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High genetic diversity but low population structure in the frog Pseudopaludicola falcipes (Hensel, 1867) (Amphibia, Anura) from the Pampas of South America

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High genetic diversity but low population structure in the frog *Pseudopaludicola falcipes* (Hensel, 1867) (Amphibia, Anura) from the Pampas of South America ☆

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ABSTRACT

Relative to South America's ecoregions, the temperate grasslands of the Pampas have been poorly studied from a phylogeographic perspective. Based on an intermediate biogeographic setting between subtropical forest (Atlantic Forest) and arid ecosystems (Chaco and Patagonia), Pampean species are expected to show unstable demographic histories due to the Quaternary climatic oscillations. Herein, we investigate the phylogenetic relatedness and phylogeographic history of *Pseudopaludicola falcipes*, a small and common frog that is widely distributed across the Pampean grasslands. First, we use molecular data to assess if *P. falcipes* represents a single or multiple, separately evolving cryptic lineages. Because *P. falcipes* is a small-size species (<20 mm) with extensive coloration and morphological variation, we suspected that it might represent a complex of cryptic species. In addition, we expected strong genetic and geographic structuring within *Pseudopaludicola falcipes* due to its large geographic distribution, potentially short dispersal distances, and multiple riverine barriers. We found that *P. falcipes* is a single evolutionary lineage with poor geographic structuring. Furthermore, current populations of *P. falcipes* have a large effective population size, maintain ancestral polymorphisms, and have a complex network of gene flow. We conclude that the demographic history of *P. falcipes*, combined with its ecological attributes and the landscape features of the Pampas, favored a unique combination among anurans of small body size, large population size, high genetic variability, but high cohesiveness of populations over a wide geographic distribution.

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1. Introduction

Miocene and Pliocene geological events in conjunction with Pleistocene climatic changes shaped a complex pattern of phylogeographic events that impacted the overall diversity of South America (Rull, 2008). Although South America is home to the greatest biodiversity on the planet, as well the largest number of amphibian species, recent reviews have shown a relative scarcity of phylogeographic studies in this region and particularly among amphibians (Beheregaray, 2008; Turchetto-Zolet et al., 2013). Anurans are informative subjects for phylogeographic research given that they are distributed worldwide, usually have genetically highly structured populations over short geographical distances, retain high-resolution signals of historical events that generated current species distributions, and are easy to sample (Zeisset and Beebee, 2008). Furthermore, anuran species are low-dispersal ectotherms, and consequently the impacts of environmental change are expected to be strong (Carnaval et al., 2014).

The uplift of the Andes resulted in allopatric speciation in several amphibian lineages (Slade and Moritz, 1998; Elmer et al., 2007; Koscinski et al., 2008; Santos et al. 2009; Kieswetter and Schneider, 2013; Castroviejo-Fisher et al., 2014). Probably associated with more humid events in Andean Altiplano, populations of some anurans (like the *Telmatobius marmoratus* species complex) may have recent population expansion and high connectivity events following the Last Glacial Maximum (Victoriano et al., 2015). Previous studies have shown that species currently associated with the Atlantic Forest survived in isolated refugia during the glacial periods (Porto et al., 2013) followed by subsequent

speciation through geographic isolation (Thome et al., 2010), sometimes mediated by sea-level fluctuations (Fitzpatrick et al., 2009; Bell et al., 2012). In the Amazon Basin, rivers played an important role in amphibian diversification in conjunction with paleogeographic events during the Miocene that led to patterns of endemism (Noonan and Wray, 2006; Santos et al., 2009; Kaefer et al., 2013; Cheng et al., 2013). The current diversity of the Guiana Shield seems to have originated from multiple and simultaneous refugia during the Pleistocene (Fouquet et al., 2012), although other studies suggest that most of the diversification may have occurred during the Pliocene (Salerno et al., 2015). In the Valdivian forests of SW Patagonia, several Pleistocene refugia, followed by subsequent expansions, have been suggested as mechanisms of diversification (Nuñez et al., 2011; Blotto et al., 2013). In contrast, very little is known about the diversification of amphibians and other taxa occurring in South American open environments, e.g., the Pampas (Turchetto-Zolet et al., 2013).

With a surface area of 760,000 km^2, the Pampas or Río de la Plata's grasslands are a unique biome with a distinctive biodiversity but with a landscape heavily modified by historical and current human development (Bilenca and Miñarro, 2004). A few papers have examined the phylogeographic patterns of organisms inhabiting this biome including plants, fishes, lizards and mammals. Weak genetic structure, possibly associated with events in the last 100 kya (Turchetto et al., 2014), and allopatric fragmentation established by population expansion after a size reduction *circa* 500 kya, were found in petunia flowering plants (Longo et al., 2014). Another flowering plant (*Calibrachoa heterophylla*) showed a major diversification occurring 400 kya and linked to sea-level oscillations (Mäder et al., 2013). There have been also a few studies on fossorial rodents of the genus *Ctenomys*, usually associated with strong genetic structure, which may have arisen after recent population expansions (Wlasiuk et al., 2003; Kittlein and Gaggiotti, 2008; Mora et al., 2006; Roratto et al., 2014). Population expansions have been found also in annual killifishes of the genus *Austrolebias* (García, 2006) and in the gecko *Homonota uruguayensis*, which may have undergone an expansion ∼250 kya (Felappi et al., 2015). Surprisingly, no publications have focused on the phylogeographic history of Pampean amphibians; however, interest in the region is increasing as evidenced by two recent doctoral dissertations (Langone, 2013; Barraso, 2014).

Currently, the genus *Pseudopaludicola* consists of 18 recognized species; almost half of them (45%) were described since 2003. The genus is distributed throughout South America, east of the Andes, from northeastern Peru, western Colombia, central-southern Venezuela, through Guiana, southwestern Surinam, eastern Bolivia, Paraguay, much of Brazil, and extending south to northeastern and central Argentina and Uruguay (Pansonato et al., 2014). *Pseudopaludicola falcipes* (Hensel, 1867) is a small frog (males 13.5–16.0 mm, females 14.7–18.1 mm) endemic to the Pampas with a wide geographical distribution that ranges from the Argentinean provinces of Buenos Aires, Corrientes, Entre Ríos, Misiones, and Santa Fé, across Uruguay to the Brazilian states of Rio Grande do Sul, Santa Catarina, and probably Paraná (Langone et al., in press). Populations and individuals across the distribution show variation in morphology and patterns of dorsal coloration (Lobo, 1994). Despite a small body size that might be associated with limited dispersal, the species usually occurs at high abundances across a continuous grassland landscape, suggesting potentially high population connectivity. Therefore, because of this strong morphological and ecological variation, *P. falcipes* could represent either a complex of cryptic lineages or a single widely distributed and polytypic lineage. For these reasons, *P. falcipes* is an ideal taxon to test for the presence of cryptic evolutionary lineages, and for exploring past environmental factors that have shaped patterns of genetic

structure, demographic history, and the distribution range across the Pampas grasslands.

2. Material and methods

2.1. Sampling

The geographic distribution of *Pseudopaludicola falcipes* is based on previous reports (Langone et al., in press). We sampled 41 localities across the distribution and collected a total of 156 individuals with a sampling effort ranging between 1 and 10 individuals per locality (average ± stand. dev. = 3.8 ± 3.0) (Fig. 1 and Table S1). The maximum geographic distance between two sampled localities was 1058 km, and the minimum was 9 km (354 km ± 210 km). Denser sampling took place in Uruguay, but the availability of samples from Argentina and Brazil was more limited. As much as possible, we included at least two individuals per locality as recommended for assessing genetic isolation among evolutionary lineages (Wiens and Penkrot, 2002). All maps were done with available public software Google Earth™ and Quatum GIS v.1.7.4.

