poor sampling of *L. asper* precludes discerning between stable or unstable populations in the south.

Besides areas protected by Michicola High, other putative refugia are located in the east and southeast of the current Chaco distribution. The Paranense Sea covered a massive land surface in the south of South America, flooding all lowlands comprised between old highs and cratons; such as Asunción and Michicola highs, and Brazilian and Rio de la Plata cratons (Fig. 9). A fossil of a recently described species of *Lepidobatrachus* (NICOLI, 2015) was found at Farola Monte Hermoso, Buenos Aires province (TOMASSINI *et al.,* 2011). This locality corresponds to Rio de la Plata Craton. At Farola Monte Hermoso fossils related with some species currently restricted to Chaco wooded areas were recovered (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 current Chaco (TONNI, 1974; PASCUAL, 1984; PASCUAL *et al*., 1996).

Other putative refugium for Chacoan fauna during marine introgressions is the Asunción high. This formation protected a large land surface during the middle-Miocene marine introgressions. This high covers parts of southern Paraguay southward along Misiones, Corrientes, and Entre Ríos provinces (HERNANDEZ *et al*., 2005). Fossil records from Hermanderías y El Bretela deposit (Entre Ríos province) supports domination of Chacoan like xerophilous wood paleo-communities at least until the Pliocene (HINOJOSA & VILLAGRAN, 1997). Several authors suggested that climatic changes generated by the final uplift of the Andes and the influence of the Humboldt Current resulted on xeric vegetation expansions in great part of south South America during the middle-late Miocene and Pliocene (SOLBRIG *et al*., 1977; AXELROD, 1979; LANDRUM, 1981, ARROYO *et al*., 1995).

We suggest several plausible refugia during the middle Miocene marine introgressions. *Lepidobatrachus llanensis* ancestor was isolated in northern Chaco in

areas protected by Michicola High with relatively recent expansions to the south (see discussion about Santiago del Estero barrier for details between north and south populations contact). The absence of a northern refugium for the *L. asper* + *L. laevis* ancestor is suggested. However, refugia in southern and eastern current Chaco, in Rio de la Plata craton, and in Asunción high are equally plausible.

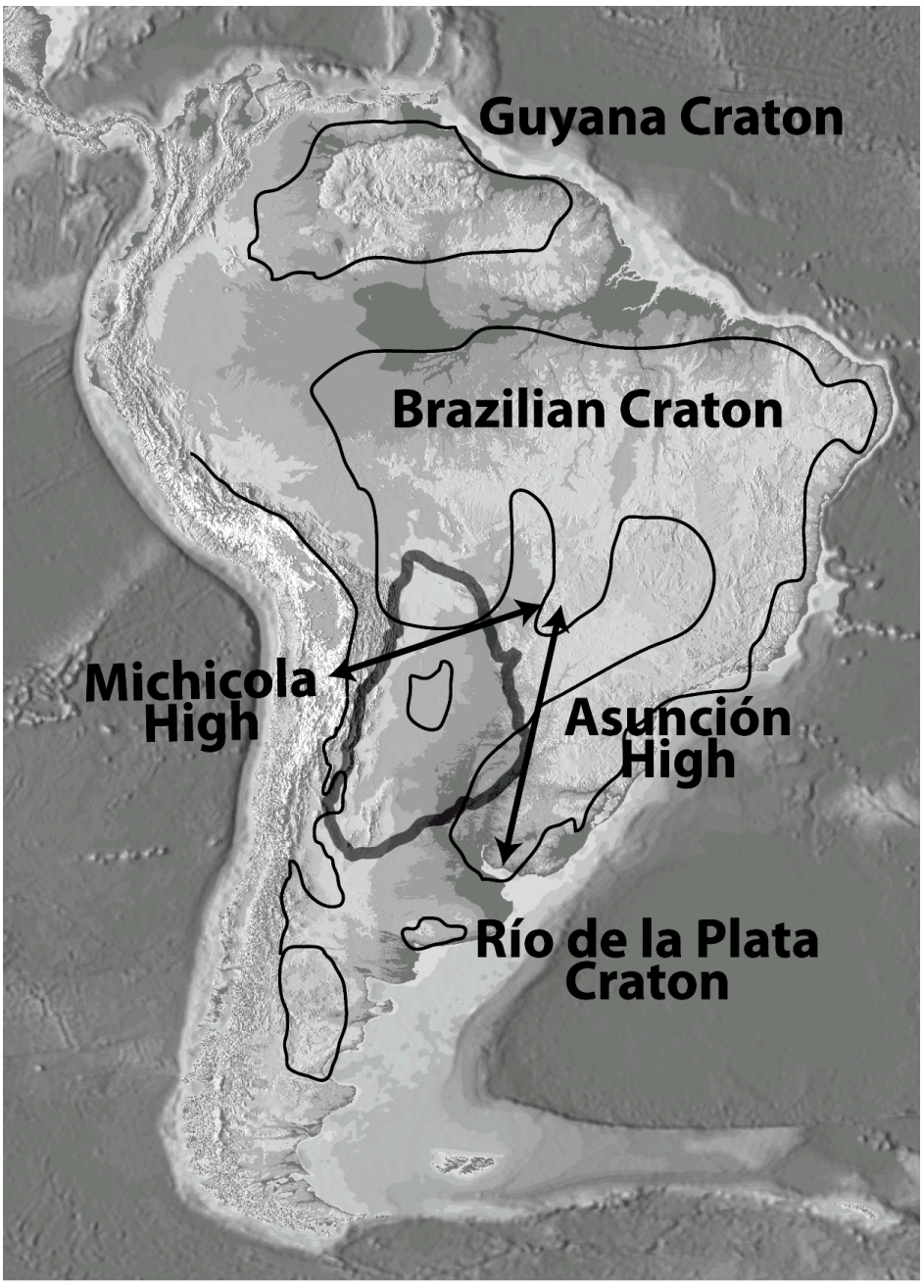

Figure 9: Approximate distribution of main highs and cratons in South America based on Hernandez *et al*. (2005). Double-headed arrows represent Michicola and Asunción highs distributions. Area enclosed in thick black denotes the limits of Chaco biome.

4.3 The influence of Santiago del Estero climatic barrier and main Chacoan rivers on genetic structure

As referred above, southwestern populations of *L. llanensis* were probably resulted from recent expansion of populations from the northern distribution of the species. Analyses under isolation-with-migration model (Fig. 8) fail to detect signs of migration in any direction after splitting, which was estimated in 0.89 Ma [95% highest posterior density interval (HPD) = 0.36–1.18 Ma]. Divergence time estimated with IMa2 matches with estimation of Brusquetti *et al*. (see Chapter 1). As expressed by these authors, Santiago del Estero gap corresponds to an area of high temperatures (PROHASKA, 1959), low precipitation regime (BOLETTA *et al*., 1989), and high salinity (RUIBAL, 1962), that may act as a dispersal barrier for some organisms. Lack of migration between populations of *L. llanensis* from north and south of the gap is concordant with the barrier hypothesis.

We identified a complex relationship among mitochondrial haplogroups, which were also evident in the mitochondrial tree inference of Brusquetti *et al*. (see Chapter 1). Defensores del Chaco (Boquerón) individuals were grouped into two haplogroups, N4 and N5, with N4 closely related to S haplogroup (southern distribution; Catamarca, La Rioja, and Córdoba). Haplogroup N4 also shows the lowest genetic diversity within *L. llanensis* haplogroups. Furthermore, based on tMRCA, N4 is the youngest haplogroup with mean tMRCA of 65,347 years (11,832–126,000 years 95% HPD confidence interval), compared to S haplogroup with mean tMRCA of 94,188 years (2,074–248,000 years 95% HPD confidence interval), and N1+N2+N3 with mean tMRCA of 563,200 years (202,200–1,014,400 years 95% HPD confidence interval) (Fig. S5). This pattern suggests at least two colonization events; first, from north to south and second, from south back to north. In contrast to mitochondrial marker, in nuclear genealogies all Defensores del Chaco haplotypes were mixed without any pattern (Fig. 3). This discordance between mitochondrial and nuclear markers is expected due to shallow history of isolation and by the differences on mutation rates and effective population sizes (PETIT & EXCOFFIER, 2009).

We found that genetic structure is more related to rivers in *L. llanensis* than in *L. laevis*. *Lepidobatrachus llanensis* CO1 genealogy shows exclusive haplotypes for

all between-rivers populations (Fig. 5B). South-Dulce populations were clearly separated; however, a long geographic distance (about 400 km) and the Santiago del Estero gap are between these populations and the remaining *L. llanensis*. North-Pilcomayo haplotypes constitute two haplogroups. One of this haplogroups, with haplotypes from almost all north-Pilcomayo localities, was separated by several mutational steps (Fig. 5B). Remaining north-Pilcomayo haplotypes and those from both sides of Bermejo River show mixed relationships. *Lepidobatrachus laevis* CO1 genealogy shows exclusive haplotypes only for south-Bermejo populations and shared haplotypes between individuals from both sides of Paraguay and Pilcomayo rivers (Fig. 5A). In *L. laevis*, genetic evidence suggest a recent colonization at the influence area of Pilcomayo and Bermejo rivers and, therefore, a shorter evolutionary history than *L. llanensis*, with older populations and longer persistence in this region of the Chaco. The lack of genetic structure of *L. laevis* in the Pilcomayo and Bermejo area of influence is possibly related with late Quaternary history of these rivers. In this Period the climatic changes resulted in interspersed times of dry and humid periods in the Chaco (IRIONDO, 1993). During the dry periods, Pilcomayo and Bermejo, like other Chacoan allochthonous rivers, were ephemeral and with highly seasonal channels. Two dry periods between the last glacial maximum (21,000 years ago) and the late Holocene were identified (IRIONDO, 1993). However, while *L. laevis* suffered recent expansions from southern regions due to lack of dispersal barriers, Pilcomayo and Bermejo rivers have been modeling *L. llanensis* populations long time ago.

The dynamic 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, resulted on 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, also can vary in size, density and location (HEY & MACHADO, 2003).

Our data support the hypothesis of recent colonization of east bank of Paraguay River by *L. laevis* as suggested by Brusquetti *et al*. (see Chapter 1). Although this river is the distribution limit for some small mammals, it is probably

not acting as an impassable barrier, but different soil structures and compositions determined different habitats at each side of the river (MYERS, 1982). However, the southwestern region of Mato Grosso do Sul, the only known record of *L. laevis* east side of the river (see SUGAI *et al*., 2013 as *L. asper*), is also considered part of the Chaco (see SOUZA *et al*., 2010).

As referred by Brusquetti *et al*. (see Chapter 1), an abrupt water level decrease was identified in the Paraguay River between the years 1960 and 1972 (COLLISCHONN *et al*., 2001). This water level decrease may have permitted the dispersion of *L. laevis* to the other side of the river. However, we do not discard vegetation rafts as responsible for the Paraguay River east bank populations. Two *L. laevis* records from riverine localities (west bank) were known in Paraguay (FAIVOVICH, 1994), one of them just in the opposite bank of the only known record of the east bank. We suggest that heavy rains may carry some individuals (tadpoles or adults) to the river, via small temporary streams, and some individuals are capable to cross 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).

5. CONCLUSIONS

Our data reinforce the importance of the middle Miocene marine introgression as one of the main diversification drivers for *Lepidobatrachus*. This internal sea possibly worked as a vicariant agent for a widely distributed *Lepidobatrachus* ancestor, which was isolated in areas protected from flooding by old highs and cratons. These highs and cratons were probably suitable refugia during marine introgressions. *Lepidobatrachus llanensis* ancestor became isolated in the north of Chaco distribution in some areas protected by Michicola High, while *L. asper* + *L. laevis* ancestor stayed in the south or east in areas protected by Asunción High or Rio de la Plata Craton. We do not detect migration between *L. llanensis* populations of north and south of Santiago del Estero gap after divergence (of about 0.9 Ma). Lack of migration between northern and southern *L. llanensis* populations supports the hypothesis of Santiago del Estero climatic barrier. The recent colonization of *L. laevis* on the influence area of the Pilcomayo and Bermejo rivers is reflected on the lack of

genetic structure. Longer persistence of *L. llanensis* in this area resulted on a complex genetic pattern shaped by recurrent connections and disconnections between populations of both sides of these rivers.

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Appendix 1: Voucher information. Abbreviations: National Route (NR), Provincial Route (PR), Parque Nacional (PN).

Samples ID	Country	Province/Department	Locality	Lat	Long	locality code	mtDNA haplogroup
Lepidobatrachus asper							
LGE 101	Argentina	Santiago del Estero	Saladillo River	-28.88	-63.98	L9	*L. asper*
LGE 102	Argentina	Santiago del Estero	Saladillo River	-28.88	-63.98	L9	*L. asper*
LGE 103	Argentina	Santiago del Estero	Saladillo River	-28.88	-63.98	L9	*L. asper*
LGE 104	Argentina	Santiago del Estero	Saladillo River	-28.88	-63.98	L9	*L. asper*
LGE 329	Argentina	Santiago del Estero	NR 9, km 1028	-28.71	-64.06	L8	*L. asper*
LGE 1347	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64.00	L7	*L. asper*
LGE 1348	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64.00	L7	*L. asper*
LGE 1349	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64.00	L7	*L. asper*
LGE 1350	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64.00	L7	*L. asper*
LGE 1351	Argentina	Santiago del Estero	NR 9, km 1013	-28.83	-64.00	L7	*L. asper*
LGE 4961	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	*L. asper*
LGE 4962	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	*L. asper*
JNL 253	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	*L. asper*
JNL 254	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	*L. asper*
JNL 255	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	*L. asper*
JNL 257	Argentina	Córdoba	8 km N from Las Saladas	-30.68	-63.18	L1	*L. asper*
Lepidobatrachus laevis							
DB 4933	Argentina	Salta	21 km SE from NR 34	-23.14	-63.73	L24	*L. laevis*
DB 4943	Argentina	Salta	21 km SE from NR 34	-23.14	-63.73	L24	*L. laevis*
DB 4942	Argentina	Salta	Pichanal	-23.36	-64.18	L28	*L. laevis*
DB 5003	Argentina	Salta	Pichanal	-25.40	-64.15	L29	*L. laevis*
DB 8562	Argentina	Chaco	Nueva Pompeya	-24.85	-61.54	L13	*L. laevis*
LGE 5275	Argentina	Chaco	Mesón de Hierro	-27.40	-60.93	L15	*L. laevis*
LGE 5277	Argentina	Chaco	Mesón de Hierro	-27.40	-60.93	L15	*L. laevis*
LGE 5289	Argentina	Chaco	Mesón de Hierro	-27.40	-60.93	L15	*L. laevis*
LGE 5292	Argentina	Chaco	Mesón de Hierro	-27.40	-60.93	L15	*L. laevis*
LGE 5295	Argentina	Chaco	Mesón de Hierro	-27.40	-60.93	L15	*L. laevis*

Samples ID	Country	Province/Department	Locality	Lat	Long	locality code	mtDNA haplogroup
LGE 5315	Argentina	Chaco	Mesón de Hierro	-27.40	-60.93	L15	*L. laevis*
LGE 5425	Argentina	Chaco	Santa Sylvina	-27.78	-61.05	L16	*L. laevis*
LGE 5426	Argentina	Chaco	Santa Sylvina	-27.78	-61.05	L16	*L. laevis*
LGE 5427	Argentina	Chaco	Santa Sylvina	-27.78	-61.05	L16	*L. laevis*
LGE 5428	Argentina	Chaco	Santa Sylvina	-27.78	-61.05	L16	*L. laevis*
IIBPH 1457	Paraguay	Boquerón	20 km E from Filadelfia	-22.34	-60.27	L31	*L. laevis*
IIBPH 1749	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	*L. laevis*
IIBPH 1750	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	*L. laevis*
IIBPH 1751	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	*L. laevis*
IIBPH 1752	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	*L. laevis*
IIBPH 1753	Paraguay	Boquerón	road to Loma Plata-NR 9	-22.58	-59.84	L37	*L. laevis*
IIBPH 1811	Paraguay	Boquerón	Chaco Boef Farm	-22.17	-60.50	L36	*L. laevis*
IIBPH 1812	Paraguay	Boquerón	Chaco Boef Farm	-22.17	-60.50	L36	*L. laevis*
IIBPH 1813	Paraguay	Boquerón	Chaco Boef Farm	-22.17	-60.50	L36	*L. laevis*
IIBPH 1884	Paraguay	Presidente Hayes	Fortín Salazar	-23.08	-59.29	L32	*L. laevis*
IIBPH 1906	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	*L. laevis*
IIBPH 2286	Paraguay	Boquerón	Infante Rivarola	-21.59	-62.12	L38	*L. laevis*
IIBPH 2287	Paraguay	Boquerón	Infante Rivarola	-21.59	-62.12	L38	*L. laevis*
IIBPH 2288	Paraguay	Boquerón	Infante Rivarola	-21.59	-62.12	L38	*L. laevis*
IIBPH 2349	Paraguay	Boquerón	Teniente Prieto Farm	-21.11	-61.37	L54	*L. laevis*
IIBPH 2350	Paraguay	Boquerón	Teniente Prieto Farm	-21.11	-61.37	L54	*L. laevis*
IIBPH 2360	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L39	*L. laevis*
IIBPH 2361	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L39	*L. laevis*
IIBPH 2362	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L39	*L. laevis*
IIBPH 2365	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L40	*L. laevis*
IIBPH 2366	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L40	*L. laevis*
IIBPH 2367	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.21	L40	*L. laevis*
IIBPH 2378	Paraguay	Boquerón	PN Defensores del Chaco	-20.52	-60.22	L41	*L. laevis*
IIBPH 2379	Paraguay	Boquerón	PN Defensores del Chaco	-20.52	-60.22	L41	*L. laevis*
IIBPH 2391	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	*L. laevis*