2.2. Molecular methodology

Tissue (muscle and liver) was taken from specimens and preserved in ethanol 95%, and total genomic DNA was isolated using Qiagen DNeasy kit (Valencia, California, USA). Fragments of the mitochondrial *12S* (∼870 bp) and *16S* rRNA (∼543 bp) and the nuclear *tyrosinase* genes (∼319 bp) were amplified using standard primers (Table 1). We used Green or Red Taq polymerase (Promega) to perform PCR amplifications with a combination of standard and touchdown thermal-cycling profiles used in frogs (de Sá et al., 2005; Streicher et al., 2012; de Sá et al., 2012). Amplified products were cleaned using USB ExoSap-IT (US78201, Amersham Biosciences, Piscataway, New Jersey, USA) and sequenced (in both primer directions) by SeqWright Corp. (Houston, Texas, USA; www.seqwright.com). Resulting chromatograms were visualized, aligned, and cleaned using Sequencher 5.1 (Gene Codes Corp., Ann Arbor, Michigan, USA). Each sequence was searched against NCBI database using BLAST (Basic Local AlignmentSearch Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1990) to eliminate potential contamination. Sequence alignment for each locus was initially produced in SATé-II (Liu et al., 2012) and subsequently visually examined to identify potential problematic areas. Sequences were deposited in GenBank (see Table S2 for accession numbers).

2.3. Phylogenetic analyses

Outgroups were selected following recent molecular phylogenetic hypotheses (Pyron and Wiens, 2011; Fouquet et al., 2013). Outgroup sequences were available in GenBank (Table S2) and included the following taxa: *Leptodactylus fuscus*, *Scythrophrys sawayae*, *Paratelmatobius* sp., *Paratelmatobius mantiqueira*, *Pleurodema brachyops*, *Engystomops petersi*, *Edalorhina perezi*, *Physalaemus cuvieri*, *Physalaemus nattereri*, as well as *Pseudopaludicola* sp. 1 and *P.* sp. 2 (from Bolivia). An additional specimen of *Pseudopaludicola* sp. from Paraguay (IIBP 834, Concepción Department, Estancia Garay Cué S.A. Cerrados del Tagatiyá) was also included in the analysis. Phylogenetic reconstructions were performed under the criteria of maximum likelihood (ML) implemented in RAxML (Randomized Accelerated Maximum Likelihood) (Stamatakis et al., 2008) and Bayesian analysis implemented in MrBayes (MrBayes using a hybrid code, Pratas et al., 2009). Both programs were
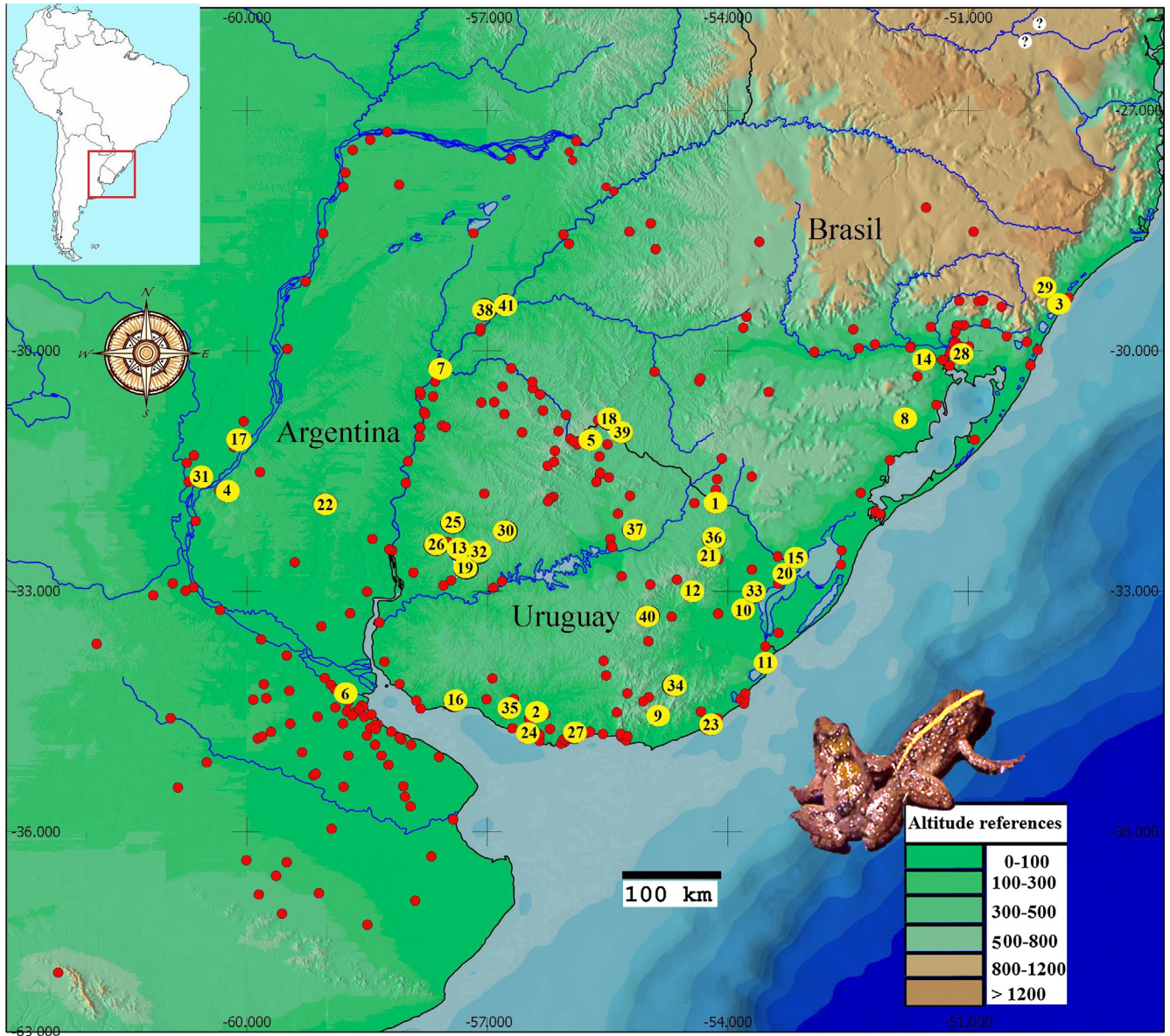
Fig. 1. Geographic distribution of *P. falcipes* based on collection points (red dots) Sampled localities across the distribution are numbered from 1 to 41 (yellow dots). See Tables S1 and S2 for locality and voucher information. At right in the figure, two individuals of *P. falcipes* with different dorsal pattern (from a photograph courtesy of Axel Kwet). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Primers used in DNA amplification and sequencing.

Primer	Gen	Direction	Sequence	Reference
16SAR	16S	F	5′-CGCCTGTTTATCAAAAACAT-3′	Palumbi et al. (1991)
16SBR	16S	R	5′-CCGGTCTGAACTCAGATCACGT-3′	Palumbi et al. (1991)
12SMVZ59	12S	F	5′-ATAGCRCTGAARAYGCTRAGATG-3′	Wiens et al. (2005)
12SMVZ50	12S	R	5′-TYTCGGTGTAAGYGARAKGCTT-3′	Wiens et al. (2005)
TyrC	Tyr	F	5′-GGCAGAGGAWCRTGCCAAGATGT-3′	Bossuyt and Milinkovitch (2000)
TyrG	Tyr	R	5′-TGCTGGCRTCTCTCCARTCCCA-3′	Bossuyt and Milinkovitch (2000)

accessed on-line through the CIPRES server website (Miller et al., 2010) running on XSEDE (National Science Foundation resource Teragrid ABE; www.phylo.org/subsections/portal). Initially, data were partitioned into mitochondrial and nuclear data and separate analyses were run; followed by concatenated analyses of all data. The general time reversible plus gamma rate-variation (GTR + G) was selected for the mitochondrial and nuclear partitions as the evolutionary model of nucleotide substitutions based on

jModelTest analysis with Akaike criteria (Posada, 2008). Separate (mitochondrial vs. nuclear) and concatenated analyses produced the same overall topology; consequently, we present results of concatenated analyses only. The concatenated matrix consisted of a total of 1738 base positions (bp). *Leptodactylus fuscus* was used to root the cladogram. Bayesian analysis was run with four Markov chains for 10 million generations and sampled every 1000 generations. The 'burn-in' was estimated empirically by plotting

the "log-likelihood" (−ln L) relative to the number of generations; the first 1000 trees were discarded, and the remaining trees were used to estimate a consensus tree of the resulting 9000 trees. Visual examination of the sampled parameter values indicates good exploration of parameter space. Supported nodes were those with bootstrap (BS) values (1000 replicates) of at least 70% in RAxML analyses and Bayesian support with posterior probability (PP) >0.95 in MrBayes analyses. Trees were visualized in FigTree v.1.3.1 (available at: http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Genetic diversity

Mitochondrial and nuclear sequences were analyzed in Arlequin v.3.5 to calculate nucleotide and haplotype diversity (Excoffier and Lischer, 2010). Neutrality test with Tajima's D statistic (Tajima, 1989) was performed for the entire sampling (i.e., not by locality or populations given the low number of individuals per locality) with a number of simulated samples of 10,000 permutations.

Sequences were collapsed into haplotypes using TCS v.1.21 (Clement et al., 2000). Gaps were treated as a "missing" data with a 95% probability-limit that the haplotypes were parsimony connected. TCS assigns different weights to each haplotype according to its frequency in the total sampling. The haplotype networks were constructed with the R package pegas 0.8-1 (Paradis, 2010). Under coalescent theory, the ancestral haplotype is expected to be the most common, i.e., represented in a greater number of populations, and to have multiple connections to low-frequency haplotypes (Crandall and Templeton, 1993). The geographic distribution of haplotypes was plotted using the on-line program PhyloGeoViz (Tsai, 2011) available at: http://phylogeoviz.org/.

2.5. Geographic structure

Mantel's tests (Mantel, 1967) were run to assess the pattern of isolation by distance in each locus using the software zt v.1.1 (Bonnet and Van de Peer, 2002). Geographic distances among localities were converted from geographic coordinates to kilometers using an online tool (The Northern California Earthquake Data Center http://www.ncedc.org/convert/distance.html). Genetic distances were based on the pairwise Φ_{ST} statistic (Excoffier et al., 1992) calculated in Arlequin after excluding localities with only one sequence. Negative values of Φ_{ST} were considered equal to zero (Barth et al., 2011). The significance of the Mantel test was obtained from the comparison between the observed and expected correlation with 10,000 random permutations.

We ran non-metric multidimensional scaling (NMDS) analyses with pairwise Φ_{ST}-distances between localities for each locus using the vegan package of R (Oksanen et al., 2015). We also used Geneland v.4.0 (Guillot et al., 2012) to assign all individuals to geographic clusters based on multilocus genotypes (combined mitochondrial and nuclear datasets) and geographic coordinates of 41 sampled localities. We ran 10 MCMC chains with 1 million iterations sampled every 100 steps using a model of correlated allele frequencies, and assuming between 1 and 5 clusters a priori. We processed posterior samples to estimate the number of clusters, and the posterior probability of membership of each individual to population clusters. In addition, we also obtained posterior probability maps showing the cluster membership of pixels defined by geographic coordinates.

2.6. Phylogeographic diffusion

We reconstructed diffusion dynamics of *P. falcipes* populations on continuous time and space using BEAST v.1.8 (Drummond et al., 2012). We analyzed the combined *12S* and *16S* data from 147 individuals representing 41 sampled localities. We applied a relaxed random walk (RRW) to model the variation in diffusion rates across branches of the genealogy and to estimate the geographic coordinates of ancestral nodes taking into account genealogical uncertainty and a flexible demographic model (Lemey et al., 2010). We specified a Bayesian Skyride model (Minin et al., 2008), the best-fit substitution model chosen by jModelTest v.2.0 (Darriba et al., 2012), and relaxed clock model with the substitution rate recently estimated for *Pseudopaludicola* (Fouquet et al., 2013). We applied a log-normally distributed prior for the substitution rate with a mean of 0.0045 (stand. dev. = 0.33). We sampled trees and diffusion-model parameters from two replicate analyses consisting of MCMC chains of 250 million steps sampled every 25,000 steps. Sampled parameters in log files were inspected with Tracer v. 1.4 (Available at: http://beast.bio.ed.ac.uk/Tracer) to check for convergence between runs, discard the burn-in, and estimate model parameters. We used TreeAnnotator in BEAST to produce a consensus tree, which was used in Spread v. 1.0.6 (Bielejec et al., 2011) for annotating diffusion-model parameters and producing an animation of the continuous diffusion process for visualization in Google Earth™.

2.7. Demographic analyses

We used Extended Bayesian Skyride Plot (EBSP) analyses implemented in BEAST v.1.8 (Heled and Drummond, 2008) to reconstruct the change in population size over time for each of the clusters detected in population structure analyses. We specified two loci (a combined mitochondrial and a nuclear dataset) with locus-specific substitution and a relaxed clock model. We applied the same prior for the substitution rate of the *12S–16S* dataset used in the diffusion analyses. We sampled trees and model parameters from MCMC chains with 50 million steps sampled every 5000 steps. We plotted the mean and 95% highest posterior density of the effective population size after checking for stationarity in Tracer and removing the burn-in samples. We compared the timing of changes in population size with the duration of glacial cycles based on the dating of marine isotope stages (MIS) from Lisiecki and Raymo (2005). In addition, we followed the nomenclature for Quaternary chronostratigraphy of Cohen and Gibbard (2011).

We also estimated the parameters of a demographic model of isolation with migration using a Bayesian approach in Lamarc v.2.1.9 (Kuhner, 2006) based on population clusters detected with Geneland. The parameters included in the model were: the mutation-scaled population size ($\theta = 4N_e\mu$, where N_e is the inbreeding effective population size of diploid individuals and μ is the mutation rate per site per generation), the exponential growth rate of each population (g, where $\theta_t = \theta_{present} \times e^{-gt}$), the mutation-scaled immigration rate between populations ($M = m/\mu$, where m is the backward immigration rate per generation), and the scaled divergence time between populations ($T = t \times u$, where t is the divergence time in generations). We ran two replicate analyses with five initial MCMC chains (500 trees sampled every 20 steps and a burn-in of 1000) and two final chains (10,000 trees sampled every 20 steps with a burn-in of 1000) and the following priors: θ between 1 and 10^{-5} substitutions per site/generation, uniform M between 1 and 2000 (in units of 1/substitutions per site), uniform g between −500 and 2000 (in units of 1/generations), and uniform T between 1×10^{-10} and 0.01 substitutions per site.

We also used an Approximate Bayesian Computation (ABC) approach (Beaumont et al., 2002; Sunnåker et al., 2013) to estimate additional parameters of the demographic model fitted with Lamarc. We were particularly interested in estimating the timing of several historical events: the divergence time between population clusters (for comparison with Lamarc's estimates), the time when migration started between clusters, and the time when

population growth started in both clusters. We implemented this approach by simulating 1 million coalescent genealogies with the program ms (Hudson, 2002) using a custom Perl script (see Supplementary Data). We used uniform prior distributions for all parameters including θ per locus (lower bound: 0.01, upper bound: 10), divergence time (td) (0.0001, 0.5), migration rate in $4N_em$ units (0, 5), time when migration started (tm) (0.0001, td), time of population growth for both clusters (t1g and t2g) (0.0001, td), and the ratio between current and ancestral population sizes (0.1, 0.9). All time parameters are expressed in $4N_e$ units. In order to match the empirical number of loci and the unequal number of gene copies per locus and per population cluster, two loci were simulated separately and θ was adjusted for the mitochondrial locus with a value of $0.25N_e$ and a rate multiplier of 20. We calculated the following summary statistics of simulations with msSS.pl (a Perl script written by N. Takebayashi, available at: http://raven.iab.alaska.edu/~ntakebay/teaching/programming/coalsim/scripts/msSS.pl) and for empirical data with DnaSp v5 (Librado and Rozas, 2009): average nucleotide pairwise distance per site (pi), number of segregating sites, Tajima's D, pi within population 1, pi within population 2, and pi between populations. Summary statistics were imported into R to perform the acceptance–rejection step with the package ABC (Approximate Bayesian Computation) (Csilléry et al., 2012) using simple rejection and a tolerance of 0.0002 to approximate posterior distributions with 200 sample points. In order to evaluate model fit and adequacy, we performed a Principal Components Analysis (PCA) in R using all summary statistics from the prior and posterior distributions, and the empirical data. Summary statistics from the posterior distribution were obtained from 10,000 predictive simulations using the parameter values estimated in the ABC analysis.