Samples ID	Country	Province/Department	Locality	Lat	Long	locality code	mtDNA haplogroup
IIBPH 2392	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	*L. laevis*
IIBPH 2393	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	*L. laevis*
IIBPH 2395	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	*L. laevis*
IIBPH 2396	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	*L. laevis*
IIBPH 2397	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	*L. laevis*
IIBPH 2398	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	*L. laevis*
IIBPH 2407	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L43	*L. laevis*
ZUFMS 2638	Brazil	Mato Grosso do Sul	Porto Murtinho	-21.69	-57.72	L30	*L. laevis*
ZUFMS 2639	Brazil	Mato Grosso do Sul	Porto Murtinho	-21.69	-57.72	L30	*L. laevis*
ZUFMS 2640	Brazil	Mato Grosso do Sul	Porto Murtinho	-21.69	-57.72	L30	*L. laevis*
ZUFMS 2641	Brazil	Mato Grosso do Sul	Porto Murtinho	-21.69	-57.72	L30	*L. laevis*
IIBPH 2819	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	*L. laevis*
IIBPH 2820	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	*L. laevis*
IIBPH 2821	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	*L. laevis*
IIBPH 2822	Paraguay	Boquerón	Campo Jurado	-22.67	-61.53	L33	*L. laevis*
IIBPH 2862	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	*L. laevis*
IIBPH 2863	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	*L. laevis*
IIBPH 2864	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	*L. laevis*
IIBPH 2865	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	*L. laevis*
IIBPH 2866	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.63	-61.47	L34	*L. laevis*
IIBPH 2884	Paraguay	Boquerón	Road Montania-Madrejón	-21.54	-59.89	L51	*L. laevis*
IIBPH 2885	Paraguay	Boquerón	Road Montania-Madrejón	-21.54	-59.89	L51	*L. laevis*
IIBPH 2886	Paraguay	Boquerón	Road Montania-Madrejón	-21.54	-59.89	L51	*L. laevis*
IIBPH 2887	Paraguay	Boquerón	Road Montania-Madrejón	-21.54	-59.89	L51	*L. laevis*
IIBPH 2888	Paraguay	Boquerón	Road Montania-Madrejón	-21.54	-59.89	L51	*L. laevis*
IIBPH 2906	Paraguay	Boquerón	Pitiantuta Farm detour	-21.41	-59.80	L52	*L. laevis*
IIBPH 2930	Paraguay	Boquerón	Pirizal, Linea 1	-22.95	-60.64	L50	*L. laevis*
LGE 8180	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	*L. laevis*
LGE 8197	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	*L. laevis*
LGE 8201	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	*L. laevis*

Samples ID	Country	Province/Department	Locality	Lat	Long	locality code	mtDNA haplogroup
LGE 8202	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	*L. laevis*
LGE 8203	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	*L. laevis*
LGE 8232	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	*L. laevis*
LGE 8247	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	*L. laevis*
BB 1953	Argentina	Salta	Pluma de Pato	-23.38	-63.08	L26	*L. laevis*
BB 1996	Argentina	Salta	Dragones	-23.26	-63.30	L25	*L. laevis*
BB 2009	Argentina	Salta	Morillo	-23.36	-63.13	L27	*L. laevis*
LL 1	Argentina	Chaco	Nueva Pompeya	-25.02	-61.52	L12	*L. laevis*
LL 2	Argentina	Chaco	Nueva Pompeya	-25.02	-61.52	L12	*L. laevis*
Lepidobatrachus llanensis							
DB 7700	Argentina	Chaco	Wichi	-24.70	-61.43	L11	*L. llanensis* N1
DB 8402	Argentina	Chaco	El Sauzal	-24.58	-61.54	L10	
DB 8702	Argentina	Chaco	Nueva Pompeya	-24.84	-61.57	L14	*L. llanensis* N3
DB 8703	Argentina	Chaco	Nueva Pompeya	-24.84	-61.57	L14	*L. llanensis* N1
LGE 220	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	
LGE 1360	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	*L. llanensis* N3
LGE 1362	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	*L. llanensis* N3
LGE 1660	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	*L. llanensis* N3
LGE 1758	Argentina	Chaco	Taco Pozo	-25.51	-63.01	L17	*L. llanensis* N3
LGE 2324	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 2325	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 2326	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 2331	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 2332	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 2333	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 5536	Argentina	Catamarca	San Martín	-29.19	-65.80	L5	*L. llanensis* S
LGE 5537	Argentina	Catamarca	San Martín	-29.19	-65.80	L5	*L. llanensis* S
LGE 5538	Argentina	Catamarca	San Martín	-29.19	-65.80	L5	*L. llanensis* S
LGE 5539	Argentina	Catamarca	San Martín	-29.19	-65.80	L5	*L. llanensis* S
LGE 5540	Argentina	Catamarca	San Martín	-29.19	-65.80	L5	*L. llanensis* S

Samples ID	Country	Province/Department	Locality	Lat	Long	locality code	mtDNA haplogroup
LGE 5541	Argentina	Catamarca	San Martín	-29.19	-65.80	L5	*L. llanensis* S
LGE 5546	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	*L. llanensis* S
LGE 5547	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	*L. llanensis* S
LGE 5548	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	*L. llanensis* S
LGE 5550	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	*L. llanensis* S
LGE 5551	Argentina	La Rioja	Bajo Hondo	-29.78	-66.59	L3	*L. llanensis* S
LGE 5587A	Argentina	Catamarca	San Martín	-29.22	-65.80	L6	*L. llanensis* S
LGE 5587B	Argentina	Catamarca	San Martín	-29.22	-65.80	L6	*L. llanensis* S
LGE 5587C	Argentina	Catamarca	San Martín	-29.22	-65.80	L6	*L. llanensis* S
LGE 5587D	Argentina	Catamarca	San Martín	-29.22	-65.80	L6	*L. llanensis* S
LGE 5593A	Argentina	Catamarca	Telarito	-29.46	-65.67	L4	*L. llanensis* S
LGE 5593B	Argentina	Catamarca	Telarito	-29.46	-65.67	L4	*L. llanensis* S
IIBPH 2380	Paraguay	Boquerón	PN Defensores del Chaco	-20.52	-60.22	L41	*L. llanensis* N4
IIBPH 2381	Paraguay	Boquerón	PN Defensores del Chaco	-20.55	-60.17	L44	*L. llanensis* N4
IIBPH 2384	Paraguay	Boquerón	PN Defensores del Chaco	-20.56	-60.13	L45	*L. llanensis* N5
IIBPH 2394	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.02	L42	*L. llanensis* N4
IIBPH 2400	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.03	L46	*L. llanensis* N4
IIBPH 2401	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.03	L46	*L. llanensis* N4
IIBPH 2402	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L47	*L. llanensis* N4
IIBPH 2403	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L47	*L. llanensis* N4
IIBPH 2404	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L47	*L. llanensis* N5
IIBPH 2405	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L48	*L. llanensis* N4
IIBPH 2406	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L48	*L. llanensis* N4
IIBPH 2408	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L48	*L. llanensis* N4
IIBPH 2409	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L48	*L. llanensis* N4
IIBPH 2410	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L48	*L. llanensis* N4
IIBPH 2412	Paraguay	Boquerón	PN Defensores del Chaco	-20.61	-60.00	L48	*L. llanensis* N5
IIBPH 2368	Paraguay	Boquerón	PN Defensores del Chaco	-20.53	-60.20	L49	*L. llanensis* N5
IIBPH 2382	Paraguay	Boquerón	PN Defensores del Chaco	-20.55	-60.17	L44	*L. llanensis* N4
IIBPH 2854	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.62	-61.45	L35	

Samples ID	Country	Province/Department	Locality	Lat	Long	locality code	mtDNA haplogroup
IIBPH 2855	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.62	-61.45	L35	*L. llanensis* N3
IIBPH 2858	Paraguay	Boquerón	Capitán Joel Estigarribia	-22.62	-61.45	L35	*L. llanensis* N3
IIBPH 2903	Paraguay	Boquerón	Pitiantuta Farm detour	-21.41	-59.81	L53	*L. llanensis* S
LGE 7416	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 7417	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 7418	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 7419	Argentina	Córdoba	Totoralejos	-29.62	-64.84	L2	*L. llanensis* S
LGE 8121	Argentina	Formosa	Las Lomitas	-24.47	-60.67	L20	*L. llanensis* N2
LGE 8148	Argentina	Formosa	Laguna Yema	-24.56	-60.64	L19	*L. llanensis* N3
LGE 8159	Argentina	Formosa	Laguna Yema	-24.56	-60.64	L19	*L. llanensis* N3
LGE 8164	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	*L. llanensis* N2
LGE 8165	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	*L. llanensis* N3
LGE 8166	Argentina	Formosa	Laguna Yema	-24.22	-61.21	L18	*L. llanensis* N3
LGE 8207	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	*L. llanensis* N6
LGE 8208	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	
LGE 8209	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	*L. llanensis* N3
LGE 8215	Argentina	Salta	Fortín Dragones	-23.38	-62.08	L22	*L. llanensis* N6
LGE 8216	Argentina	Salta	Fortín Dragones	-23.38	-62.08	L22	*L. llanensis* N3
LGE 8217	Argentina	Salta	Fortín Dragones	-23.38	-62.08	L22	*L. llanensis* N6
LGE 8224	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	*L. llanensis* N6
LGE 8225	Argentina	Salta	Fortín Dragones	-23.43	-62.97	L21	*L. llanensis* N3
BB 1891	Argentina	Salta	Fortín Dragones	-23.25	-63.34	L23	*L. llanensis* N6
BB 1892	Argentina	Salta	Fortín Dragones	-23.25	-63.34	L23	*L. llanensis* N6
LLL 2	Argentina	Chaco	Nueva Pompeya	-25.02	-61.52	L12	*L. llanensis* N1
LGE 5854	Argentina	Catamarca	Telarito	-29.46	-65.67	L4	*L. llanensis* S
LGE 5858	Argentina	Catamarca	Telarito	-29.46	-65.67	L4	*L. llanensis* S
LGE 5856	Argentina	Catamarca	San Martín	-29.22	-65.80	L6	*L. llanensis* S
LGE 5857	Argentina	Catamarca	San Martín	-29.22	-65.80	L6	*L. llanensis* S

Appendix 2: GenBank accession of sequences used in this article. (Upon acceptance)

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
Lepidobatrachus asper							
LGE 101	x	x	x	x	x		x
LGE 102	x	x	x	x	x		x
LGE 103	x						
LGE 104	x						
LGE 105	x						
LGE 329	x	x	x	x	x		x
LGE 1347	x	x	x	x	x		x
LGE 1348	x	x	x	x	x		x
LGE 1349	x						
LGE 1350	x						
LGE 1351	x						
LGE 4961		x	x	x			x
LGE 4962		x	x		x		
JNL 253	x						
JNL 254	x						
JNL 255	x						
JNL 257	x						
Lepidobatrachus laevis							
DB 4933	x		x			x	
DB 4943	x	x	x	x	x	x	x
DB 4942	x						
DB 5003	x						
DB 8562	x		x	x	x	x	x
LGE 5275	x		x	x			x
LGE 5277	x		x	x			x
LGE 5289	x						
LGE 5292	x						
LGE 5295	x						
LGE 5315	x						
LGE 5425	x		x	x			x
LGE 5426	x		x	x			x
LGE 5427	x						
LGE 5428	x						
IIBPH 1457	x			x			
IIBPH 1749	x		x	x	x	x	
IIBPH 1750	x		x	x			x
IIBPH 1751	x						
IIBPH 1752	x						
IIBPH 1753	x						
IIBPH 1811	x				x		x
IIBPH 1812	x	x	x	x		x	
IIBPH 1813	x						
IIBPH 1884	x	x	x	x	x		x
IIBPH 1906	x						
IIBPH 2749	x						
IIBPH 2286	x		x	x			x
IIBPH 2287	x		x				x
IIBPH 2288	x						
IIBPH 2349	x		x	x	x	x	

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
IIBPH 2350	x	x	x	x		x	x
IIBPH 2360	x						
IIBPH 2361	x						
IIBPH 2362	x						
IIBPH 2365	x	x	x	x	x		
IIBPH 2366	x						
IIBPH 2367	x						
IIBPH 2378	x						
IIBPH 2379	x		x	x	x		
IIBPH 2391	x						
IIBPH 2392	x						
IIBPH 2393	x		x	x			
IIBPH 2395	x						
IIBPH 2396	x	x	x		x		x
IIBPH 2397	x						
IIBPH 2398	x						
IIBPH 2407	x						
ZUFMS 2638	x	x	x	x		x	x
ZUFMS 2639	x	x	x	x	x	x	x
ZUFMS 2640	x						
ZUFMS 2641	x						
IIBPH 2819	x		x		x		x
IIBPH 2820	x			x	x		x
IIBPH 2821	x						
IIBPH 2822	x						
IIBPH 2862	x		x		x		x
IIBPH 2863	x				x	x	x
IIBPH 2864	x						
IIBPH 2865	x						
IIBPH 2866	x						
IIBPH 2884	x		x	x	x	x	x
IIBPH 2885	x	x	x				x
IIBPH 2886	x						
IIBPH 2887	x						
IIBPH 2888	x						
IIBPH 2906	x	x	x	x	x	x	x
IIBPH 2930	x			x	x	x	x
LGE 8180	x		x	x	x	x	x
LGE 8197	x	x	x	x	x		x
LGE 8201	x						
LGE 8202	x						
LGE 8203	x						
LGE 8232	x	x	x	x		x	x
LGE 8247	x	x	x	x	x	x	x
BB 1953	x		x	x	x	x	x
BB 1996	x	x	x	x		x	
BB 2009	x						
LL 1	x		x	x	x	x	
LL 2	x		x	x	x	x	x
Lepidobatrachus llanensis							
DB 8402		x	x	x		x	

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
DB 7700	x		x	x	x		x
DB 8702	x	x	x		x		x
DB 8703	x	x	x		x	x	
LGE 220			x	x			
LGE 1360	x			x			x
LGE 1362	x						
LGE 1660	x						
LGE 1758	x						
LGE 2324	x		x	x	x	x	
LGE 2325	x		x	x	x		x
LGE 2326	x						
LGE 2331	x						
LGE 2332	x						
LGE 2333	x						
LGE 5536	x						
LGE 5537	x			x			
LGE 5538		x	x	x	x		x
LGE 5539	x						
LGE 5540	x						
LGE 5541	x						
LGE 5546	x	x	x				
LGE 5547	x	x	x	x	x	x	x
LGE 5548	x						
LGE 5550	x						
LGE 5551	x						
LGE 5587A	x		x	x			
LGE 5587B	x	x		x	x	x	
LGE 5587C	x						
LGE 5587D	x						
LGE 5593A	x	x	x	x	x		x
LGE 5593B	x	x	x	x		x	x
IIBPH 2380	x	x	x	x	x	x	x
IIBPH 2381	x						
IIBPH 2384	x	x	x	x	x	x	
IIBPH 2394	x						
IIBPH 2400	x	x	x	x	x	x	
IIBPH 2401	x						
IIBPH 2402	x						
IIBPH 2403	x						
IIBPH 2404	x			x			
IIBPH 2405	x						
IIBPH 2406	x						
IIBPH 2408	x						
IIBPH 2409	x						
IIBPH 2410	x						
IIBPH 2412	x			x			
IIBPH 2368	x						
IIBPH 2382	x						
IIBPH 2854			x	x			x
IIBPH 2855	x	x	x	x	x	x	x
IIBPH 2858	x						

Samples ID	CO1	MVZ 15-16	MVZ 27-28	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
IIBPH 2903		x	x	x	x		
LGE 7416	x						
LGE 7417	x						
LGE 7418	x						
LGE 7419	x						
LGE 8121	x	x	x	x	x	x	x
LGE 8148	x		x	x	x	x	x
LGE 8159	x	x	x	x			x
LGE 8164	x	x		x	x	x	
LGE 8165	x			x	x	x	x
LGE 8166	x						
LGE 8207	x		x		x	x	
LGE 8208			x	x	x	x	
LGE 8209	x						
LGE 8215	x		x		x		
LGE 8216	x		x	x	x	x	x
LGE 8217	x						
LGE 8224	x						
LGE 8225	x						
BB 1891	x	x		x	x	x	x
BB 1892	x			x	x		x
LLL 2	x		x	x			
LGE 5854	x						
LGE 5858	x						
LGE 5856	x						
LGE 5857	x						

Supporting Information:

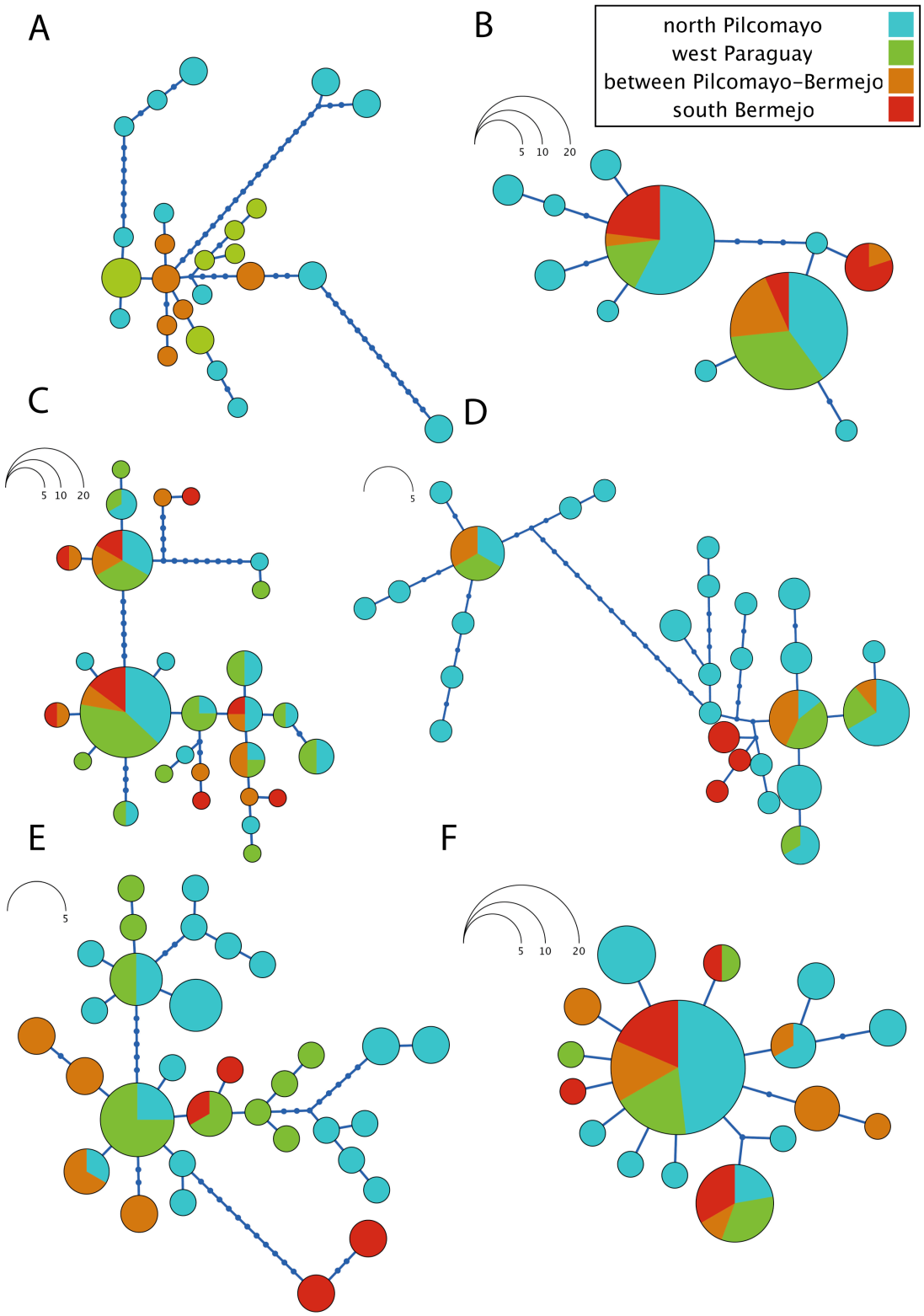

Figure S1: *Lepidobatrachus laevis* nuclear haplotype network. A) MVZ 15-16, B) MVZ 27-28, C) MVZ 29-30, D) MVZ 39-40, E) MVZ 47-48, and F) RPL3. Small blue dots indicate unsampled mutations. Colors represent populations separated by rivers. Circle size corresponds to frequency. Locus names follow Table 1.