3. Results

3.1. Phylogenetic analysis

All samples of *Pseudopaludicola* formed a clade with high support (BS = 100%, PP = 1.0, Fig. S1). The analyses also recovered two sister clades: one clade consisting of all *P. falcipes* samples (RAxML, BS = 100%; MrBayes, PP = 1.0, Fig. S1), and the other clade consisting of all other *Pseudopaludicola* included in the analyses. In all analyses, no significant support was found for any intraspecific cluster within *P. falcipes*.

3.2. Genetic diversity

Values of genetic diversity found in *Pseudopaludicola falcipes* are shown in Table 2. Pairwise values of Φ_{ST} between localities were 0.15 ± 0.25 (mean ± stand. dev.) for the mitochondrial data, and 0.08 ± 0.12 for the *tyrosinase*. There is large variation in genetic distances including high and zero distances between nearby locations as well as localities that are far apart (Tables S3 and S4). The Mantel test found no significant correlation between geographic (Table S5) and genetic distances in the mitochondrial genes ($r = -0.01$; $p = 0.47$) or in the nuclear *tyrosinase* ($r = 0.01$, $p = 0.47$) (Fig. S2).

Most mitochondrial and nuclear haplotypes were restricted to a single or a few localities (Table S6). Among the 81 distinct *12S–16S* haplotypes, 84% correspond to single-locality haplotypes (68), and of the 50 distinct *tyrosinase* haplotypes, 86% were single-locality haplotypes (43). On the other hand, single haplotypes in the mitochondrial and *tyrosinase* datasets were the most frequent and they were widely distributed among many localities (haplotypes 1 in

Table 2
Values of genetic diversity in *P. falcipes*.

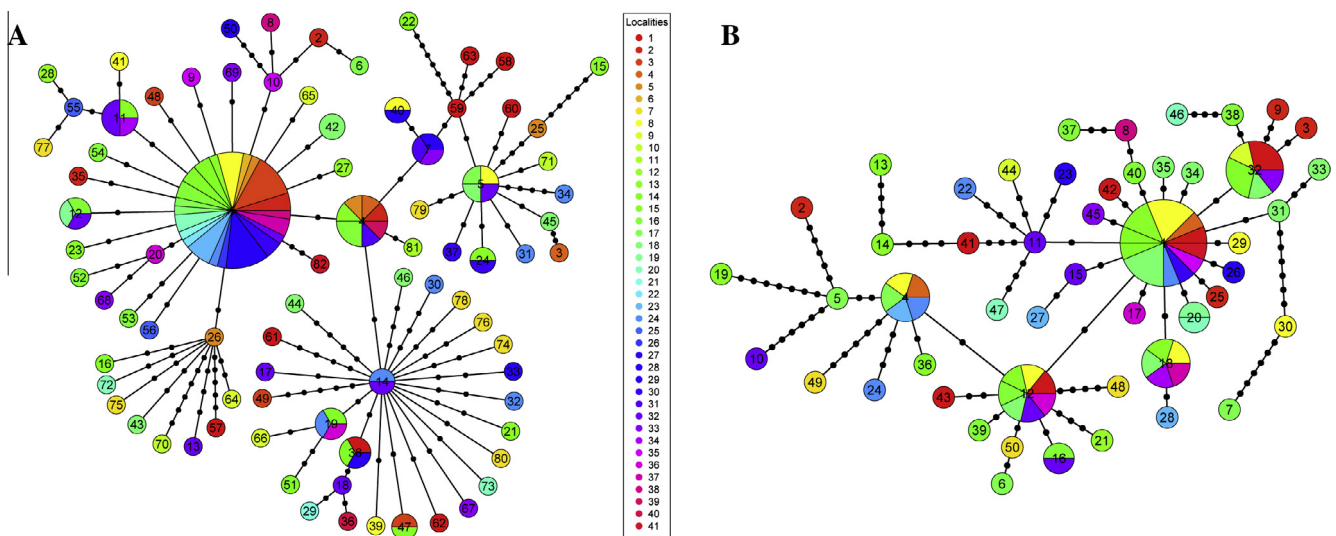| Gene	Sequence number	Sequence length	Haplotype number	Polymorphic sites	Haplotype diversity (h)	Nucleotide diversity (π)
12S-16S	148	1563	81	1385	0.9194 ± 0.0196	0.2119 ± 0.1008
Tyr	87	319	50	93	0.9508 ± 0.0142	0.0235 ± 0.0122

Fig. 2. Haplotype networks. (A) 12S and 16S, (B) tyrosinase. Pie size is proportional to the haplotype frequency; missing intermediate haplotypes are shown as blacks dots. Alternative links between haplotypes are not shown after specifying the threshold option to zero in pegas. Color codes represent the localities (see Table S1) and numbers within pies represent haplotype codes (see Table S6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table S6). Under a 95% parsimony threshold and specifying gaps as "missing" data in the program TCS, both mitochondrial and nuclear data each produced a single network connecting all haplotypes (Fig. 2). Ancestral haplotypes of high frequency and connected to many other haplotypes, are present in much of the geographical distribution of *P. falcipes* (Fig. 3). Tajima's neutrality tests were significant for both the mitochondrial ($D = -2.49$, $p < 0.01$) and the tyrosinase data ($D = -2.18$, $p < 0.01$).

3.3. Phylogeographic diffusion

The Bayesian phylogeographic reconstruction suggests that *Pseudopaludicola falcipes* originated in North-Eastern Uruguay (currently corresponding to Treinta y Tres and Cerro Largo Departments) about 1.76 million years ago (mya) in the Early Pleistocene (Fig. S3A). Subsequently, the species dispersed towards the West (towards Paysandú and Río Negro Departments, Uruguay), and then South (Colonia Department). Slightly more recently, about 1.3 mya, another lineage dispersed towards the northeast to colonize the State of Rio Grande do Sul (Brazil) (Fig. S3B). Furthermore, about 950 kya, the populations in central Uruguay expanded North ('Rivera' Department) and South (San José, Canelones, and Maldonado Departments) (Fig. S3C). This range expansion was followed by additional northern and western events of colonization towards Argentina (Corrientes and Entre Ríos Provinces) about 800 kya (Fig. S3D). A new wave of dispersal between populations in
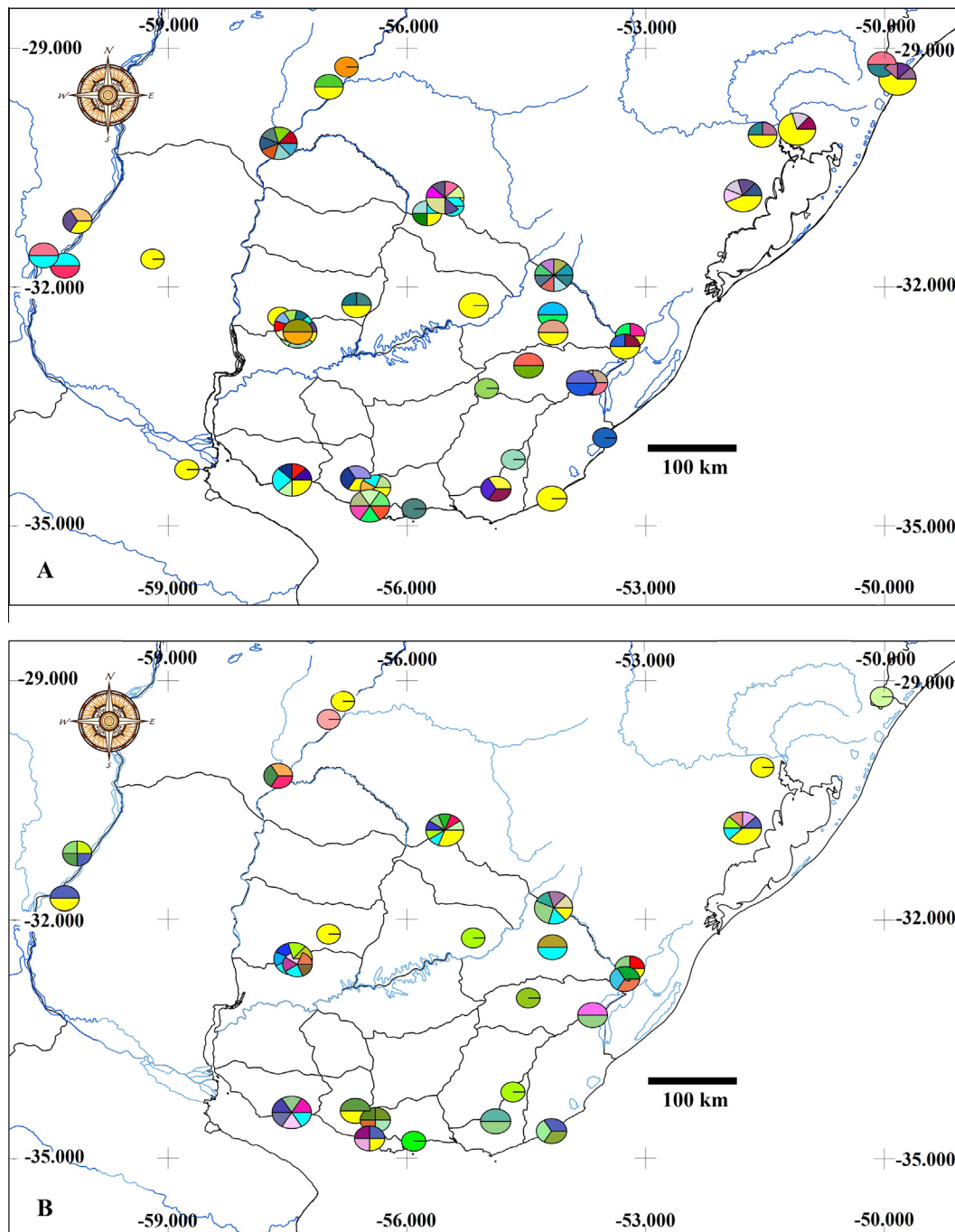

Fig. 3. Geographic distributions of haplotypes. (A) *12S* and *16S*, (B) *tyrosinase*. Ancestral haplotypes (coded as 1 in Table S6) are shown in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
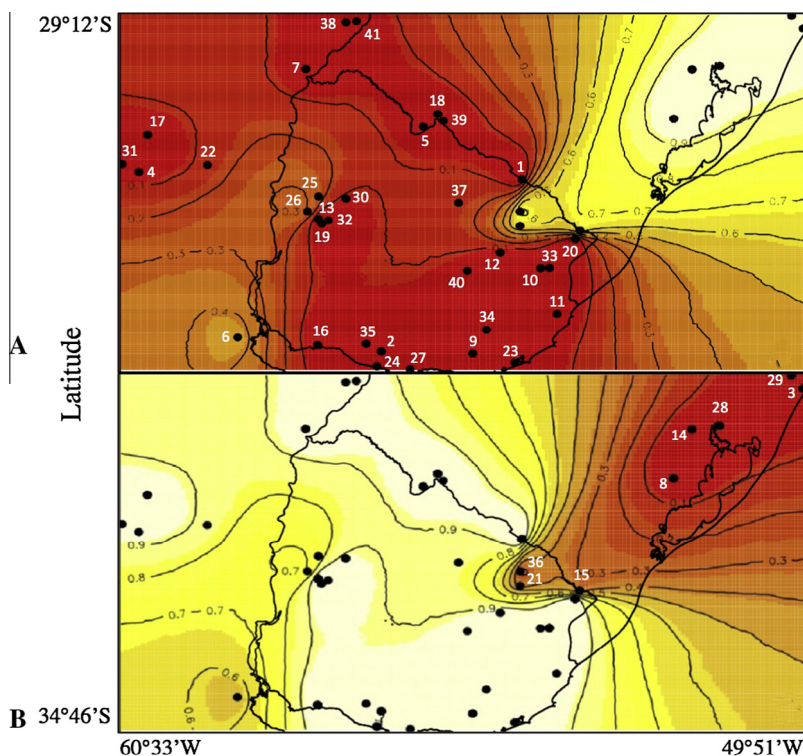
Fig. 4. Map showing the posterior probability of pixel membership of two population clusters: (A) Brazil, and (B) Uruguay–Argentina. The probability of membership represents the modal probability assignment for each pixel to each cluster. The color contours show regions of high (yellow) to low (red) posterior probability of membership. Continuous black lines represent the country boundaries and the black dots are the sampled localities identified with the codes used in Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Uruguay and more long-distance localities in Brazil and Argentina occurred around 500 kya (Fig. S3E). Finally and more recently (about 165 kya), the species reached the boundaries of its range, maintained to the present, with additional expansion of populations occurring in Entre Ríos and from southern Uruguay to Buenos Aires Province, Argentina (Fig. S3F). The mean diffusion rate throughout the phylogeographic history of *P. falcipes* was of 950 km per million years (~1 m/year).

3.4. Geographic structure

The ordination of sampled localities in the NMDS analysis did not reveal any clear geographic clustering of populations (Fig. S4). On the other hand, Geneland consistently detected, with high probability, two population clusters across 10 replicate runs (Fig. S5). One population included most localities from the State of Rio Grande do Sul, Brazil (except locality 15), while the other population contained all sampled localities from Uruguay (except localities 21 and 36) and Argentina (Fig. 4). There is a sharp transition zone between the ranges of these two clusters in the area corresponding to the political boundary between Uruguay and the State of Rio Grande do Sul, where some localities showed ambiguous assignment of individuals to clusters (localities 15, 21, and 36) (Fig. S6).

3.5. Demographic analyses

The reconstruction of population size over time using EBSP shows stable and small sizes until 250 kya in the State of Rio Grande do Sul with subsequent exponential growth to the present (Fig. 5A). The Uruguay–Argentina (Uru–Arg) population remained stable in size until a sudden growth started about 1 mya to reach

a larger final size for this population than for the one in the State of Rio Grande do Sul (RGS) (Fig. 5B).

The demographic model estimated with Lamarc showed that the scaled divergence time between populations of *Pseudopaludicola falcipes* is 0.0059 (most probable estimate = MPE) with a 95%-highest posterior density (HPD) between 0.0049 and 0.0095 (Fig. 6A). Based on the substitution rate used in the demographic EBSP analysis (0.0045 subs./site/million years), this divergence time corresponds to ~1.31 mya (=5.9E−03/4.5E−09), which agrees with the colonization time of the State of Rio Grande do Sul found in the diffusion analysis. Population size is larger for the Uru–Arg population (MPE θ = 1.564) than for the State of Rio Grande do Sul (MPE θ = 0.008); it was even much larger for their ancestral population (MPE θ = 13.380, Fig. 6B). Growth rates were positive for all descendant and ancestral populations (MPE g = 1,030 for Uru–Arg, 1737 for State of Rio Grande do Sul, and 495 for the ancestral population) (Fig. 6C). In addition, there has been a higher migration rate from RGS to Uruguay–Argentina (MPE = 385) than in the opposite direction (MPE = 286) (Fig. 6D). These migration rates are equivalent to 150 effective migrants per generation ($N_e m = M \times \theta/4 = 385 \times 1.564/4$) from RGS to Uru–Arg, and <1 migrant in the opposite direction (=286 × 0.008/4).