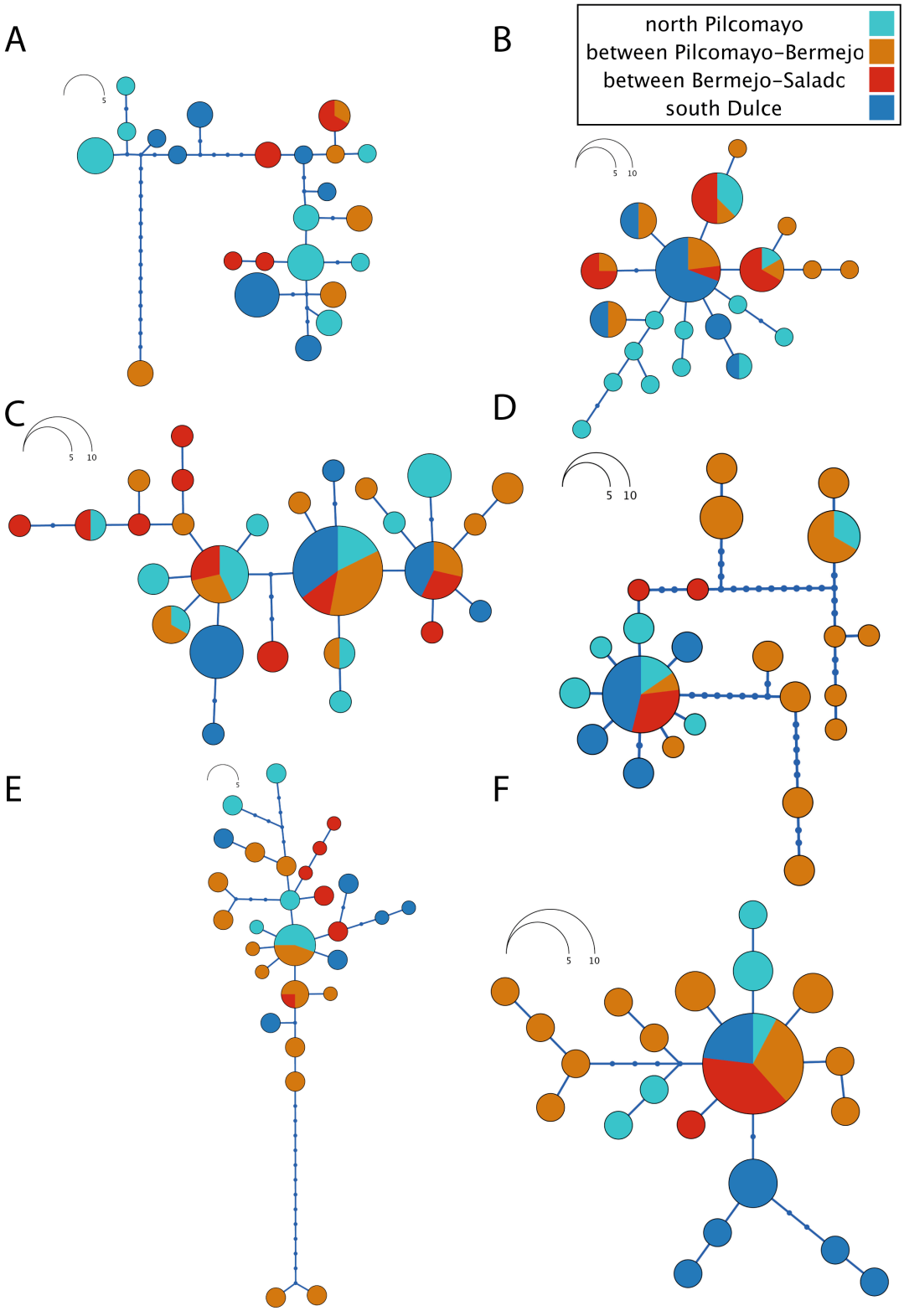

Figure S2: *Lepidobatrachus llanensis* nuclear haplotype network. A) MVZ 15-16, B) MVZ 27-28, C) MVZ 29-30, D) MVZ 39-40, E) MVZ 47-48, and F) RPL3. Small blue dots indicate unsampled mutations. Colors represent populations separated by rivers. Circle size corresponds to frequency. Locus names follow Table 1.

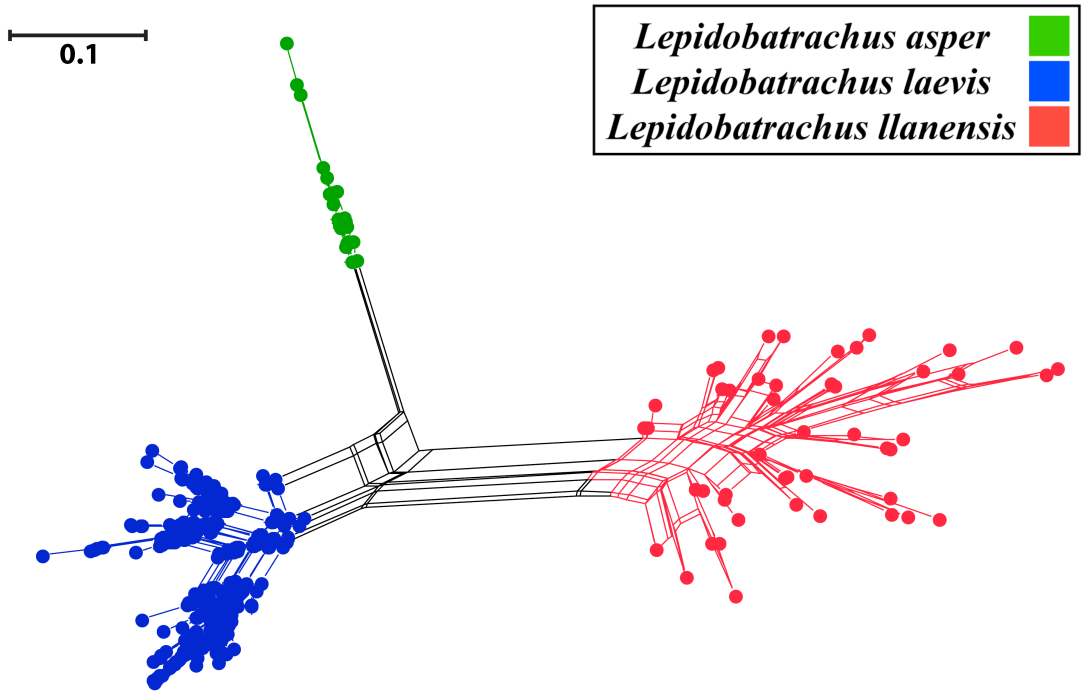

Figure S3: Nuclear multilocus genetic distance network of *Lepidobatrachus* species based on all nuclear fragments. Colors represent the three species.

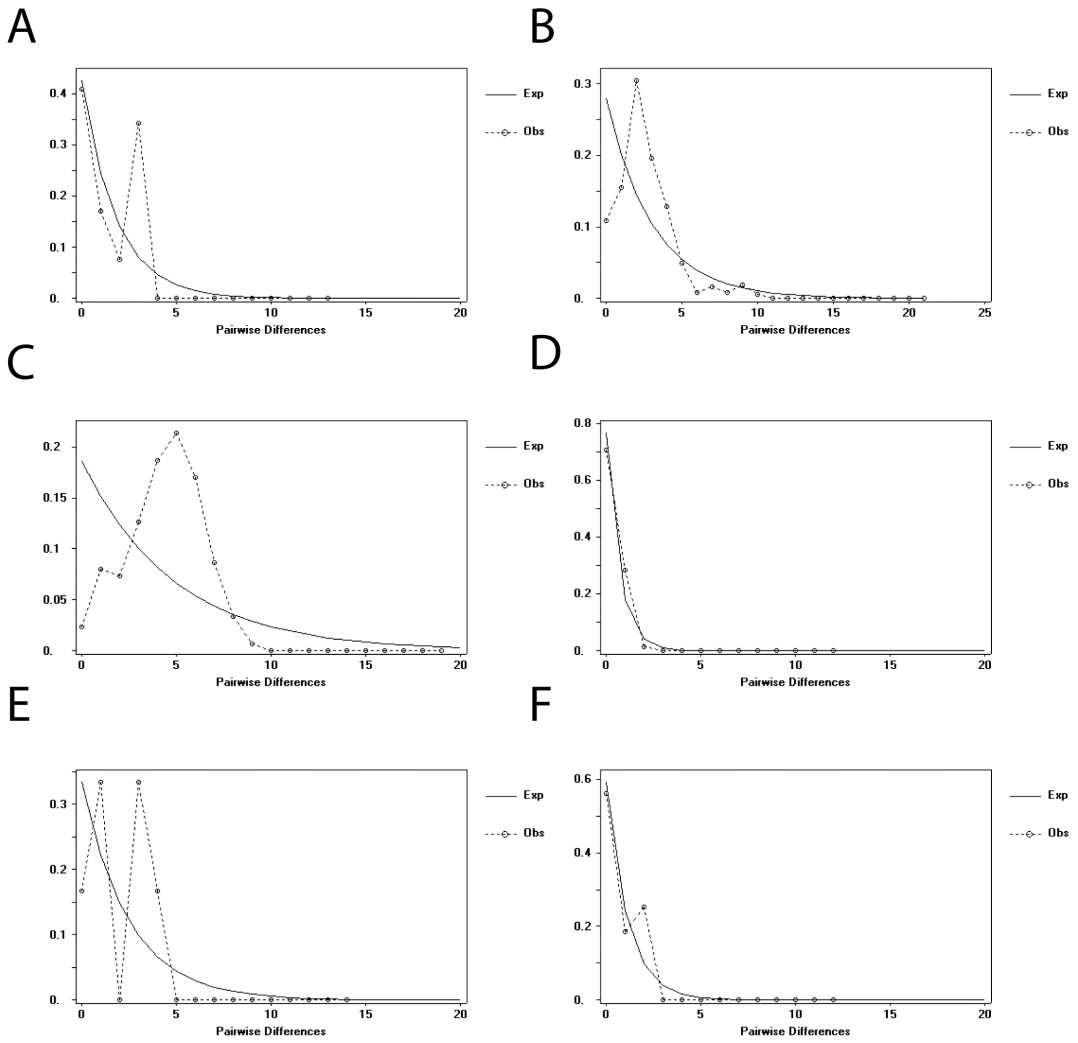

Figure S4: Mismatch distribution analyses (MDA) of *Lepidobatrachus* main haplogroups. A) *Lepidobatrachus asper*, B) *Lepidobatrachus laevis*, C) *Lepidobatrachus llanensis* N3, D) *Lepidobatrachus llanensis* N4, E) *Lepidobatrachus llanensis* N5, and F) *Lepidobatrachus llanensis* S. Continuous and dotted lines correspond to expected (Exp) and observed (Obs) distributions of pairwise differences among haplotypes under a recent population expansion model.

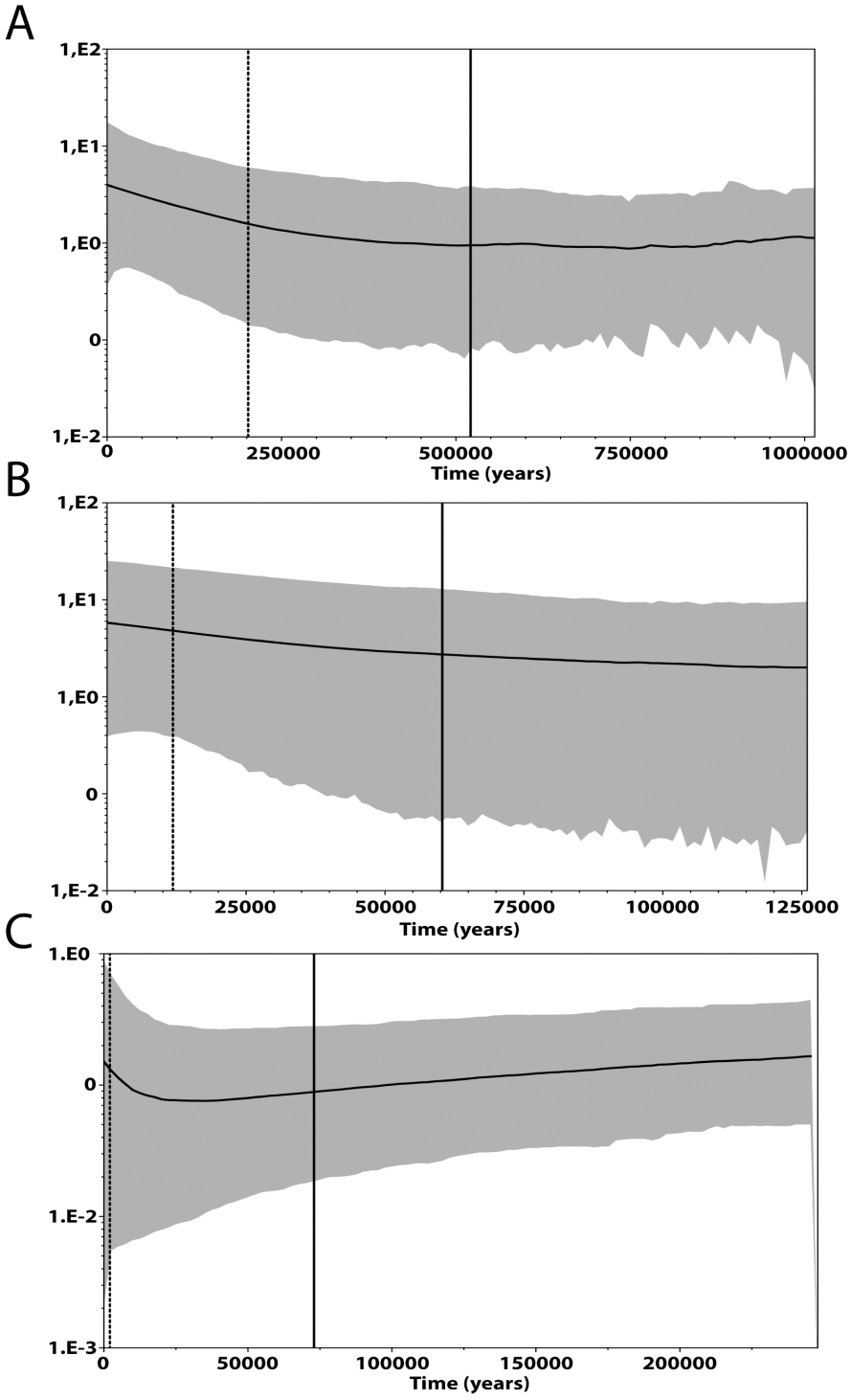

Figure S5: Bayesian skyline plots (BSP) of mitochondrial data of *Lepidobatrachus llanensis* main haplogroups. A) *Lepidobatrachus llanensis* N1+N2+N3 B) *Lepidobatrachus llanensis* N4, C) *Lepidobatrachus llanensis* S. Haplogroup names follow Figure 2. Horizontal continuous line and surrounded gray area corresponds to mean and 95% highest posterior density limits for the effective population size, respectively. Vertical continuous and dotted lines correspond to mean and lowest estimated tMRCA, respectively. X axis is time in years and Y axis is effective population sizes.

CAPITULO 3

Middle-Pleistocene expansion and current gene flow in the Chaco Vizcacheras' White-lipped Frog, *Leptodactylus bufonius* Boulenger, 1894 (Anura, Leptodactylidae)

Abstract

Neotropical species diversification cannot be restricted to a specific period of time or mechanism, since it is an ongoing process happening from Eocene to Pleistocene and is related to several processes and events. The Chaco biome was influenced by several of these events, from old marine introgressions to relatively recent glaciations. *Leptodactylus bufonius* is a widespread distributed Chacoan species, used here to access the recent evolutionary history of this biome. We used mitochondrial and nuclear markers to assess genetic structure and demographic history of this species. We found evidence to support recent range expansion events and current gene flow among populations. Range expansions are related with Pleistocene inter-glacial periods. Current gene flow is maintained by short distance dispersion that follows a stepping-stone model allowing high connectivity, even among distant populations. This pattern matches diversification of frogs in other semiarid regions of the world.