The ABC analysis produced a θ estimate for the mitochondrial locus of 33.75 per locus (95%-range: 2.79–9.67), which is equivalent to a value of 0.022 per site. Based on this θ estimate and a per-linage substitution rate of 0.0045 per site per million years, $4N_e$ time units represents about 19 million generations. Divergence time was 0.070 time units (0.015–0.400) or 1.34 million generations, and migration between clusters began 0.014 time units (0.002–0.126) or 260,000 generations ago. The Uru–Arg cluster began its population growth at 0.039 time units (0.005–0.251) representing 745,000 generations, whereas the Brazil population began to grow at 0.017 time units (0.002–0.174) which equals
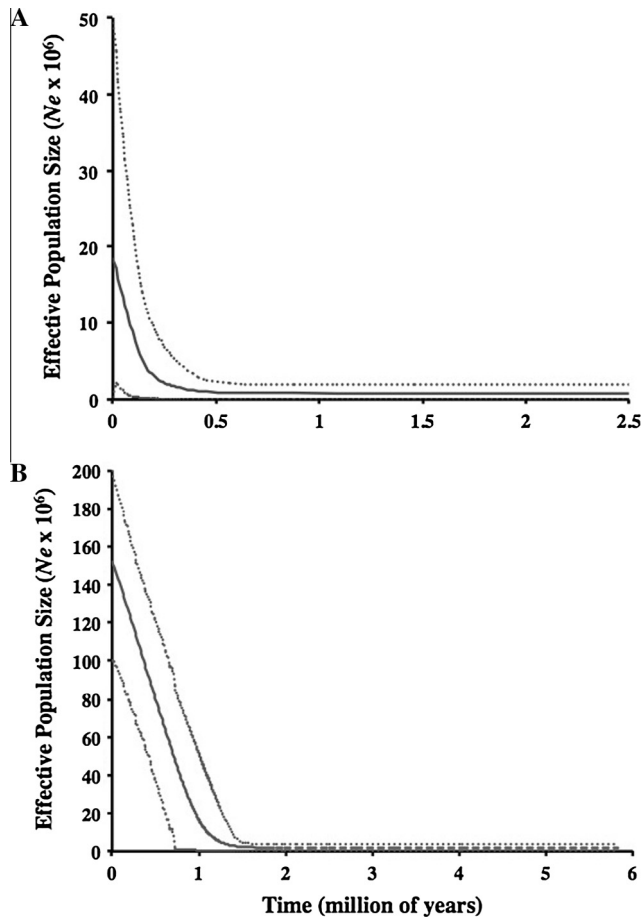
Fig. 5. Reconstruction of the effective population size over time based on EBSP analyses for each of two population clusters: (A) Brazil and (B) Uruguay–Argentina. Dotted lines above and below the continuous line (average value) enclose the 95%-highest posterior density.

∼320,000 generations (Fig. 7). The PCA analysis of simulated and observed summary statistics reveals a good model fit and adequacy given that the plot of the first two components shows the empirical data point within the prior and posterior point clouds (Fig. S7).

4. Discussion

4.1. Phylogenetic analysis

The relationships of the genus *Pseudopaludicola* based on recent molecular phylogenies have been controversial, and alternative hypotheses have been proposed (Frost et al., 2006; Grant et al., 2006; Santos et al., 2009; Pyron and Wiens, 2011; Faivovich et al., 2012; Fouquet et al., 2013; Veiga-Menoncello et al., 2014). Although, our sampling was not designed to test monophyly of *Pseudopaludicola*, our results agree with previous studies that support the monophyly of *Pseudopaludicola* (Pyron and Wiens, 2011; Fouquet et al., 2013; Veiga-Menoncello et al., 2014). Our phylogenetic analyses reveal several haplotype clades within *Pseudopaludicola*, which could not be assigned unambiguously to previously recognized species-level taxa, and their taxonomic resolution is outside the scope of the present study. Furthermore, none of our phylogenetic analyses reveal significant support for intra-specific clustering within *P. falcipes*, and consequently, we consider it a single species across its wide geographic distribution.

4.2. Genetic diversity

The genetic diversity of *Pseudopaludicola falcipes* is among the highest in comparison with the estimates of phylogenetic and population studies of other frogs using *12S*, *16S*, and/or *tyrosinase* genes (Table 3). The surprisingly high genetic variation of *P. falcipes* based on the extent of its geographic range might be a consequence of the high abundance of this frog in the temporary ponds scattered across the Pampean grasslands (Barrio, 1945; Gallardo, 1968). High levels of nucleotide polymorphism within populations have been associated with large effective population sizes over long periods of time (Charlesworth, 2009; Pabijan et al., 2012), which is consistent with our results of steady demographic growth and current population sizes of tens of millions (Fig. 5). The sparse sampling in some parts of the distribution range (e.g., Buenos Aires Province, Argentina, and central Rio Grande do Sul, Brazil) suggests that there is substantial genetic diversity still hidden within *P. falcipes*. In addition, secondary contact among formerly allopatric and differentiated lineages could also increase genetic diversity (Grant and Bowen, 1998). This scenario may apply to *P. falcipes* because it contains two population clusters that diverged more than one mya and subsequently contacted again permitting gene flow (∼260 kya). Overall, our results for *P. falcipes* are congruent with several of Frankham's (1996) predictions about the positive correlation between genetic diversity and population size, and the expected high genetic variation in widely spread, small-sized species with low risk of extinction. These predictions apply well to *P. falcipes* based on the results of our genetic analyses, and are consistent with the low conservation threat of this species in the region (Lavilla et al., 2004).

4.3. Geographic structure

Although the molecular markers used in our study revealed high genetic variation in *Pseudopaludicola falcipes*, ordination and correlation analyses found poor phylogeographic structure. This lack of phylogeographic structure and limited lineage differentiation within *P. falcipes* are probably not a result of insufficient signal because the same genes have been used to resolve interspecific differentiation in *Pseudopaludicola* (Veiga-Menoncello et al., 2014).

The admixed individuals at the border region between the two population clusters of *P. falcipes* suggest the possibility of former isolation and differentiation with subsequent and more recent geographic contact. In fact, the Lamarc and ABC analyses allow reconstructing a scenario of initial divergence, subsequent population growth in both populations, and more recent gene flow between them (Figs. 6 and 7). These demographic processes might be responsible for the presence of individuals with mixed ancestry in the geographic boundary between both populations as a result of sex-biased dispersal (Canestrelli et al., 2007; Helfer et al., 2012) or asymmetric hybridization (Liu et al., 2010; Qi et al., 2014). While genealogical stochasticity could have allowed the retention and sharing of ancestral polymorphisms between populations, particularly in the nuclear locus, our coalescent-based analyses in Lamarc and ABC strongly suggest that shared haplotypes in this border region are consistent with recent gene flow between the population clusters.

Pseudopaludicola falcipes displays an unusual pattern of low geographic structure but high genetic variation within population clusters. In contrast, other frogs are either weakly structured phylogeographically and have low genetic variation, or are highly structured and exhibit high genetic variation (Vences et al., 2013). The pattern in *P. falcipes* rejects the traditional view that small frogs have limited dispersal ability and are strongly philopatric (Beebee, 2005; Blaustein et al., 1994; Gamble et al., 2007). In general, behavioral and ecological studies reveal that frogs have
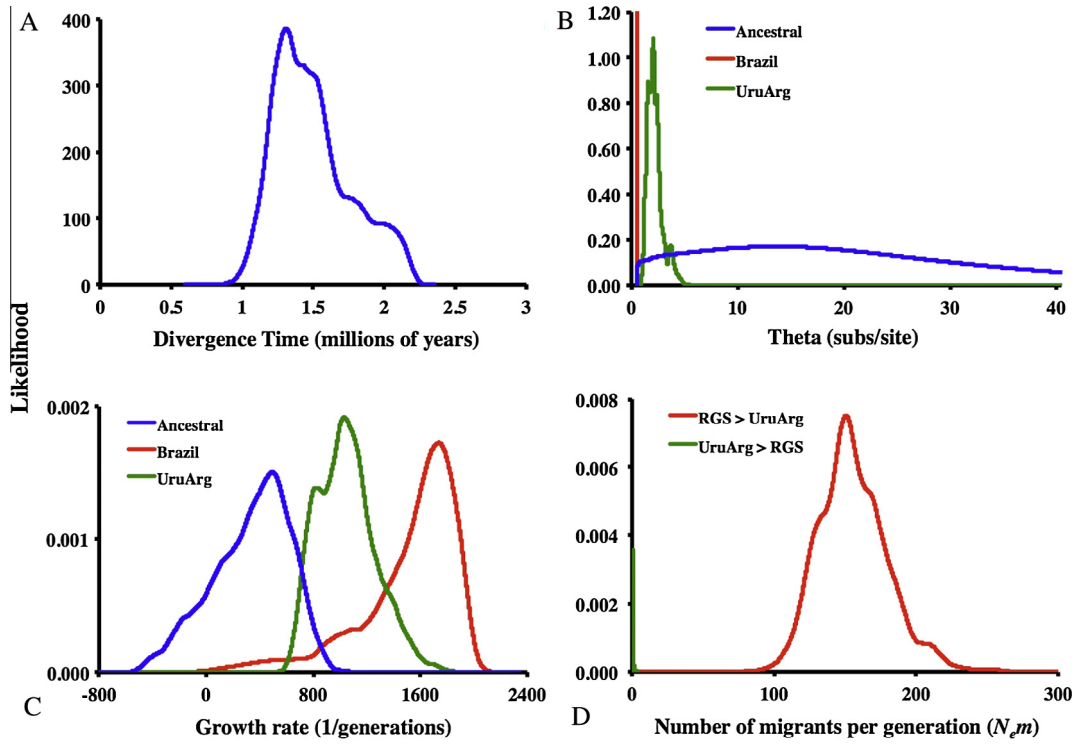
Fig. 6. Density plots for the posterior probability distributions of the demographic parameters estimated in Lamarc analyses for two populations (Brazil and Uruguay–Argentina): (A) divergence time (in millions of years), (B) theta (in substitutions per site), (C) growth rates (in units of 1/generations), and (D) number of effective migrants per generation ($N_e m$).
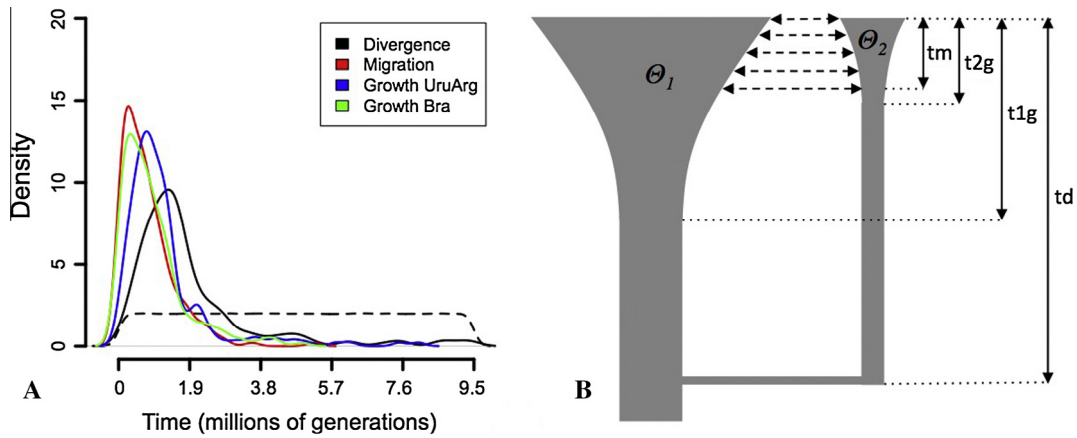