1. Introduction

It is increasingly clear that Neotropical diversification cannot be restricted to a particular timeframe or mechanism (RULL, 2011). Diversification in the Neotropics is related to events and processes that have taken place from late Eocene/early Oligocene to Pleistocene (RULL, 2008). The Chaco biome, due to its geographic situation, has been influenced by several of these events, having then a rich history of events, like marine introgressions, glaciations, and associated climatic changes (HERNANDEZ *et al*., 2005; ORTIZ-JAUGUERIZAR & CLADERA, 2006).

Diversification of the Chaco endemic frog genus *Lepidobatrachus* (Ceratophryidae) was recently assessed by Brusquetti *et al*. (Chapter 1 and 2). These authors proposed an important role of the middle Miocene marine introgressions and the climatic changes of the late Miocene on these frogs diversification and a putative dispersal barrier on central Santiago del Estero province (Argentina) that may affected the genetic structure of one of the species of this genus, *Lepidobatrachus llanensis*. This barrier corresponds to a highly arid area known as the "South American heat pole", which shows high temperatures (PROHASKA, 1959), low precipitation regime (BOLETTA *et al*., 1989), high soil salinity (RUIBAL, 1962), and act as a climatic barrier for populations of north and south of this area (BRUSQUETTI *et al*., Chapter 1 and 2). Brusquetti *et al*. (Chapter 1 and 2) also found some effects of the main Chacoan rivers although in only one species, *Lepi. llanensis*. This fact would be associated with the longer persistence time on main Chacoan rivers influence area of this species than *Lepidobatrachus laevis*, the other species distributed in this area.

The early history of the Chaco, in the Quaternary period, differs from Tertiary mainly because the dynamics of the environmental changes, which occurred with higher amplitude and frequency and modified distributional ranges of biomes, species, and populations (ORTIZ-JAUREGUIZAR & CLADERA, 2006). The main factors that affected the South American fauna in the Pleistocene were the glaciations and the massive immigrants from North America (see ORTIZ-JAUREGUIZAR & CLADERA, 2006). Although glaciers did not pass southern South America latitudes and have only persisted in the Andes, climatic and environmental changes associated with glaciation events resulted on cyclic distributional shifts of southern biomes,

including the Chaco (COSACOV *et al*., 2010; ORTIZ-JAUREGUIZAR & CLADERA, 2006).

The Chaco is a semiarid biome with a marked climatic seasonality (PRADO, 1993; CABRERA, 1994). Pond-breeding frogs of other arid or semiarid environments show low levels of genetic structure and differentiation between populations (e.g., CHAN & ZAMUDIO, 2009; PABIJAN *et al*., 2015). This low genetic structure suggests that arid-adapted amphibians have to be highly vagile and with low site fidelity (CHAN & ZAMUDIO, 2009), contrary to general expectations about amphibians. In small animals with high risk of desiccation, like frogs, low dispersal abilities are the more reasonable expectation (BLAUSTEIN *et al*., 1994).

The speciose genus *Leptodactylus* comprises 74 recognized species and occupies a wide range of environments, occurring in southern North America, South America, and West Indies (FROST, 2015). However, only two *Leptodactylus* species are considered as endemic of the Chaco, *Lept. laticeps* and *Lept. bufonius*. *Leptodactylus bufonius* is a very common species widely distributed in this biome but also is known by some records in the Pantanal (PANSONATO *et al*., 2011), however in the transition area between Chaco and Pantanal localities.

Although some species of the genus *Leptodactylus* show trends towards terrestriality (e.g., foam nest on underground chambers in *Lept. fuscus* group) (DE SÁ *et al*., 2014), the genus is not supposed to have diversified in semiarid environments like the ceratophryids (FAIVOVICH *et al*., 2014). Different to *Leptodactylus* species, ceratophryids show morphological and behavioral characteristics associated with living in arid environments (see FAIVOVICH *et al*., 2014). Then, besides having a shorter evolutionary history in the Chaco when compared to the ceratophryid genus *Lepidobatrachus* (used by Brusquetti *et al*., Chapter 1 and 2), *Lept. bufonius* also presents a different biology being a great model to assess the most recent evolutionary history of this biome and test previously hypothesized dispersal barriers. To do this, we used mitochondrial and nuclear markers to assess the genetic structure and demographic history of this species. This widely distributed species allows us to test if Chacoan species shows low genetic structure, as known for species adapted to other semiarid biomes. We also investigated the potential mechanism that contributes to

this pattern and tested the effects of potential dispersal barriers previously hypothesized for *Lepi. llanensis*.

2. Material and methods

2.1 Sampling

We analyzed DNA sequences of 184 individuals collected from 96 localities across the species distribution range, covering almost the entire Chaco biome (Fig. 1, Appendix 1). Vouchers were housed at the Herpetological collections of Instituto de Investigación Biológica del Paraguay (IIBPH), Asunción, Paraguay; Laboratorio de Ecología y Evolución (LGE, DB, and GS), Posadas, Argentina; and in Amphibian Collection Célio F. B. Haddad (CFBH), Departamento de Zoologia, I.B., Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil.

2.2 Laboratory procedures and molecular methods

We used the DNeasy extraction kit (Qiagen, Valencia, CA, USA) to extract total genomic DNA from tissue samples (liver or muscle conserved on 95% ethanol). Via polymerase chain reaction (PCR), we amplified one mitochondrial fragment and four nuclear introns using specific primers (Table 1) and a commercial kit (Master Mix, Fermentas). We amplified the mitochondrial fragment using the following protocol: an initial denaturation step of 3 min at 94 °C; 10 cycles of 15 s of denaturation at 95 °C + 20 s of annealing at 45 °C + 50 s of extension at 60 °C; 26 cycles of 15 s of denaturation at 95 °C + 20 s of annealing at 50 °C + 50 s of extension at 60 °C; and a final extension of 5 min at 60 °C. Nuclear fragments amplification protocol were as follows: an initial denaturation step of 3 min at 95 °C (94 ºC in RPL3); 35 cycles (45 cycles for some MVZ 47-48 and all RPL3) of 30 s of denaturation at 95 °C + 30 s of annealing at 50-57 °C + 45 s of extension at 72 °C; and a final extension step of 7 min at 72 °C (see Table 1 for details). We used ExoSAP (Fermentas) to purify PCR products. Sequencing was made at Macrogen Inc. (Seoul, Korea). We edited sequences in CodonCode Aligner v. 3.5.4 (Codon Code Corporation).

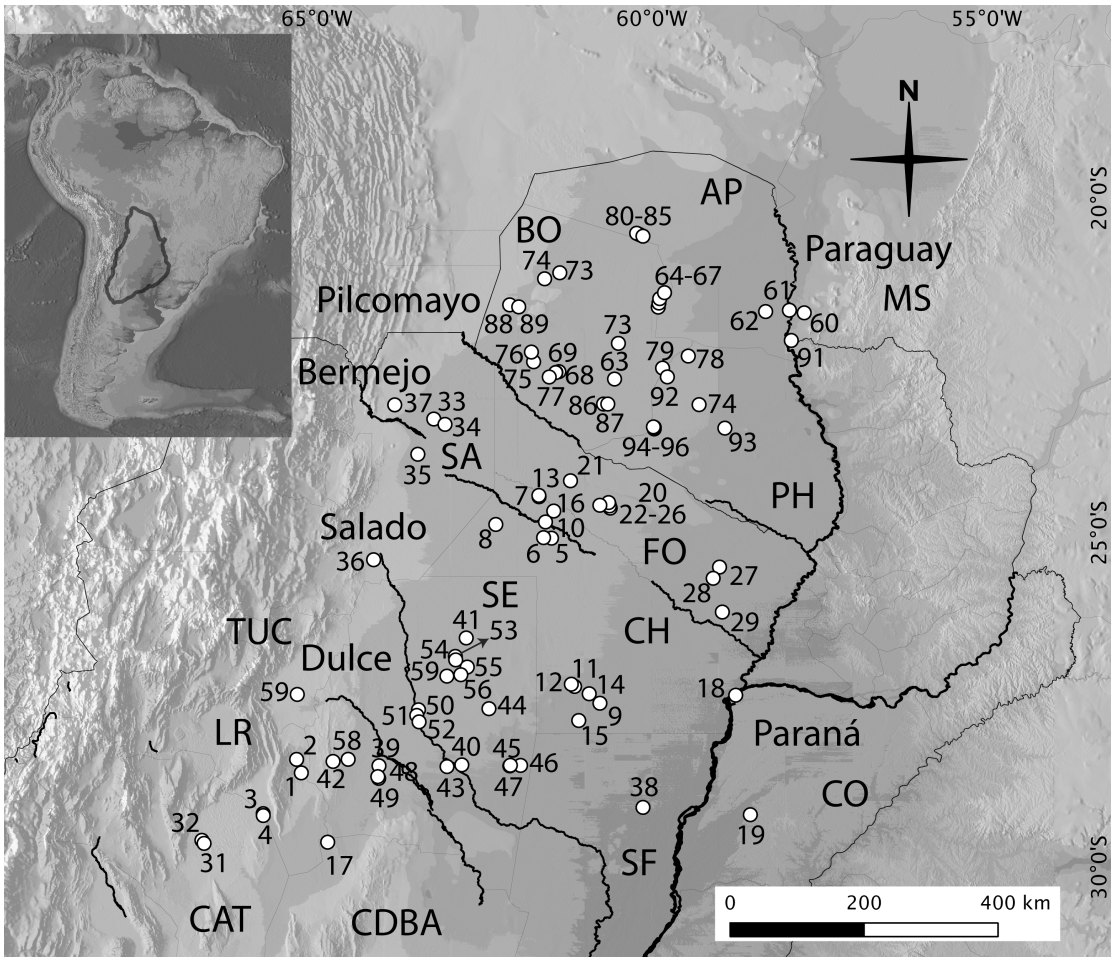

Figure 1: Sampling localities of *Leptodactylus bufonius*. Area enclosed in black in South America map encompasses the limits of Chaco biome, according to Morrone (2001). Main rivers are highlighted with their respective names. Detailed locality information represented by numbers is shown in Appendix 1. Department/province/state abbreviations: CAT, Catamarca; CDBA, Córdoba; CH, Chaco, CO, Corrientes; FO, Formosa; LR, La Rioja; SA, Salta; SF, Santa Fé; SE, Santiago del Estero; TUC, Tucumán (Argentina); MS, Mato Grosso do Sul (Brazil); AP, Alto Paraguay; BO, Boquerón; PH, Presidente Hayes (Paraguay).

We aligned sequences with MUSCLE (EDGAR, 2004) in MEGA 6 (TAMURA *et al*., 2013) and checked by eye. We estimated evolutionary models that best fit our data set with JModeltest 0.1.1 (POSADA, 2008) under the Akaike information criterion (AKAIKE, 1973). To verify absence of recombination we used PhiTest implemented in Splitstree v4.2 (HUSON & BRYANT, 2006); and GARD (Genetic Algorithm Recombination Detection) (KOSAKOVSKY POND *et al*., 2006), MAXCHI (SMITH, 1992; POSADA & CRANDALL, 2001), and CHIMAERA (POSADA & CRANDALL, 2001) implemented in the program RDP3 (HEATH *et al*., 2006). We separated sequences of individuals with heterozygous indels with CodonCode Aligner v. 3.5.4 (Codon Code Corporation) and to resolve haplotypes of

heterozygous individuals we used Phase 2.1 (STEPHENS *et al*., 2001) implemented in DnaSP 5.10 (LIBRADO & ROZAS, 2009), discarding unresolved ones (< 0.90 of posterior probability). We generated unique haplotypes data sets in DnaSP.

2.3 Mitochondrial phylogenetic analysis

We estimated mitochondrial tree under Bayesian Inference (BI) with MrBayes (RONQUIST & HUELSENBECK, 2003). We applied two independent runs, with four chains each, for 10 million generations sampling every 1000 generations, with priors under default settings. We verified convergence in TRACER 1.5 (RAMBAUT *et al*., 2013) and by examining standard deviation of split frequencies between independent runs (< 0.01). The first 2500 trees were discarded as burn-in. We partitioned our data set by codon position. We used *Leptodactylus troglodytes* to root the tree.

2.4 Haplotype network genealogies

We generated haplotype genealogies for each locus using Haploviewer (SALZBURGER *et al.*, 2011). Haploviewer turns phylogenetic trees into haplotype genealogies. We used DNAPARS available in PHYLIP v.3.695 package (FELSENSTEIN, 2005) to generate a Maximum Parsimony tree. We treated indels as fifth state and inferred branch lengths with "Rearrange on one best tree" as search method. In order to assess the influence of Santiago del Estero gap and main Chacoan rivers on *Lept. bufonius* genetic structure we used three data sets: first without *a-priori* population designation, second with populations separated by rivers, and third with populations of north and south of Santiago del Estero gap.

Table 1: Primers and annealing temperature (°C) used to amplify each locus.

Locus ID (length on base pair number)	Primer sequence 5'- 3'	Annealing	Reference
CO1 (637 bp)	ANF1 ACHAAYCAYAAAGAYATYGG	45/50	**Jungfer** *et al.*, 2013
Cytochrome c oxidase subunit 1	ANR1 CCGGTCTGAACTCAGATCACGT		**Jungfer** *et al.*, 2013
MVZ 29-30 (221 bp)	MVZ29 ATCCTCCATACTACTTAAGGAGACC	57	**Bell** *et al.*, 2011
Y Box Binding (Intron 1)	MVZ30 CTGAAGCCTCTGTACATGTTTTG		**Bell** *et al.*, 2011
MVZ 39-40 (188 bp)	MVZ39 GGATCTGCTAGAGACCTGTCACTTC	57	**Bell** *et al.*, 2011
X. laevis MGC82783 protein (Intron 2)	MVZ40 ACAGAGTCTTCAAACCCAGCAATAC		**Bell** *et al.*, 2011
MVZ 47-48 (349 bp)	MVZ47 AGTGAAAGATACAGTCACAGTGCTAGG	54.7/56.7/59	**Bell** *et al.*, 2011
X. laevis Fibrinogen, A alpha polypeptide (Intron 1)	MVZ48 GGAGGATATCAGCACAGTCTAAAAAG		**Bell** *et al.*, 2011
RPL3 (418 bp)	RPL35F AAGAAGTCYCACCTCATGGAGAT	50/53/64.3	**Pinho** *et al.*, 2009
Ribosomal Protein L3 (Intron 5)	RPL36RA AGTTTCTTTGTGTGCCAACGGCTAG		**Pinho** *et al.*, 2009

2.5 Bayesian genetic assignment

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 multi-locus genotypic data, STRUCTURE divides individuals into genetic clusters (K) that minimize both deviations from Hardy–Weinberg and linkage equilibrium within each cluster and also calculates fractional membership of individuals to each cluster (Q).

For this analysis we included only nuclear loci and only samples with sequences for at least three loci (see Appendix 2). To convert sequences to STRUCTURE input we used the program xmfa2struct (available at: http:// www.xavierdidelot.xtreemhost.com/clonalframe.htm); 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. We performed for each K, ranging from 1 and 15, $5x10^5$ iterations as burn-in and $5x10^5$ additional iterations. Most likely K was based on the method of Evanno *et al*. (2005) 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 & ROSENBERG, 2007) and visualize with DISTRUCT v.1.1 (ROSENBERG, 2004).

2.6 Isolation by distance (IBD), genetic diversity, and gene flow

We tested isolation by distance with Mantel test in Alleles in Space 1.0 (MILLER, 2005) for total data set. Gene flow and genetic distance were estimated between populations of north and south of Santiago del Estero gap. We estimated gene flow with *Fst*, and genetic distance with *Da* (net mean distances) between groups of populations (TAMURA & NEI, 1993), both in DnaSP.

We also assessed basic genetic diversity for complete data set and for north and south of Santiago del Estero gap populations, separately. To assess genetic diversity we estimated haplotype diversity (*Hd*) and per site nucleotide diversity (*Pi*), both estimations were made in DnaSP software.

2.7 Demographic estimations

We tested for recent population expansions on *Lept. bufonius* by mismatch distribution analyses (MDA) (ROGERS & HARPENDING, 1992), Tajima's *D*

(TAJIMA, 1989), Fu's *Fs* (FU, 1997), and Ramos-Onsins and Rozas's *R2* tests (RAMOS-ONSINS & ROZAS, 2002). We used Harpending's raggedness statistic (*r*) (HARPENDING, 1994) to assess goodness of fit between expected and observed distribution on MDA. We estimated significance levels of *Fs*, *R2*, and *r* with 10,000 coalescent simulation replicates. All statistics and significance analyses to test population expansions were made in DnaSP. In MDA plots, unimodal pattern corresponds to sudden demographic expansions and multimodal topology to populations at equilibrium or bottleneck (SLATKIN & HUDSON, 1991; ROGERS & HARPENDING, 1992).

We used Bayesian Skyline plot (BSP) implemented in Beast 1.8.2 (DRUMMOND & RAMBAUT, 2007) to estimate changes in effective population size through time and the age of the most recent common ancestor (tMRCA). For BSP analyses we estimated evolutionary models that best fit our data set with JModeltest, resulting on TN93 + I + G. We used a lognormal relaxed clock model with 0.0078 as substitution rate (normal distribution, mean = 0.0078, Stdev = 0.0005), based on the 0.78% per linage per million years mutation rate estimated by Freilich (2014). We used five groups for the Coalescent Bayesian Skyline with Piecewise-constant model. We run 200 millions of Markov chain Monte Carlo simulations sampling every 20,000 chains. Chain convergence was assessed in Tracer 1.5 (RAMBAUT & DRUMMOND, 2007) by effective sample size (ESS) examination (> 200).

3. Results

3.1 Mitochondrial phylogenetic analysis

Mitochondrial gene tree did not resolve the relationships within *Lept. bufonius* haplotypes; basically it resulted on a large polytomy with only one supported clade (Fig. 2). This clade groups haplotypes from almost all sampled localities.

3.2 Haplotype network genealogies

Mitochondrial haplotype genealogy did not show any geographical pattern, without any genetic structure corresponding neither to Santiago del Estero gap nor to

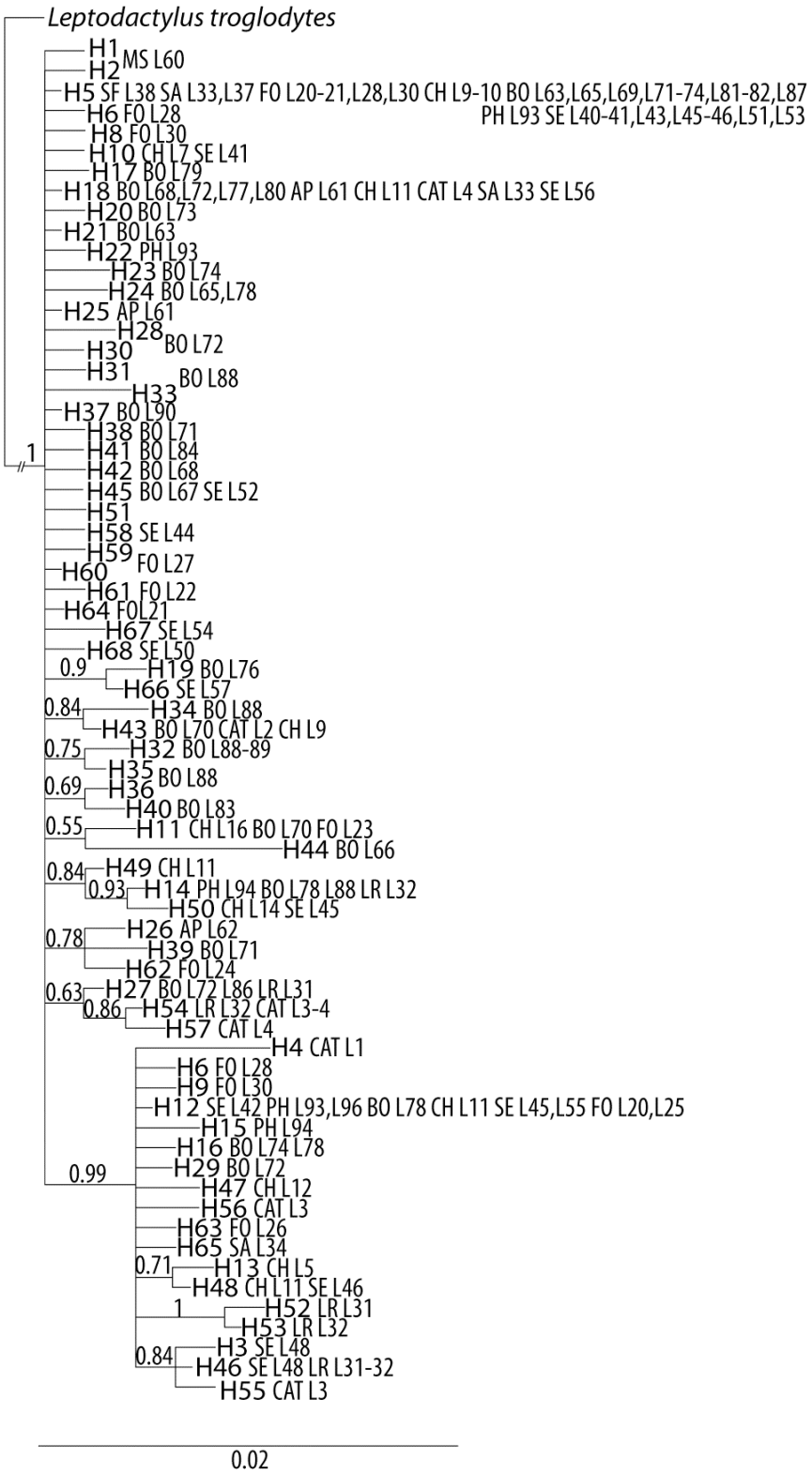

Figure 2: Fifty percent majority rule consensus tree from Bayesian inference analysis of mitochondrial (CO1) fragment of *Leptodactylus bufonius*. Numbers on nodes are Bayesian posterior probabilities. Terminal names correspond to haplotype number followed by province, department or state code and locality code (see Fig. 1 and Appendix 1). Scale bar corresponds to number of substitution/site.

the main rivers (Fig. 3). A larger more frequent haplotype is shared by individuals from almost all *Lept. bufonius* distribution, with the exceptions of southwestern localities of Catamarca, La Rioja, and southwestern Santiago del Estero provinces, in Argentina. Two other relatively more frequent haplotypes, as the larger described above, are surrounded by several rare haplotypes, almost all connected by a single mutational step. Although it is possible to identify five haplogroups, each one is connected with its neighbor by only one mutational step, showing high degree of localities overlap.

Genealogies of nuclear fragments MVZ 39-40 and RPL3 are basically represented by two haplotypes, which in fact correspond to each individual allele (Fig. 4). Loci MVZ 29-30 and MVZ 47-48 show a little more of genetic structure, with some differences between them. In MVZ29-30 almost all haplotypes are rare, with only few relatively more frequent. Haplotypes are connected between them by a wide range of mutational steps (1–15) without any pattern of organization. In contrast, in MVZ 47-48 there are more frequent haplotypes than rare haplotypes. Some genetic structure is evident, with some haplotypes forming haplogroups, but each one of these is connected with its neighbor by only one mutational step, forming a continuous and unique haplogroup (Fig. 4).

3.3 Bayesian genetic assignment

According to Evannos' ΔK the most likely number of clusters obtained in STRUCTURE was K = 2. This is supported by the fact that from K = 3 almost all individuals are admixed indicating no true geographic structure. On K = 2 barplot, except one individual from the Argentinian Tucumán province (L59), all others correspond to the same cluster with only an ephemeral admixture in some individuals (Fig. 5).

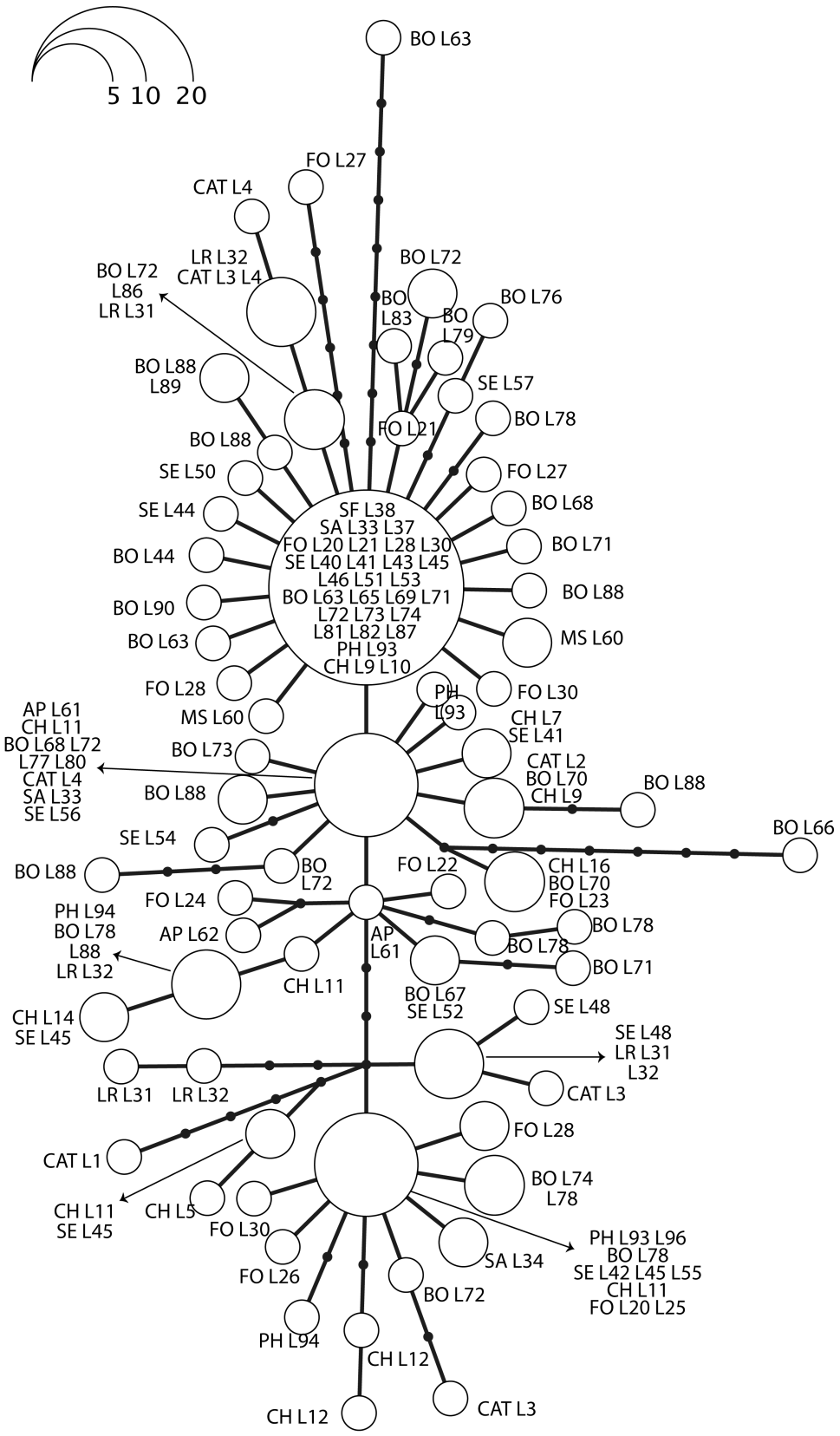

Figure 3: Mitochondrial haplotype network of *Leptodactylus bufonius*. Black dots indicate unsampled mutations. Circle size corresponds to haplotype frequency. Provinces or departments and sampling localities (L) follow Figure 1 and Appendix 1.

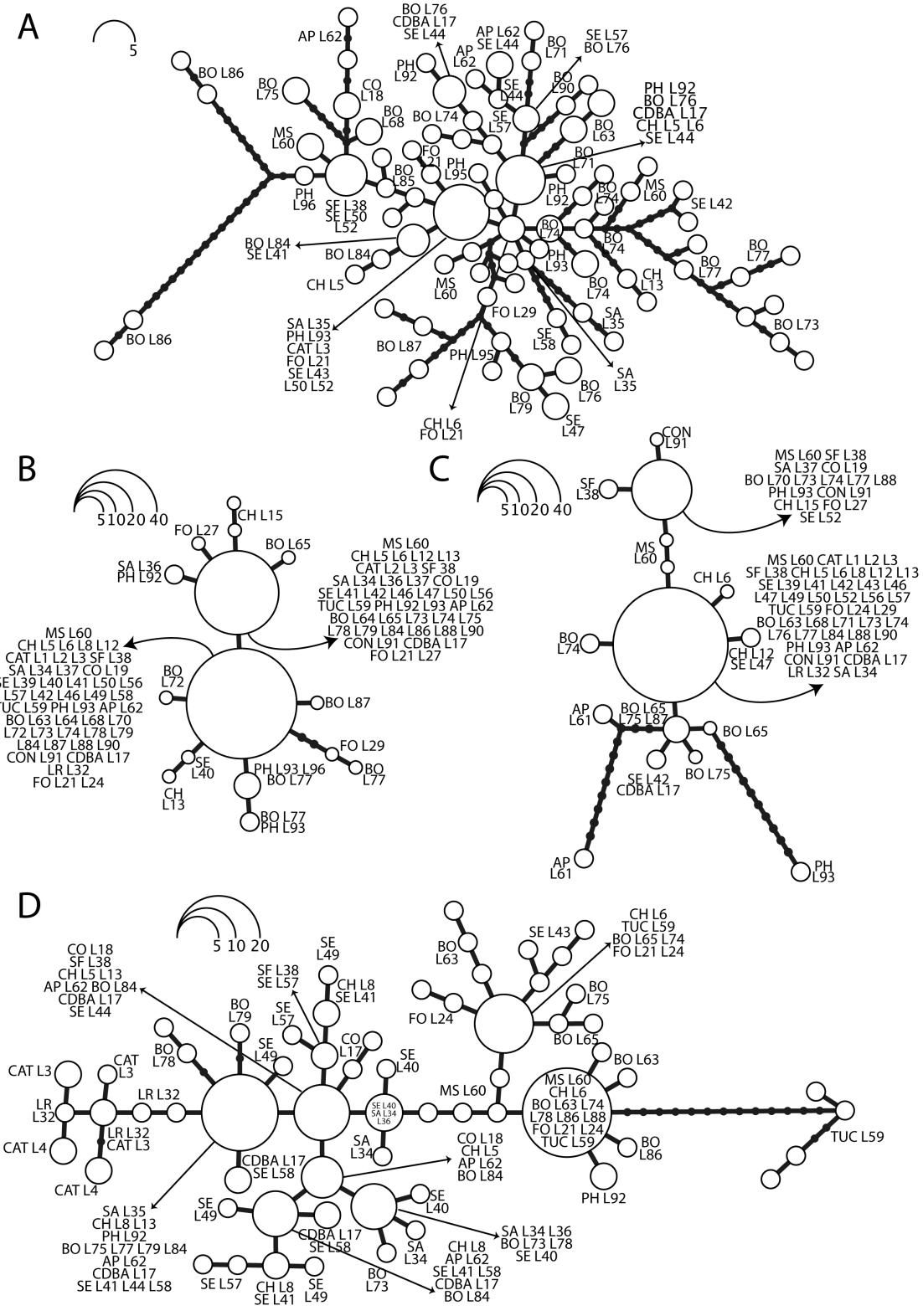

Figure 4: Nuclear haplotypes networks of *Leptodactylus bufonius.* A) MVZ 29-30, B) MVZ 39-40, C) RPL3, D) MVZ 47-48. Black dots indicate unsampled mutations. Circle size corresponds to haplotype frequency. Provinces or departments and sampling localities (L) follow Appendix 1. Locus names follow Figure 1 and Table 1.

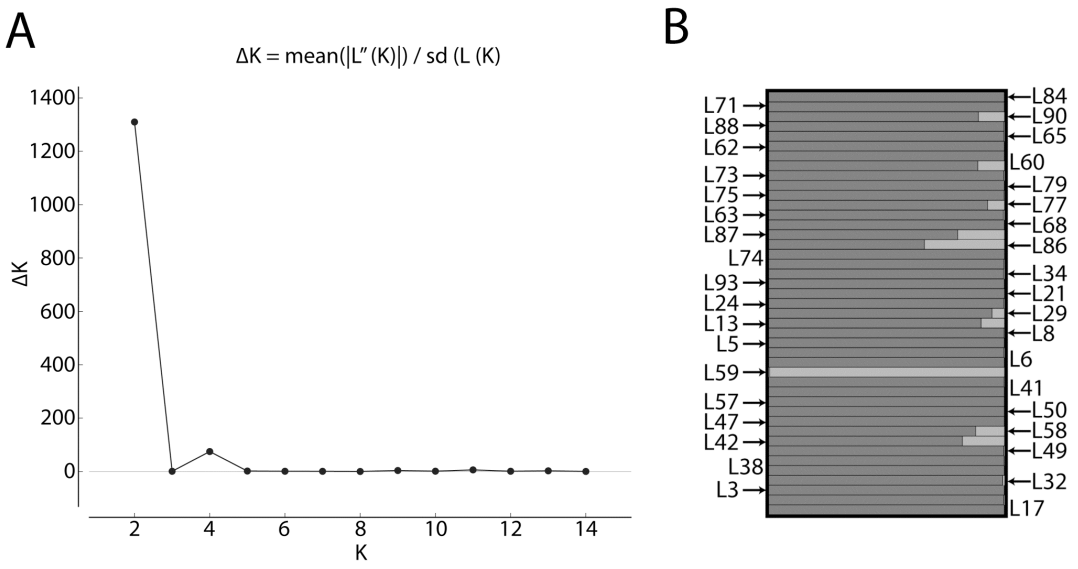

Figure 5: Bayesian clustering and specimen assignment using STRUCTURE. A) Value of ΔK as a function of K = 1-15. B) K = 2, bars correspond to each specimen and their membership coefficient (q). Sampling sites are plotted by specimen, following Appendix 1.

3.4 Isolation by distance (IBD), genetic diversity, and gene flow and genetic distance between populations of north and south of the Santiago del Estero gap

Mantel test shows positive correlation between genetic distance and geographic distance (r = 0.09258, p = 0.002), which corresponds to isolation-by-distance pattern. Genetic diversity is almost identical on all three analyses: complete data set, only populations from north of Santiago del Estero gap, and only populations from south of Santiago del Estero gap (Table 2). Haplotype diversity is relatively high (*Hd* = 0.923–0.927) and nucleotide diversity is low (*Pi* = 0.00712–0.00811). Among populations of north and south of Santiago del Estero gap, *Fst* and *Da* values are low (*Fst* = 0.01690, *Da* = 0.00013), without evidence of genetic differentiation between these groups of populations.

3.5 Demographic estimations

For all the analyses (complete data set, and populations from north and south of Santiago del Estero gap), MDA plots were bimodal with significant values (Fig. 6), supporting deviation from the model of sudden demographic expansion (Table 2). For complete data set and for populations of north of Santiago del Estero gap recent

population expansions were supported by all neutrality tests (*D*, *F*, and *R2*). However, for south of Santiago del Estero gap populations a recent expansion is only supported by Fu's *F*, with non-significant values for *D* and *R2* (Table 2).

Table 2: Genetic diversity (*Hd* and *Pi*), neutrality tests (Tajima's *D*, Fu's *Fs*, *R2* test), and Harpending's raggedness tests of goodness of MDA fit (*r*) within complete data set (*Leptodactylus bufonius*), and within populations north (*Leptodactylus bufonius* north) and south of Santiago del Estero gap (*Leptodactylus bufonius* south). Significant values ($p < 0.05$) are highlighted in bold.