Fig. 7. Density plot of the demographic parameters estimated in ABC analyses. (A) Density plot for divergence time (black), migration time (red), time of growth for Uruguay–Argentina (blue), and time of growth for Brazil (green). (B) Schematic representation of the demographic model estimated in ABC (td = divergence time, tm = migration time, t1g = time of growth for Uruguay–Argentina, and t2g = time of growth for Brazil). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

small home ranges, short migratory distances, and continuous-habitat requirements (Wells, 2007; Semlitsch, 2008; Funk et al., 2005). These life-history attributes of frogs combined with several demographic characteristics (e.g., high variability in reproductive success among individuals, frequent bottlenecks, and typical metapopulation dynamics) contribute to the development of a fine-scale genetic subdivision via local genetic drift (Newman and Squire, 2001; Andersen et al., 2004; Rowe and Beebee, 2004). Consequently, frog populations are expected to be genetically structured and to show limited gene flow, which facilitates isolation, divergence, and subsequent speciation via vicariance (Avise, 2000; Vences and Wake, 2007). However, there is evidence of frogs

dispersing through geographic barriers including oceans and arid, unsuitable habitats, which otherwise are effective barriers because of strong physiological constraints (Vences et al., 2003; Measey et al., 2007a, 2007b; Chan and Zamudio, 2009; Kuchta et al., 2009; Bell et al., 2015). Our analyses indicate that *P. falcipes* is a good disperser in the Pampean lowlands consisting of almost continuous, open grasslands, which is congruent with the higher genetic connectivity of amphibians in homogeneous landscapes (Newman and Squire, 2001). The average diffusion rate for *P. falcipes* throughout its phylogeographic history was about one m/year, suggesting a good dispersal ability of this species in spite of its small body size. In addition, several life history and ecological

Table 3
Average nucleotide diversity of *12S*, *16S*, and *tyrosinase* genes in anurans.

Taxa	SVL (mm)	12S	16S	Tyr	References	Data
Agalychnis callydrias	57–79	–	0.0017–0.0157	–	Robertson and Zamudio (2009)	*16S + ND1*
Ansonia spp.	32–50	–	0.0569	–	Sanguila et al. (2011)	*ND1 + 16S + rRNA + tRNALeu*
Bufo spp.	80–130	–	0.0010–0.0160	–	Recuero et al. (2011)	*16S + Cyt b*
Gastrophryne olivacea	22–41	–	0.0040	–	Streicher et al. (2012)	*16S*
Mantellidae spp.	21–90	–	0–0.1090	–	Pabijan et al. (2012)	*16S*
Myxophies spp.	59–104	–	0.0034–0.0080	–	Oza et al. (2012)	*16S + ND2*
Polypedates leucomystax	50–80	–	0.0088–0.0209	–	Brown et al. (2010)	*16S*
Sooglosidae spp.	6–22	–	0.0017–0.0153	–	Taylor et al. (2012)	*16S*
Strongylopus grayii	25–50	–	0.0300	–	Tolley et al. (2010)	*16S + ND2*
Gastrophryne carolinensis	22–38	0.0190	–	–	Makowsky et al. (2009)	*12S*
Pelophylax spp.	120–170	0.0100–0.0600	0.0100–0.1100	–	Hofman et al. (2012)	*12S/16S*
Engystomops petersi	21–39	0–0.0117		–	Funk et al. (2007)	*12S + 16S + tRNAVal*
Ameerega bassleri	32–42	0.0280		–	Roberts et al. (2006)	*12S + 16S + Cyt b*
Hyla spp.	30–47	0.4200–1.0200		0.3400–0.6100	Gvoždík et al. (2010)	*12S + 16S*
Lithobates spp.	44–127	0.0004–0.0048		0–0.0085	Newman et al. (2012)	*12S + tRNAVal + 16S + ND2*
Smilisca fodiens	38–64	0.0360		0.0240	Cox et al. (2012)	*12S + 16S*
Pseudopaludicola falcipes	14–18	0.2119		0.0235	This study	*12S/16S/Tyr*

attributes of *P. falcipes* further facilitate its dispersal ability including: association with open habitats, reproduction in temporal pools, large clutch size, and fast larval development (Van Bocxlaer et al., 2010; Streicher et al., 2012).

In addition to the present study and not formally published, there is one phylogeographic analysis of frogs from the Pampas that included *Physalaemus fernandezae* and *P. henselii* (Barraso, 2014). *Physalaemus fernandezae* occurs in the lowlands of Buenos Aires Province and does not show a marked phylogeographic structure, while *P. henselii* occupies more diverse environments in Uruguay and southern Brazil, and does show a strong phylogeographic pattern. The contrasting phylogeographic structures of *Pseudopaludicola falcipes* and *Physalaemus henselii*, despite their similar distributions in the Pampas, suggest that these co-distributed species have different population histories or ecological requirements. This type of incongruence in phylogeographic histories between co-distributed taxa has appeared in other studies of amphibians due to either subtle differences in microhabitat distribution (Steele and Storfer, 2007), or different population histories of ecologically similar species (Austin and Zamudio, 2008). We suggest that additional studies at a finer spatial scale would be necessary to evaluate if higher connectivity and dispersal at a local scale are consistent with the lack of geographic structuring at a regional scale as revealed in the phylogeographic patterns.