	Hd	*Pi*	*D*	*Fs*	*R2*	*r*
Leptodactylus bufonius	0.924	0.00763	**-2.0238**	**-65.6964**	**0.0312**	**0.0113**
Leptodactylus bufonius north	0.924	0.00716	**-1.9337**	**-43.2410**	**0.0386**	**0.0157**
Leptodactylus bufonius south	0.925	0.00811	-1.3345	**-12.5412**	0.0622	**0.0140**

BSP analysis shows a pronounced increase on effective population size, estimated between 350,000 and 400,000 years ago (Fig. 7). Mean tMRCA is 915,500 years (95 % HPD 378,000–1,792,300 years).

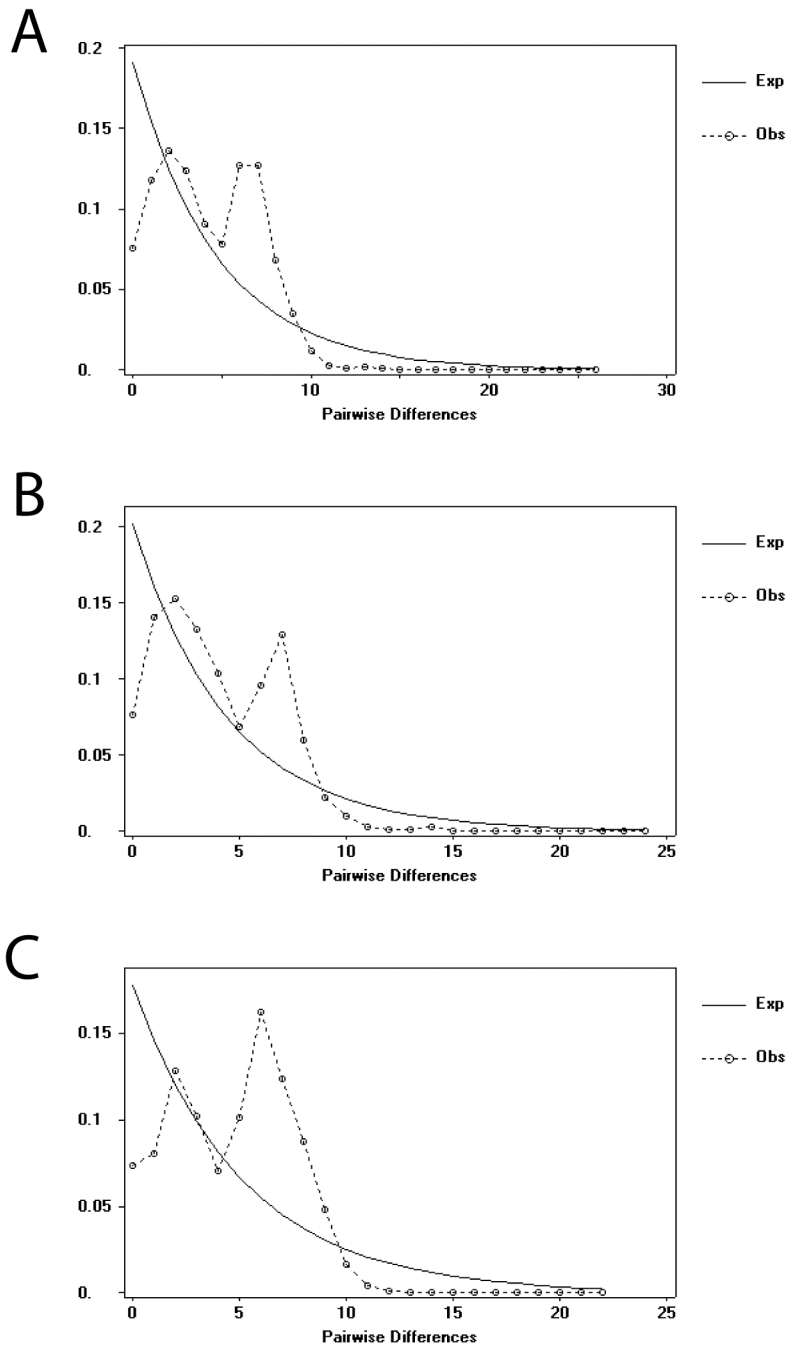

Figure 6: Mismatch distribution analyses (MDA) of *Leptodactylus bufonius*. A) complete data set, B) populations of north, and C) south of Santiago del Estero gap. Continuous and dotted lines correspond to expected (Exp) and observed (Obs) distributions of pairwise differences among haplotypes under a recent population expansion model.

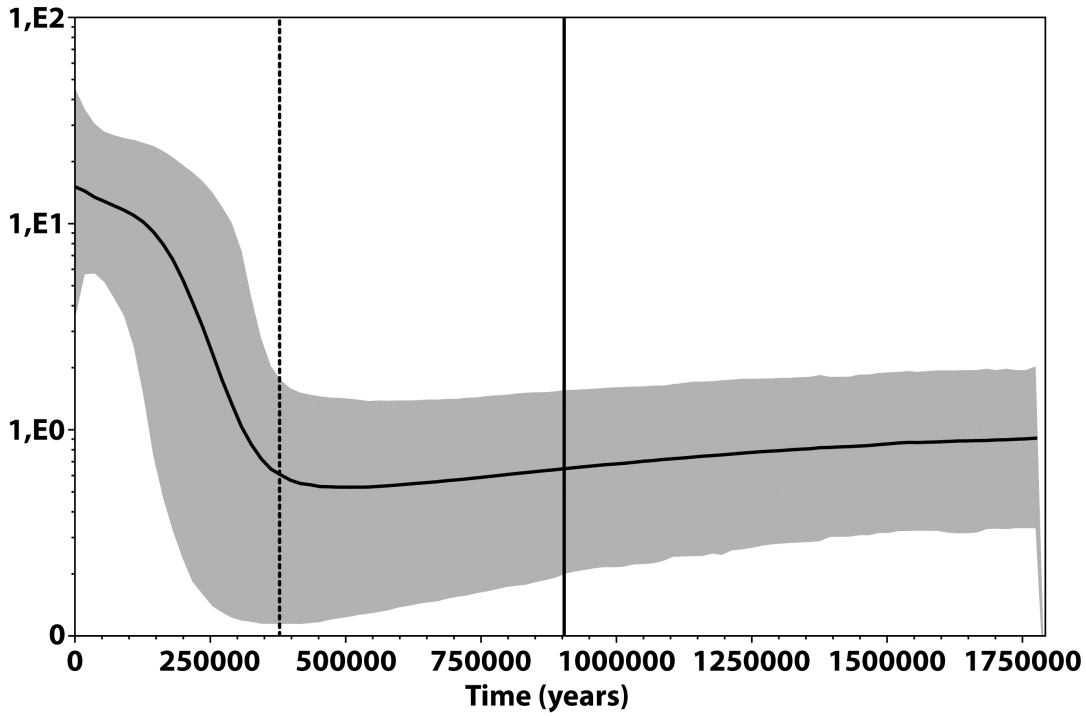

Figure 7: Bayesian skyline plots (BSP) of mitochondrial data of *Leptodactylus bufonius*. Horizontal continuous line and surrounded gray area correspond to mean and 95% highest posterior density limits for the effective population size, respectively. Vertical continuous and dotted lines correspond to mean and lowest estimated tMRCA, respectively. X axis is time in years and Y axis is effective population sizes.

4. Discussion

Here we used different methods to assess the genetic diversity of *Lept. bufonius*. All results support low genetic structure, without any genetic differentiation through the entire species distribution, with the exception of STRUCTURE analysis, which support two clusters (K = 2). However, one of these clusters corresponds to only one individual (L59 from Tucumán) and shows an ephemeral admixture in few others (Fig. 5). These admixtures are present in individuals from different areas, geographically distant from each other. An ephemeral admixture without any geographical pattern suggests some role of ancestral polymorphism (SLATKIN & HUDSON, 1991). The complete differentiation of the individual from Tucumán might be an artifact of our analysis due to the low number of loci and/or our sampling (YANG *etal.,* 2005). However, the use of low number of markers on Bayesian clustering analyses decrease the power of detects fine-scale genetic structure (e.g., THOMÉ *et al*., 2012; BRUNES *et al*., 2014), but judging for the results of all other

analyses this is not the case. On the other hand, we do not include individuals of this locality in the analyses with mitochondrial data (BI and haplotype network) that would allow to test the idea of a genetic break on this area. Furthermore, Tucumán is at the Chaco limit; marginal populations are expected to show high genetic differentiation and lower genetic diversity than populations of core area due to the lower effective population size and the low gene flow caused by isolation (ECKERT *et al*., 2008; HARDIE & HUTCHINGS, 2010). However, we cannot test any hypothesis about this geographic area with our current sampling.

The shallow intraspecific phylogeny (Fig. 2), together with high haplotype diversity (*Hd*), and low nucleotide diversity (*Pi*) correspond to recent population expansion after bottleneck events (EIZIRICK *et al*., 2001; ALTHOFF & PELLMYR, 2002; JOSEPH *et al*., 2002; STAMATIS *et al*., 2004). High values of haplotype diversity and low nucleotide diversity results from an accumulation of mutations, which consequently results on a high number of closely related haplotypes (i.e. many recently evolved haplotypes). A recent population expansion is also supported by network genealogies. A star-like pattern is present in almost all genealogies (mitochondrial and nuclear) (Figs. 3 and 4). This star-like pattern reflects a recent size population increase from a small population source (SLATKIN & HUDSON, 1991). The center of the star is the most frequent haplotype, being the ancestral haplotype, recent derivative haplotypes are connected to it independently by a low number of mutations (AVISE, 2000).

However, although with recent population expansion in their evolutionary history, species with current restricted population connectivity maintain evidence of local population differentiation (e.g., PALO *et al*., 2004; PABIJAN & BABIK, 2006). In *Lept. bufonius,* both mitochondrial and nuclear markers show high levels of admixture among populations, without any evidence of genetic differentiation through sampled localities. This suggests that low genetic structure of *Lept. bufonius* could be related to a combination of recent historical range expansion and current gene flow between populations (e.g., CHAN & ZAMUDIO, 2009; PABIJAN *et al*., 2015).

4.1 Santiago del Estero gap and main Chacoan rivers

We did not detect any genetic discontinuity along the geographic distribution of the species, and any evidence that supports the effects of Santiago del Estero gap and the Chacoan rivers on *Lept. bufonius* genetic structure.

Fst and *Da* between populations of north and south of the Santiago del Estero gap did not show any differentiation, supporting a continuous landscape without any dispersal barrier for *Lept. bufonius*. The occurrence of this species covers homogeneously the entire Chaco (Fig. 1), including a continuous distribution at the east of the Santiago del Estero gap. On the other hand, *Lepi*. *llanensis* shows a disjunct distribution separated by the Santiago del Estero gap, which could be explained by its narrower and more western distribution when compared to *Lept. bufonius* (see BRUSQUETTI *et al*., Chapters 1 and 2). The lack of geographical dispersal barriers on the east side of the gap and the current gene flow may mask the effect of the Santiago del Estero gap in *Lept. bufonius*.

Besides the geographic distribution, these two species present very distinct biologies. *Lepidobatrachus* species are highly specialized in living on semiarid environments (FAIVOVICH *et al*., 2014). These frogs are mostly aquatic when active in the rainy season, the reproductive mode is entirely aquatic, and spend dry periods buried and protected against desiccation by a dead-skin cocoon. These characteristics make these frogs clearly less vagile than *Lept. bufonius*, which exhibits some specializations to semiarid environments but these are mainly related with larvae survival (WELLS, 2007; SHOEMAKER & MCCLANAHAM, 1973), do not compromising the dispersal abilities of juveniles and adults (see discussion below).

Brusquetti *et al*. (see Chapter 1 and 2) showed different levels of influence of Pilcomayo and Bermejo Rivers between the two closely related *Lepi. laevis* and *Lepi. llanensis* species. Genetic structure differences between these species was related to different persistence time on the area influenced by these rivers. For *Lept. bufonius* we found a similar pattern of *Lepi. laevis*, without any signal of influence of these rivers on genetic structure (Fig. 5). In addition to the relatively short and recent persistence in the Chaco (tMRCA of about 0.9 Ma), the main difference between

Lept. bufonius and *Lepidobatrachus* species is the current gene flow on *Lept. bufonius*. Through the Quaternary, climatic changes promoted dry and humid periods, with at least two dry periods since the last glacial maximum (21,000 years ago). In dry periods the allochtonous rivers were highly ephemeral and seasonal (IRIONDO, 1993). This dynamic, including high levels of gene flow, was able to hide the traces of past effects of these rivers, as those still discernible in *Lepi. llanensis* genetic structure (see BRUSQUETTI *et al*., Chapter 1 and 2).

Although the Paraguay is a much more important river, we found a similar pattern to that found for *Lepi. laevis* (see Brusquetti *et al*. Chapter 2); exclusive haplotypes were connected by only one mutational step with the most frequent haplotype. This pattern suggests a recent colonization of the east side of the river. Myers (1982) suggested that the effect of this river on the geographic distribution of small mammals is related to habitat differences in each side of the river, and not because it is an impassable barrier. However, our locality records of the east side of the river are considered as part of the Chaco, like southern Mato Grosso do Sul state in Brazil (see SOUZA *et al*., 2010) and western Corrientes province in Argentina (MORRONE, 2001), making the hypothesis of habitat differences apparently unrealistic, at least in this specific case.

4.2 Recent range expansion of *Leptodactylus bufonius*

Besides phylogenies (BI and haplotype network) and genetic diversity, neutrality tests also support recent range expansion for *Lept. bufonius*. On MDA (Fig. 6) a multimodal topology may indicate either populations at equilibrium or in decline, or may correspond to signs of demographic bottleneck (SLATKIN & HUDSON, 1991; ROGERS & HARPENDING, 1992). As discussed above, we found evidence of recent population expansions from small source stock, and then we interpreted MDA results as evidence of bottleneck events. On BSP analysis (Fig. 7) we found an exponential increase on effective population size corresponding to the late Pleistocene (350,000–400,000 years). According to Ortiz-Jaguerizaguar & Cladera (2006), in southern South America (in latitudes superior to 15°), from the early Paleocene to the late Pleistocene climatic conditions became colder, dryer, and seasonal; but this cold and dry climate were intermittent during the Quaternary as a consequence of glacial

cycles. Main Pleistocene glaciations in South America include: (1) the most extensive Andean glaciation or Greatest Patagonian Glaciation (GPG, 1–1.2 Ma); (2) the coldest Pleistocene glaciation (0.7 Ma); (3) the last southern Patagonian glaciation (180 kya); and (4) the Last Glacial Maximum (LGM, 25–23 Ka) (RABASSA & CLAPPERTON, 1990; RUZZANTE *et al*., 2008). Although glaciers did not pass much more than 40º S northwards and persisted only in the Andes (see COSACOV *et al*., 2010), their cyclic advance and retreat has had considerable effects on the climate, resulting in expansions and contractions of arid and humid biomes (ORTIZ-JAUGUERIZAGUAR & CLADERA, 2006). Cold and dry climates resulted on contraction of the subtropical and tropical biomes (KALIN-ARROYO *et al*., 1988). Interglacial periods, posterior to main glaciations (GPG and coldest Pleistocene glaciation; ~1.1–0.7 Ma), match with *Lept. bufonius* range expansion. Several evidences support that GPG was significantly greater than LGM and the other last glaciations (McCULLOCH *et al*., 2000; RABASSA, 2008).

We hypothesize that these bigger glacial periods (GPG and coldest Pleistocene glaciation) has had a strong influence on the Chaco distribution with stronger effects on current southern Chaco due to glacial proximities and expansions of Patagonian Steppe to the north (COSACOV *et al*., 2010). Furthermore, during Pleistocene glaciations, a narrow area with high arid conditions, known as "Arid Diagonal", was distributed from the Atlantic coast of the Argentinian province of Chubut to central Tucumán at the eastern Andes flank (COSACOV *et al*., 2010), covering the current southwestern distribution of the species (La Rioja, Catamarca, and southwestern Santiago del Estero). Higher number of exclusive haplotypes on central-north distribution than south distribution (42 and 21 exclusive haplotypes, respectively) supports that during the Pleistocene, central and north Chaco were more stable than the south and especially more than southwestern Chaco. Paradoxically, in the southwestern we found relatively high haplotype diversity. However, this high diversity may be a result of a long persistence time, which could be responsible to maintain diversity through the time, or of distinct and recent colonization events by different lineages (e.g., HEWITT, 1996; PETIT *et al*., 2002; MRAZ *et al*., 2007). We considered the second scenario more plausible, taking into account that the "Arid Diagonal" reached the southwestern distribution of the species, and individuals of this area share several haplotypes with individuals from central and north populations.

4.3 Gene flow and dispersal abilities of *Leptodactylus bufonius*

Dispersal is defined as unidirectional movements from natal sites to breeding sites, but considering that breeding sites are not the pond of birth and also not part of the local population (SEMLITSCH, 2007). Different frog species show high dispersal capabilities at different life stages (juvenile or adult) and sometimes with a sex bias (SMITH & GREEN, 2005). Adults of Australian *Litoria aurea* frequently disperse only 500 m however some individuals were recorded at distances of about 10 km (PYKE & WHITE, 2001). In *Rana luteiventris* juveniles are great dispersers (> 5 km) (Funk *et al*., 2005), as well as juveniles of some *Rhinella* species that inhabit semiarid environments, like *Rh. schneideri* and *Rh. granulosa* in the Caatinga biome (NAVAS *et al*., 2004). Besides dispersal during juvenile and adult stages, tadpoles of pond-breeding anurans can be displaced by heavy rain floods as described for tadpoles of the Malagasy arid-adapted *Laliostoma labrosum* (PABIJAN *et al*., 2015).

Leptodactylus bufonius reproduces during the rainy season (CEI, 1949). At the border of temporally rain ponds males build a very characteristic mud chamber where the eggs are deposited in a foam nest (HEYER, 1969; MARTINS, 1988). Both, the subterranean chamber and the foam nest, protect the eggs and tadpoles against desiccation and high temperatures (DUELLMAN, 1992; DOWNIE & SMITH, 2003). Subsequent rains drag advanced stage tadpoles to breeding ponds where they complete their development (CEI, 1949). This reproductive biology results on high dependence on temporary ponds, which year after year can differ on surface and time of persistence, depending of annual rain variation (LESCANO *et al*., 2015). *Leptodactylus bufonius* occurs in virtually all the Chaco biome (Fig. 1), which presents a high variety of environmental conditions (PRADO, 1993). Although all Chaco extension generally shows the same stationary regime, with rainy summers and dry winters, this regime shows variation through the Chaco distribution, showing different periods of time for rainy and dry seasons, temperatures, and also rain volumes (AB'SABER, 1977; PRADO, 1993; CABRERA, 1994), becoming more arid in east-west direction (BUCHER, 1982). Besides this high ecological amplitude, rainfall at the Chaco is very variable in distribution and volume, generally represented by unpredictable and highly local rains, known as "aguaceros", resulting on extremely dry years in different regions (BUCHER, 1982). This stochasticity in occurrence and

persistence of breeding ponds may favor dispersal against philopatry; then, in explosive pond-breeding amphibians inhabiting arid biomes, like the Chaco, a low genetic structure is expected, but this expectation includes high vagility (PABIJAN, *et al*., 2015; CHAN & ZAMUDIO, 2009; RODRÍGUEZ *et al*., 2015; VENCES *et al*., 2002).

Levels of gene flow among populations depend on specific characteristics related to dispersion, like physiological tolerances and behavior, always with the landscape component included (CHAN & ZAMUDIO, 2009). For pond-breeding amphibians, ponds can be considered as habitat patches, nested inside a terrestrial less suitable matrix (HAMER & PARRIS, 2011; MARSH & TRENHAM, 2001; MAZEROLLE & DESROCHERS, 2005; RIBEIRO *et al*., 2011). Low levels of hostility of the terrestrial matrix would result on high levels of dispersion among closer populations and consequently high levels of gene flow.

Lescano *et al*. (2015) assess the anuran richness in natural (rain ponds) and artificial ponds (with cattle breeding purposes) in a Dry Chaco area, finding a positive correlation between abundance and well-preserved forests for *Lept. bufonius*. These authors interpreted that well-preserved forests are an advantage for persistence around breeding ponds, mainly due to the higher availability of refuges. However, besides refuge opportunities, if this well-preserved forest connects different breeding ponds, dispersal capabilities of this species may increase substantially because forest provide cover from predators and decreases risk of desiccation. That is, forest cover makes the terrestrial matrix less hostile. Then, the positive correlation between high abundance of *Lept. bufonius* and forested ponds can be interpreted as the result of high levels of dispersion among ponds due to a less hostile matrix. In degraded areas of the Chaco (mainly for cattle breeding purposes) soil moisture decreases and salinity increases (ABRIL & BUCHER, 1999), both characteristics that enhance the risk of desiccation in frogs. Some Chacoan frogs have morphological and behavioral adaptations related to water balance on arid environments, like thick dorsal skin, pelvic patch, burrowing behavior, cocoon formation, and water-conserving posture (WELLS, 2007; FAIVOVICH *et al*., 2014). However, only thick dorsal skin and pelvic patch would be related with dispersal capabilities on a semiarid environment, and in *Lept. bufonius* only pelvic patch is present. The pelvic patch is a highly vascularized area at the

ventral surface of the body specialized in absorbing high volumes of water in short periods of time (HILLMAN et al., 2009), but it is totally useless in habitats with extremely low levels of moisture.

Anthropogenic changes may decrease individuals dispersion among populations by habitat loss and forest modification, but may facilitate generalist species occurrence. Artificial ponds are constantly constructed in the Chaco for cattle breeding, increasing the density of breading ponds for anurans. Lescano *et al*. (2015) found that *Lept. bufonius* were the most abundant species in artificial ponds, which suggests a high adaptability to breed in this kind of habitat. Despite of the density of breeding ponds, increase in gene flow may be associated to species that, besides high dispersion abilities, also show low site fidelity (CHAN & ZAMUDIO, 2009). The fact that males of *Lept. bufonius* construct nests and call close or inside of them (CRUMP, 1995; READING & JOFRÉ, 2003) suggests some territoriality and consequently some philopatry. However, other aspects of the reproductive behavior of this species may influence on levels of site-fidelity. To achieve good water insulation, mud chambers are only used after being dried (at least 24 hours), during this drying time chambers are abandoned. Males return to call close or inside the chambers, but it is not known if the male returns to the chamber he built or if males construct more than one chamber. Furthermore, chambers are used only once because after the deposit of the eggs the female closes the chamber and subsequent rains destroy it and drag tadpoles into ponds (READING & JOFRÉ, 2003). This behavior suggests temporary territoriality and site fidelity on males, which would be able to disperse after each reproductive event. Furthermore, *Lept. bufonius* spermatogenesis lasts almost all year (CEI, 1949) indicating that males are capable to reproduce across the entire rainy season (SCHALK & SAENZ, 2015) and also after.

Finally, the system of short distance dispersal or limited dispersal that we suggest for *Lept. bufonius* is supported by the positive correlation among genetic and geographic distances (PABIJAN *et al*., 2015). This pattern of isolation-by-distance suggests that the connections among populations are maintained by dispersion between closer breeding ponds following a stepping-stone model (CHAN & ZAMUDIO, 2009). Populations separated by long distances are connected through

high and continuous levels of gene flow among neighboring populations (CHAN & ZAMUDIO, 2009).

5. CONCLUSIONS

As other species of frogs associated to semiarid environments, *Lept. bufonius* shows low genetic structure and none differentiation among populations. This pattern could be the result of recent range expansions and current gene flow through a continuous landscape. We did not find evidences of any influence of putative barriers in *Lept. bufonius* genetic structure. Range expansions match with the interglacial periods of the larger Pleistocene glaciations. These glaciations displaced the southern limits of Chaco distribution to the north. Furthermore, between the current Tucumán and Chubut Argentinian provinces a highly arid diagonal was formed covering the current southwestern distribution of the species, where we found evidence of posterior expansions. Gene flow in *Lept. bufonius* follows a stepping-stone model, with high levels of short distance dispersal between closer populations, and consequently increasing the connectivity among long distance populations.

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Appendix 1: Voucher information. Abbreviations: Ministerio de Agricultura y Ganadería (MAG), National Route (NR), Provincial Route (PR), Reserva Natural (RN), Parque Nacional (PN).

Id	Country	Province/Department	Locality	Haplotype	Locality code	Lat	Long
DB 2655	Argentina	Catamarca	Albigasta, 6 km S from El Corralito	H4	L1	-28.585	-65.230
LGE 2321	Argentina	Catamarca	Las Tunas	H43	L2	-28.383	-65.300
LGE 5544	Argentina	Catamarca	3.5 km NW from San Martín	H55	L3	-29.194	-65.799
LGE 5545	Argentina	Catamarca	3.5 km NW from San Martín	H56	L3	-29.194	-65.799
LGE 5563	Argentina	Catamarca	3.5 km NW from San Martín	H54	L3	-29.194	-65.799
LGE 5564	Argentina	Catamarca	3.5 km NW from San Martín	H54	L3	-29.194	-65.799
LGE 5588A	Argentina	Catamarca	2 km W from San Martín	H54	L4	-29.222	-65.798
LGE 5588B	Argentina	Catamarca	2 km W from San Martín	H57	L4	-29.222	-65.798
LGE 5588C	Argentina	Catamarca	2 km W from San Martín	H18	L4	-29.222	-65.798
DB 8551	Argentina	Chaco	16 km S from Misión Nueva Pompeya	H13	L5	-25.078	-61.494
DB 6927	Argentina	Chaco	25 km NE from Fuerte Esperanza		L6	-25.069	-61.613
DB 6936	Argentina	Chaco	25 km NE from Fuerte Esperanza		L6	-25.069	-61.613
DB 6849	Argentina	Chaco	3 km S from El Sauzalito	H10	L7	-24.458	-61.681
DB 6986	Argentina	Chaco	35 km S from Comandancia Frías		L8	-24.873	-62.326
LGE 5325	Argentina	Chaco	6.7 km NE from Villa Ángela	H43	L9	-27.544	-60.771
LGE 5326	Argentina	Chaco	6.7 km NE from Villa Ángela	H5	L9	-27.544	-60.771
DB 8514	Argentina	Chaco	15 km NW from Misión Nueva Pompeya	H5	L10	-24.835	-61.584
LGE 5235	Argentina	Chaco	Charata, 6 km SE from PR Nº 94	H48	L11	-27.293	-61.150
LGE 5236	Argentina	Chaco	Charata, 6 km SE from PR Nº 94	H49	L11	-27.293	-61.150
LGE 5249	Argentina	Chaco	Charata, 6 km SE from PR Nº 94		L11	-27.293	-61.150
LGE 5250	Argentina	Chaco	Charata, 6 km SE from PR Nº 94	H50	L11	-27.293	-61.150
LGE 5231	Argentina	Chaco	Charata, PR Nº 94	H47	L12	-27.260	-61.198

Id	Country	Province/Department	Locality	Haplotype	Locality code	Lat	Long
LGE 5232	Argentina	Chaco	Charata, PR Nº 94	H47	L12	-27.260	-61.198
DB 6844	Argentina	Chaco	El Sauzalito		L13	-24.439	-61.683
LGE 5262	Argentina	Chaco	Mesón de Hierro	H50	L14	-27.403	-60.932
LGE 5420	Argentina	Chaco	6 km NW from Santa Sylvina		L15	-27.802	-61.088
DB 7728	Argentina	Chaco	4 km NW from Wichi	H11	L16	-24.672	-61.461
LGE 2318	Argentina	Córdoba	Totoralejos		L17	-29.621	-64.836
LGE 2319	Argentina	Córdoba	Totoralejos		L17	-29.621	-64.836
DB 1574	Argentina	Corrientes	Paraje Perichón		L18	-27.424	-58.744
DB 5305	Argentina	Corrientes	Perugorría, Estancia El Oscuro		L19	-29.210	-58.526
LGE 8155	Argentina	Formosa	Laguna Yema	H12	L20	-24.564	-60.637
LGE 8177	Argentina	Formosa	Laguna Yema	H5	L20	-24.564	-60.637
LGE 8191	Argentina	Formosa	Laguna Yema	H64	L21	-24.220	-61.211
LGE 8192	Argentina	Formosa	Laguna Yema	H5	L21	-24.220	-61.211
LGE 8091	Argentina	Formosa	Las Lomitas	H61	L22	-24.566	-60.637
LGE 8092	Argentina	Formosa	Las Lomitas	H11	L23	-24.549	-60.642
LGE 8095	Argentina	Formosa	Las Lomitas	H62	L24	-24.628	-60.618
LGE 8101	Argentina	Formosa	Las Lomitas	H12	L25	-24.595	-60.629
LGE 8118	Argentina	Formosa	Las Lomitas	H63	L26	-24.546	-60.643
LGE 8050	Argentina	Formosa	Pirané	H59	L27	-25.509	-58.986
LGE 8051	Argentina	Formosa	Pirané	H60	L27	-25.509	-58.986
DB 6280	Argentina	Formosa	Pirané, 1.5 km NW from PR Nº 3	H5	L28	-25.677	-59.081
DB 6282	Argentina	Formosa	Pirané, 1.5 km NW from PR Nº 3	H6	L28	-25.677	-59.081
DB 6283	Argentina	Formosa	Pirané, 1.5 km NW from PR Nº 3	H6	L28	-25.677	-59.081
DB 6284	Argentina	Formosa	Pirané, 1.5 km NW from PR Nº 3	H7	L28	-25.677	-59.081

Id	Country	Province/Department	Locality	Haplotype	Locality code	Lat	Long
GS 3580	Argentina	Formosa	Reserva El Bagual		L29	-26.181	-58.944
DB 6328	Argentina	Formosa	7 km SE from Juan G. Bazán	H8	L30	-24.586	-60.772
DB 6329	Argentina	Formosa	7 km SE from Juan G. Bazán	H9	L30	-24.586	-60.772
DB 6330	Argentina	Formosa	7 km SE from Juan G. Bazán	H5	L30	-24.586	-60.772
LGE 5516	Argentina	La Rioja	24 km SE from La Rioja	H27	L31	-29.592	-66.714
LGE 5517	Argentina	La Rioja	24 km SE from La Rioja	H52	L31	-29.593	-66.714
LGE 5518	Argentina	La Rioja	24 km SE from La Rioja	H46	L31	-29.593	-66.714
LGE 5519	Argentina	La Rioja	24 km SE from La Rioja	H46	L31	-29.593	-66.714
LGE 5524	Argentina	La Rioja	30 km S from La Rioja	H14	L32	-29.639	-66.682
LGE 5525	Argentina	La Rioja	30 km S from La Rioja	H53	L32	-29.639	-66.682
LGE 5526	Argentina	La Rioja	30 km S from La Rioja	H54	L32	-29.639	-66.682
LGE 5528	Argentina	La Rioja	30 km S from La Rioja	H46	L32	-29.639	-66.682
LGE 5529	Argentina	La Rioja	30 km S from La Rioja		L32	-29.639	-66.682
LGE 8228	Argentina	Salta	Fortín Dragones	H18	L33	-23.297	-63.252
LGE 8229	Argentina	Salta	Fortín Dragones	H5	L33	-23.297	-63.252
LGE 8238	Argentina	Salta	Fortín Dragones	H65	L34	-23.377	-63.085
LGE 8239	Argentina	Salta	Fortín Dragones	H65	L34	-23.377	-63.085
DB 4864	Argentina	Salta	10 km W from El Ocultar		L35	-23.824	-63.490
DB 4798	Argentina	Salta	Pichanal		L36	-25.400	-64.150
DB 4859	Argentina	Salta	11 km SE from NR Nº 34	H5	L37	-23.085	-63.835
DB 4430	Argentina	Santa Fe	Reconquista	H5	L38	-29.101	-60.129
DB 4437	Argentina	Santa Fe	Reconquista	H5	L38	-29.101	-60.129
GS 3105	Argentina	Santiago del Estero	13 km W from Estación Atamisqui		L39	-28.479	-64.070
LGE 5498	Argentina	Santiago del Estero	Añatuya	H5	L40	-28.466	-62.834

Id	Country	Province/Department	Locality	Haplotype	Locality code	Lat	Long
LGE 8261	Argentina	Santiago del Estero	Campo Gallo	H5	L41	-26.569	-62.768
LGE 8262	Argentina	Santiago del Estero	Campo Gallo	H10	L41	-26.569	-62.768
LGE 8263	Argentina	Santiago del Estero	Campo Gallo	H5	L41	-26.569	-62.768
LGE 8269	Argentina	Santiago del Estero	Campo Gallo	H5	L41	-26.569	-62.768
LGE 8270	Argentina	Santiago del Estero	Campo Gallo		L41	-26.569	-62.768
DB 8003	Argentina	Santiago del Estero	Laprida	H12	L42	-28.382	-64.530
DB 8005	Argentina	Santiago del Estero	Laprida		L42	-28.382	-64.530
LGE P16A	Argentina	Santiago del Estero	NR 34	H5	L43	-28.491	-63.054
LGE P16B	Argentina	Santiago del Estero	NR 34		L43	-28.491	-63.054
LGE 5627	Argentina	Santiago del Estero	2.5 km N from Quimilí	H58	L44	-27.625	-62.429
LGE 5450	Argentina	Santiago del Estero	2 km S from Los Juries	H50	L45	-28.481	-62.117
LGE 5451	Argentina	Santiago del Estero	2 km S from Los Juries	H12	L45	-28.481	-62.117
LGE 5453	Argentina	Santiago del Estero	2 km S from Los Juries	H5	L45	-28.481	-62.117
LGE 5436	Argentina	Santiago del Estero	15 km E from Los Juries	H48	L46	-28.473	-61.956
LGE 5443	Argentina	Santiago del Estero	15 km E from Los Juries	H5	L46	-28.473	-61.956
LGE 5455	Argentina	Santiago del Estero	Los Juries	H5	L47	-28.471	-62.108
DB 1028	Argentina	Santiago del Estero	NR Nº 9	H3	L48	-28.667	-64.075
LGE 128	Argentina	Santiago del Estero	NR Nº 9	H46	L48	-28.667	-64.075
LGE 108	Argentina	Santiago del Estero	NR Nº 9		L49	-28.641	-64.087
LGE 8342	Argentina	Santiago del Estero	Suncho Corral	H68	L50	-27.642	-63.481
LGE 8346	Argentina	Santiago del Estero	Suncho Corral	H5	L51	-27.726	-63.506
LGE 8347	Argentina	Santiago del Estero	Suncho Corral	H45	L52	-27.822	-63.472
LGE 8285	Argentina	Santiago del Estero	Tintina	H5	L53	-26.845	-62.930
LGE 8288	Argentina	Santiago del Estero	Tintina	H67	L54	-26.896	-62.924

Id	Country	Province/Department	Locality	Haplotype	Locality code	Lat	Long
LGE 8291	Argentina	Santiago del Estero	Tintina	H12	L55	-27.004	-62.752
LGE 8278	Argentina	Santiago del Estero	Tintina, road to Libertad		L56	-27.118	-62.848
LGE 8281	Argentina	Santiago del Estero	Tintina, road to Libertad	H18	L56	-27.118	-62.848
LGE 8283	Argentina	Santiago del Estero	Tintina, road to Libertad	H66	L57	-27.139	-63.056
LGE 2515	Argentina	Santiago del Estero	Villa La Punta		L58	-28.414	-64.756
DB 8024	Argentina	Tucumán	Atahona		L59	-27.413	-65.290
DB 8025	Argentina	Tucumán	Atahona		L59	-27.413	-65.290
CFBH 30490	Brasil	Mato Grosso do Sul	20 km E from Porto Murtinho	H1	L60	-21.710	-57.721
CFBH 30491	Brasil	Mato Grosso do Sul	20 km E from Porto Murtinho	H2	L60	-21.710	-57.721
CFBH 30492	Brasil	Mato Grosso do Sul	20 km E from Porto Murtinho	H2	L60	-21.710	-57.721
IIBPH 2209	Paraguay	Alto Paraguay	Carmelo Peralta	H25	L61	-21.671	-57.943
IIBPH 2213	Paraguay	Alto Paraguay	Carmelo Peralta	H18	L61	-21.671	-57.943
IIBPH 2240	Paraguay	Alto Paraguay	Carmelo Peralta	H26	L62	-21.691	-58.298
IIBPH 1848	Paraguay	Boquerón	Buzarquis	H21	L63	-22.699	-60.553
IIBPH 1849	Paraguay	Boquerón	Buzarquis	H5	L63	-22.699	-60.553
IIBPH 1850	Paraguay	Boquerón	Buzarquis	H5	L63	-22.699	-60.553
IIBPH 1852	Paraguay	Boquerón	Buzarquis	H5	L63	-22.699	-60.553
IIBPH 2871	Paraguay	Boquerón	Teniente Montanía-Madrejón road		L64	-21.625	-59.901
IIBPH 2882	Paraguay	Boquerón	Teniente Montanía-Madrejón road	H24	L65	-21.564	-59.890
IIBPH 2883	Paraguay	Boquerón	Teniente Montanía-Madrejón road	H5	L65	-21.564	-59.890
IIBPH 2898	Paraguay	Boquerón	Teniente Montanía-Madrejón road	H44	L66	-21.498	-59.879
IIBPH 2904	Paraguay	Boquerón	Teniente Montanía-Madrejón road, Pitiantuta	H45	L67	-21.408	-59.807
IIBPH 2823	Paraguay	Boquerón	road to Puesto Militar Capitán Joel Estigarribia	H42	L68	-22.589	-61.384
IIBPH 2824	Paraguay	Boquerón	road to Puesto Militar Capitán Joel Estigarribia	H18	L68	-22.589	-61.384