4.4. Dispersal mechanisms

Long-distance colonization events between clusters of populations suggest that besides natural dispersal, *Pseudopaludicola falcipes* could also have used passive mechanisms of dispersion. One inferred dispersal route from the upper Río Negro in northeastern Uruguay to the basin of Laguna dos Patos, which occurred about 1.76 mya, is consistent with a basin-capture hypothesis based on geological and geomorphological evidence (Ribeiro, 2006). This hypothesis asserts that the upper sections of rivers in the crystalline shield of South America were often captured by rivers flowing to the Atlantic basin, which accounts for the presence of *Austrolebias* killifish in the currently isolated basins of the Río Negro and the Laguna Merin (Loureiro et al., 2011). Passive dispersal via hydrochory is a common transportation mechanism in larval and adult frogs (Martof, 1953; Carpenter, 1954; Smith and Green, 2006; Merrell, 1970), which could have facilitated the transportation of *P. falcipes* to distant basins and lagoons after flooding events. In addition, this mechanism is more efficient in species occurring near water courses and after heavy rains (Crottini et al., 2007; Measey et al., 2007a; Correa et al., 2010); *P. falcipes* frequently reproduces in temporary pools close to small streams

(Gallardo, 1968) that are occasionally flooded after heavy rains (JAL pers. obs.).

Another potential mechanism for hydrochory in *Pseudopaludicola falcipes,* and other amphibians located near large Pampean river systems (e.g., Uruguay, Paraná, and de la Plata Rivers) is via vegetation rafts. The massive arrival of mats of "camalotes" (*Eichornia* sp.) in vegetation rafts on the shores of de la Plata River, originating in the upper and middle Paraná River, have been reported frequently during the last century after heavy floods (Ihering, 1911; Achaval et al., 1979; Prigioni and Langone, 1983; Sarli et al., 1992). Several species of frogs have been transported in "camalotes" rafts including *Pseudopaludicola falcipes* (Prigioni and Langone, 1983).

Other species of frogs transported in these rafts is *Leptodactylus podicipinus*, and because the southern distribution limit of *L. podocipinus* along the Paraná River in Argentina is Santa Fé city (Heyer et al., 2004), the transportation of this species on vegetation rafts to the shores of Montevideo implies a journey of approx. 600 km (JAL and RdS, pers. obs.). Similar long-distance passive dispersal through vegetation rafts has also been found in other major freshwater rivers (Schiesari et al., 2003; Upton et al., 2011) and even across the ocean (Vences et al., 2003; Measey et al., 2007b; Camargo et al., 2009). However, we did not find evidence of colonization events from the middle Paraná (localities 4, 17, and 31; Fig. 1) to the shores of the Río de la Plata in the diffusion analyses. Instead, the area of northeastern Buenos Aires Province was colonized from the Uruguayan coast approximately 176 kya (Fig. S3F). A denser sampling of localities on both shores of the Río de la Plata, and more precise time estimates, are needed to assess whether passive dispersal via vegetation rafts has been a potential mechanism of long-distance colonization in *P. falcipes.*

4.5. Population history

The Patagonia region of South America witnessed several glacial and interglacial cycles during the Quaternary that profoundly affected the open habitats of the Pampas with biotic replacement and exchange (Rabassa, 2011). The timing of these events suggests that the origin of *Pseudopaludicola falcipes* (1.76 mya) occurred during the glaciation stage 'Cerro del Fraile VI' between 1.78 and 1.43 mya in Patagonia (Rutter et al., 2012). Our inferred area of origin of *P. falcipes* is located in Cerro Largo Department, Uruguay (Fig. S2A), and is similar to the postulated place of origin of another Pampean frog, *P. henselii* (Barraso, 2014), as well as for annual killifishes of the *Austrolebias adloffii* group (M. Loureiro, pers. com.). Although the timing of these events is probably different among taxa, additional studies should be conducted to assess if the border

region between northeastern Uruguay and southern Rio Grande do Sul is a shared center of origin that could have acted as a refugium of the Pampean biota during the Pleistocenic glaciations.

The major colonization events of *Pseudopaludicola falcipes* occurred during interglacial periods: (1) 1.3 mya: interglacial in Patagonia between 1.3 and 1.1 mya (Rutter et al., 2012), (2) 950 kya: the end of the Great Patagonian Glaciation (GPG) corresponding to Interglacial Leerdam from North West European Stages (MIS 25), (3) 800 kya: between Post GPG I and Post GPG II (~760 kya), corresponds to Interglacial I, Cromerian Complex from North West European Stages (MIS 19), and (4) 500 kya: between Post GPG II and Post GPG III (760–260 kya) corresponding to Interglacial IV, Cromerian Complex from North West European Stages (MIS 14). Despite the possible influence of Pleistocene interglacial phases on lineage differentiation and long-distance colonization, it is less clear if populations of *P. falcipes* experienced any dramatic demographic change (e.g., exponential growth or bottlenecks) as a result of glacial cycles. In fact, demographic growth in both population clusters of *P. falcipes* was constant and steady during several glacial cycles (Fig. 5). These results are in contrast with the known patterns for the Northern Hemisphere, where climatic oscillations during the Quaternary had a major influence on range contraction to glacial refugia and post-glacial expansions (Avise, 2000; Hewitt, 2000, 2003, 2004). Nevertheless, our results are congruent with the patterns found in other Pampean frogs, *Physalaemus fernandezae* and *P. henselii* (Barraso, 2014), and in lizards and rodents from the arid Monte and Patagonia at higher latitudes, which were not affected demographically by recent glacial cycles (Camargo et al., 2013).

The patterns found for *Pseudopaludicola falcipes*, together with studies of other frogs in open habitats of South America (Gehara et al., 2014; Prado et al., 2012), may help to understand the patterns of wide geographic distribution found in other Pampean anurans. For example, a historical demographic analysis of the treefrog *Hypsiboas albopunctatus* suggested that this species underwent a significant population expansion associated with the invasion of open habitats formed during the Pleistocene in central Cerrado and southern Brazil (Prado et al., 2012). Therefore, the accumulated evidence across a range of vertebrate taxa suggests that recent glacial cycles had a more moderate impact in South America due in part to its lower latitude and continentality (Fraser et al., 2012), and also because open habitats might not have been altered much in comparison with the tropical forests that were repeatedly reduced to isolated refugia (Carnaval et al., 2009; Fitzpatrick et al., 2009). In addition, our results on *P. falcipes* support the idea that the ephemeral and unpredictable puddles used for reproduction in open formations (such as the Pampean plains) facilitate the dispersal of vagile and generalist species, which may reduce intraspecific divergence (Rodríguez et al., 2015).

Indirect evidence about the climatic conditions of southern South America during the Pleistocene (e.g., climatic modeling, geology, isotopes, phylogeny and community composition of terrestrial mammals) suggests that this region of the continent was dominated by open habitats including grasslands, steppes, and scrublands (Ortiz-Jaureguizar and Cladera, 2006). Towards the end of the Pleistocene, when *P. falcipes* reached its current distribution range ~230 kya (Fig. S3F), the Pampas had basically the same geographic extent as today (França et al., 2015), supporting the idea that the open Pampean habitats favored the spread of *P. falcipes* across the region. However, the species may have been absent from vast areas affected by marine transgressions during the Quaternary in the southern portion of the Argentinean Mesopotamia (Aceñolaza, 2004). Based on the diffusion analyses, the area occupied by the marine transgressions (northeast of Buenos Aires Province) was colonized from the Uruguayan coast approximately 176 kya (Fig. S3F). After this colonization, Holocene marine

transgressions (10–5 kya; Aceñolaza, 2004) should have extirpated these recently arrived populations, or they could have persisted in marginal habitats until the marine transgressions receded. In any scenario, denser sampling in Argentinean and Uruguayan shores of de la Plata River are necessary to pinpoint the origin and colonization time of the current populations in Buenos Aires Province.

5. Conclusions

Our study is one of the first phylogeographic investigations of an amphibian species from the Pampas biogeographic province. *Pseudopaludicola falcipes* shows high genetic diversity, small body size, and a large distribution range, but a poor geographic structuring. The historical dispersal ability of *P. falcipes* (~1 m/year) combined with large population sizes, passive mechanisms of dispersal, and the landscape homogeneity of the Pampean grasslands, have allowed the maintenance of high genetic variation without significant differentiation across its distribution range. As revealed in the diffusion analyses, these demographic attributes and landscape features have facilitated the occurrence of long-distance colonization among and within two large population clusters, especially during the interglacial periods of the Pleistocene. We expect that additional phylogeographic studies of Pampean frogs will help to elucidate if the patterns found here for *P. falcipes* are part of a more general pattern or if other population histories (i.e., demographic stability and lineage diversification) have also occurred in this temperate region of South America.

A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.11.012. These data include Google maps of the most important areas described in this article.

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