Id	Country	Province/Department	Locality	Haplotype	Locality code	Lat	Long
IIBPH 2838	Paraguay	Boquerón	road to Puesto Militar Capitán Joel Estigarribia	H5	L69	-22.600	-61.428
IIBPH 2845	Paraguay	Boquerón	road to Puesto Militar Capitán Joel Estigarribia	H11	L70	-22.603	-61.432
IIBPH 2846	Paraguay	Boquerón	road to Puesto Militar Capitán Joel Estigarribia	H43	L70	-22.603	-61.432
IIBPH 2340	Paraguay	Boquerón	Estancia Teniente Prieto		L71	-21.111	-61.371
IIBPH 2341	Paraguay	Boquerón	Estancia Teniente Prieto	H5	L71	-21.111	-61.371
IIBPH 2342	Paraguay	Boquerón	Estancia Teniente Prieto	H38	L71	-21.111	-61.371
IIBPH 2343	Paraguay	Boquerón	Estancia Teniente Prieto	H39	L71	-21.111	-61.371
IIBPH 2256	Paraguay	Boquerón	PN Teniente Enciso	H27	L72	-21.201	-61.597
IIBPH 2280	Paraguay	Boquerón	PN Teniente Enciso	H28	L72	-21.201	-61.597
IIBPH 2281	Paraguay	Boquerón	PN Teniente Enciso	H29	L72	-21.201	-61.597
IIBPH 2282	Paraguay	Boquerón	PN Teniente Enciso	H30	L72	-21.201	-61.597
IIBPH 2283	Paraguay	Boquerón	PN Teniente Enciso	H5	L72	-21.201	-61.597
IIBPH 2284	Paraguay	Boquerón	PN Teniente Enciso	H28	L72	-21.201	-61.597
IIBPH 2285	Paraguay	Boquerón	PN Teniente Enciso	H18	L72	-21.201	-61.597
IIBPH 1789	Paraguay	Boquerón	15 km S from Mariscal Estigarribia		L73	-22.166	-60.496
IIBPH 1790	Paraguay	Boquerón	15 km S from Mariscal Estigarribia	H20	L73	-22.166	-60.496
IIBPH 1791	Paraguay	Boquerón	15 km S from Mariscal Estigarribia	H5	L73	-22.166	-60.496
IIBPH 1649	Paraguay	Boquerón	RN Estancia Fortín Salazar	H16	L74	-23.082	-59.291
IIBPH 1650	Paraguay	Boquerón	RN Estancia Fortín Salazar	H5	L74	-23.082	-59.291
IIBPH 1651	Paraguay	Boquerón	RN Estancia Fortín Salazar	H16	L74	-23.082	-59.291
IIBPH 1653	Paraguay	Boquerón	RN Estancia Fortín Salazar		L74	-23.082	-59.291
IIBPH 1690	Paraguay	Boquerón	RN Estancia Fortín Salazar		L74	-23.082	-59.291
IIBPH 1739	Paraguay	Boquerón	Estancia Aguada Siete		L75	-22.442	-61.762
IIBPH 1743	Paraguay	Boquerón	Estancia Cotorrita		L76	-22.296	-61.795

Id	Country	Province/Department	Locality	Haplotype	Locality code	Lat	Long
IIBPH 1744	Paraguay	Boquerón	Estancia Cotorrita	H19	L76	-22.296	-61.795
IIBPH 1721	Paraguay	Boquerón	Campo Jurado	H18	L77	-22.669	-61.528
IIBPH 1722	Paraguay	Boquerón	Campo Jurado		L77	-22.669	-61.528
IIBPH 2173	Paraguay	Boquerón	Loma Plata		L78	-22.356	-59.452
IIBPH 2174	Paraguay	Boquerón	Loma Plata	H23	L78	-22.356	-59.452
IIBPH 2175	Paraguay	Boquerón	Loma Plata	H14	L78	-22.356	-59.452
IIBPH 2181	Paraguay	Boquerón	Loma Plata	H24	L78	-22.356	-59.452
IIBPH 2182	Paraguay	Boquerón	Loma Plata		L78	-22.356	-59.452
IIBPH 2183	Paraguay	Boquerón	Loma Plata	H12	L78	-22.356	-59.452
IIBPH 2185	Paraguay	Boquerón	Loma Plata	H16	L78	-22.356	-59.452
IIBPH 1702	Paraguay	Boquerón	Transchaco road-Loma Plata cross		L79	-22.535	-59.836
IIBPH 1703	Paraguay	Boquerón	Transchaco road-Loma Plata cross	H17	L79	-22.535	-59.836
IIBPH 2363	Paraguay	Boquerón	PN Defensores del Chaco	H18	L80	-20.527	-60.210
IIBPH 2364	Paraguay	Boquerón	PN Defensores del Chaco	H5	L81	-20.525	-60.213
IIBPH 2370	Paraguay	Boquerón	PN Defensores del Chaco	H5	L82	-20.532	-60.199
IIBPH 2373	Paraguay	Boquerón	PN Defensores del Chaco	H40	L83	-20.544	-60.173
IIBPH 2376	Paraguay	Boquerón	PN Defensores del Chaco	H41	L84	-20.521	-60.224
IIBPH 2385	Paraguay	Boquerón	PN Defensores del Chaco		L85	-20.565	-60.129
IIBPH 2912	Paraguay	Boquerón	Pirizal	H27	L86	-23.073	-60.717
IIBPH 2919	Paraguay	Boquerón	Pirizal	H5	L87	-23.073	-60.654
IIBPH 2289	Paraguay	Boquerón	La Patria-Infante Rivarola road	H14	L88	-21.591	-62.118
IIBPH 2295	Paraguay	Boquerón	La Patria-Infante Rivarola road	H31	L88	-21.591	-62.118
IIBPH 2296	Paraguay	Boquerón	La Patria-Infante Rivarola road	H31	L88	-21.591	-62.118
IIBPH 2297	Paraguay	Boquerón	La Patria-Infante Rivarola road	H32	L88	-21.591	-62.118

Id	Country	Province/Department	Locality	Haplotype	Locality code	Lat	Long
IIBPH 2302	Paraguay	Boquerón	La Patria-Infante Rivarola road	H33	L88	-21.591	-62.118
IIBPH 2305	Paraguay	Boquerón	La Patria-Infante Rivarola road	H34	L88	-21.591	-62.118
IIBPH 2306	Paraguay	Boquerón	La Patria-Infante Rivarola road	H35	L88	-21.591	-62.118
IIBPH 2307	Paraguay	Boquerón	La Patria-Infante Rivarola road	H36	L88	-21.591	-62.118
IIBPH 2315	Paraguay	Boquerón	La Patria-Infante Rivarola road	H32	L89	-21.621	-61.984
IIBPH 2316	Paraguay	Boquerón	La Patria-Infante Rivarola road	H37	L90	-21.621	-61.984
IIBPH 921	Paraguay	Concepción	Vallemí		L91	-22.123	-57.915
IIBPH 922	Paraguay	Concepción	Vallemí		L91	-22.123	-57.915
IIBPH 1491	Paraguay	Presidente Hayes	MAG Experimental Station		L92	-22.668	-59.765
IIBPH 1492	Paraguay	Presidente Hayes	MAG Experimental Station		L92	-22.668	-59.765
IIBPH 2015	Paraguay	Presidente Hayes	Parador Bufalo Bill		L93	-23.433	-58.906
IIBPH 2016	Paraguay	Presidente Hayes	Parador Bufalo Bill	H5	L93	-23.433	-58.906
IIBPH 2018	Paraguay	Presidente Hayes	Parador Bufalo Bill	H22	L93	-23.433	-58.906
IIBPH 2019	Paraguay	Presidente Hayes	Parador Bufalo Bill	H12	L93	-23.433	-58.906
IIBPH 1603	Paraguay	Presidente Hayes	Montana Ranch	H14	L94	-23.445	-59.961
IIBPH 1604	Paraguay	Presidente Hayes	Montana Ranch	H15	L94	-23.445	-59.961
IIBPH 1619	Paraguay	Presidente Hayes	Montana Ranch		L95	-23.421	-59.960
IIBPH 1623	Paraguay	Presidente Hayes	Montana Ranch	H12	L96	-23.414	-59.973

Appendix 2: GenBank accession of *Leptodactylus bufonius* sequences used in this article. * corresponds to *Leptodactylus troglodytes* sequence used as outgroup. (Upon acceptance)

Samples ID	CO1	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
DB 2655	x		x		x
LGE 2321	x		x		x
LGE 5544	x				
LGE 5545	x				
LGE 5563	x	x	x	x	x
LGE 5564	x				
LGE 5588A	x				
LGE 5588B	x				
LGE 5588C	x			x	
DB 8551	x	x	x	x	x
DB 6927		x	x		x
DB 6936			x	x	x
DB 6849	x				
DB 6986			x	x	x
LGE 5325	x				
LGE 5326	x				
DB 8514	x				
LGE 5235	x				
LGE 5236	x				
LGE 5249	x				
LGE 5250	x				
LGE 5231	x				
LGE 5232	x		x		x
DB 6844		x	x	x	x
LGE 5262	x				
LGE 5420			x		x
DB 7728	x				
LGE 2318		x	x	x	x
LGE 2319			x	x	x
DB 1574		x		x	
DB 5305			x		x
LGE 8155	x				
LGE 8177	x				
LGE 8191	x				
LGE 8192	x	x	x	x	
LGE 8091	x				
LGE 8092	x				
LGE 8095	x		x	x	x

Samples ID	CO1	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
LGE 8101	x				
LGE 8118	x				
LGE 8050	x				
LGE 8051	x		x		x
DB 6280	x				
DB 6282	x				
DB 6283	x				
DB 6284	x				
GS 3580		x	x		x
DB 6328	x				
DB 6329	x				
DB 6330	x				
LGE 5516	x				
LGE 5517	x				
LGE 5518	x				
LGE 5519	x				
LGE 5524	x				
LGE 5525	x				
LGE 5526	x				
LGE 5528	x				
LGE 5529			x	x	x
LGE 8228	x				
LGE 8229	x				
LGE 8238	x				
LGE 8239			x	x	x
DB 4864		x		x	
DB 4798			x	x	
DB 4859	x		x		x
DB 4430	x	x	x		x
DB 4437	x		x	x	x
GS 3105			x		x
LGE 5498	x		x	x	
LGE 8261	x	x	x		x
LGE 8262	x		x	x	x
LGE 8263	x				
LGE 8269	x				
LGE 8270	x				
DB 8003	x				
DB 8005		x	x		x
LGE P16A	x	x			x
LGE P16B				x	x

Samples ID	CO1	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
LGE 5627	x	x		x	
LGE 5450	x				
LGE 5451	x				
LGE 5453	x				
LGE 5436	x		x		x
LGE 5443	x				
LGE 5455	x	x	x		x
DB 1028	x				
LGE 128	x				
LGE 108			x	x	x
LGE 8342	x	x	x		x
LGE 8346	x				
LGE 8347	x	x			x
LGE 8285	x				
LGE 8288	x				
LGE 8291	x				
LGE 8278			x		x
LGE 8281	x				
LGE 8283	x	x	x	x	x
LGE 2515		x	x	x	
DB 8024			x	x	
DB 8025			x	x	x
CFBH 30490	x	x	x	x	x
CFBH 30491	x	x	x	x	x
CFBH 30492	x				
IIBPH 2209	x				x
IIBPH 2213	x				
IIBPH 2240	x	x	x	x	x
IIBPH 1848	x		x	x	x
IIBPH 1849	x	x		x	
IIBPH 1850	x				
IIBPH 1852	x				
IIBPH 2871			x	x	
IIBPH 2882			x	x	x
IIBPH 2883	x				
IIBPH 2898	x				
IIBPH 2904	x				
IIBPH 2823	x				
IIBPH 2824	x	x	x	x	x
IIBPH 2838	x				
IIBPH 2845	x				

Samples ID	CO1	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
IIBPH 2846	x		x		x
IIBPH 2340		x		x	x
IIBPH 2341	x				
IIBPH 2342	x				
IIBPH 2343	x				
IIBPH 2256	x				
IIBPH 2280	x		x		
IIBPH 2281	x				
IIBPH 2282	x				
IIBPH 2283	x				
IIBPH 2284	x				
IIBPH 2285	x				
IIBPH 1789			x		x
IIBPH 1790	x	x	x	x	x
IIBPH 1791	x				
IIBPH 1649	x				
IIBPH 1650	x				
IIBPH 1651	x				
IIBPH 1653		x	x		x
IIBPH 1690		x	x	x	x
IIBPH 1739		x	x	x	x
IIBPH 1743		x			x
IIBPH 1744	x				
IIBPH 1721	x				
IIBPH 1722		x	x	x	x
IIBPH 2173			x	x	
IIBPH 2174	x				
IIBPH 2175	x				
IIBPH 2181	x				
IIBPH 2182					
IIBPH 2183	x				
IIBPH 2185	x				
IIBPH 1702		x	x	x	
IIBPH 1703	x				
IIBPH 2363	x				
IIBPH 2364	x				
IIBPH 2370	x				
IIBPH 2373	x				
IIBPH 2376	x	x	x	x	x
IIBPH 2385		x			
IIBPH 2912	x	x		x	

Samples ID	CO1	MVZ 29-30	MVZ 39-40	MVZ 47-48	RPL3
IIBPH 2919	x	x	x		x
IIBPH 2289	x				
IIBPH 2295	x				
IIBPH 2296	x				
IIBPH 2297	x				
IIBPH 2302	x				
IIBPH 2305	x				
IIBPH 2306	x		x	x	x
IIBPH 2307	x				
IIBPH 2315	x				
IIBPH 2316	x	x	x		x
IIBPH 921			x		x
IIBPH 922			x		x
IIBPH 1491		x		x	
IIBPH 1492		x	x		
IIBPH 2015			x		x
IIBPH 2016	x	x	x		x
IIBPH 2018	x				
IIBPH 2019	x				
IIBPH 1603	x				
IIBPH 1604	x				
IIBPH 1619		x			
IIBPH 1623	x	x	x		
MTCT 870*	x				

CONCLUSÕES GERAIS

1) Identificamos certo grau de estruturação genética nas espécies do gênero *Lepidobatrachus* que pode estar relacionado a algumas quebras ao longo da paisagem. *Lepi. llanensis* foi a espécie que apresentou maior estruturação.

2) A principal quebra identificada corresponde a uma área extremamente árida localizada no centro do Chaco que estaria atuando como uma barreira climática para as populações de *Lepi. llanensis,* resultando na distribuição disjunta desta espécie. Não encontramos evidência de migrações entre as populações do norte e sul dessa barreira desde a sua separação no Pleistoceno médio.

3) Os rios Pilcomayo e Bermejo influenciaram mais na estrutura genética de *Lepi. llanensis* do que na de *Lepi. laevis* que poderia estar associado ao fato de *Lepi. llanensis* ter um tempo de persistência bem maior na área de influência desses rios. Devido a sua dinâmica histórica, esses rios alóctones promoveram eventos vicariantes curtos e intermitentes permitindo conexões temporais entre as populações de ambas margens desses rios.

4) De acordo com nossas datações, o gênero *Lepidobatrachus* diversificou-se entre o Mioceno médio e o Mioceno superior. Os principais eventos associados a esse período são as introgressões marinhas e as mudanças climáticas do Mioceno superior.

5) Durante as introgressões marinhas do Mioceno médio as antigas formações continentais estáveis (arcos estruturais e crátons) poderiam ter atuado como refúgios isolando populações do *Lepidobatrachus* ancestral dando origem as diferentes espécies do gênero.

6) As mudanças climáticas do Mioceno superior favoreceram a expansão de áreas abertas, levando à fragmentação do habitat florestal que as espécies do gênero *Lepidobatrachus* estão associadas. Esta fragmentação, ao igual que as introgressões marinhas, poderia ter provocado o isolamento de populações em áreas florestadas levando à divergência destas espécies.

7) A baixa estrutura genética apresentada por *Leptodactylus bufonius* já foi registrada para espécies de anuros de outros ambientes áridos e semiáridos do mundo e poderia ser o resultado da combinação de eventos de expansão recente e de um constante fluxo gênico entre as populações.

9) Os eventos de expansão estariam relacionados aos períodos interglaciais das principais glaciações Pleistocênicas. Essas glaciações tiveram uma forte influência na distribuição do Chaco, principalmente ao sul deste bioma devido a expansões da Estepa Patagônica e à formação de corredores de extrema aridez desde a costa atlântica até a base dos Andes.

10) O fluxo gênico em *Lept. bufonius* segue um modelo de "stepping-stone" com alto grau de dispersão entre populações próximas, permitindo assim uma alta conectividade, inclusive entre populações distantes.