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Pleistocene climatic cycling and diversification of the Andean treefrog, *Hypsiboas andinus*

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Abstract

Our understanding of the causes of diversification of Neotropical organisms lags behind that of Northern Hemisphere biota, especially for montane and temperate regions of southern South America. We investigated the mitochondrial DNA genealogical patterns in 262 individuals of the frog *Hypsiboas andinus* from 26 sites across the eastern ranges of the Andes Mountains in Argentina and Bolivia. Our phylogenetic analyses indicate at least three distinct lineages: one representing *H. andinus* from Northwestern Argentina and southern Bolivia, at least one *H. andinus* lineage from northern Bolivia, and one clade containing both *H. andinus* (from the southern portion of the species range) and its putative sister taxon *Hypsiboas riojanus*. *Hypsiboas andinus* samples from northern Bolivia are well differentiated and may represent distinct species. The northern Argentine *H. andinus* lineage and southern *H. andinus* /*H. riojanus* lineage likely diverged between 2 and 6 million years ago; their current sympatry may be the result of secondary contact due to range expansion after isolation during Andean uplift or may reflect cryptic species. Within the geographically extensive northern *H. andinus* clade, we found significant geographical structuring consistent with historical fragmentation and subsequent range expansion. The timing of this fragmentation and range expansion coincide with the Pleistocene, a time of extensive climatic cycling and vegetational shifts. Average divergence among clades is lower than those found for other Neotropical taxa, highlighting the potential importance of recent climatic history in diversification in the southern Andes.

Keywords: Andes, Argentina, *Hypsiboas*, phylogeography, refugia

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Introduction

South America encompasses a disproportionately large fraction of global biodiversity (World Conservation Monitoring Centre 1992), yet our understanding of the mechanisms responsible for its origins lags far behind that of the northern temperate biota of North America and Europe (e.g. Hewitt 2000; Soltis *et al*. 2006). Comparatively fewer molecular phylogeographical studies of Neotropical compared to Holarctic species exist, with a skew in the former towards lowland taxa of lower latitudes (e.g. Patton *et al*. 1994; Lougheed *et al*. 1999; Costa 2003; Lougheed *et al*. 2006). Average divergence for Neotropical taxa appears to be deeper among lineages within 'species', as well as among species (Hackett & Rosenberg 1990; Chek *et al*. 2003). However, such conclusions derive from broad-scale comparisons between biogeographical realms, rather than between comparable biomes, latitudes or regions within realms (but see Martin & McKay 2004). Montane regions are proposed as important centres of species diversification (Simpson 1979; Fjeldså 1994; Moritz *et al*. 2000). Thus, research on factors that may have facilitated or impeded the genetic differentiation of taxa inhabiting Andean habitats is relevant to our understanding of diversification and speciation in the Neotropics. The extensive latitudinal span of the Andes, the distinct orogenic and climatic histories of different regions (Simpson 1979), and particularly the

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evidence that the southern Andes were greatly affected by the last glacial maximum (Clapperton 1993; McCulloch *et al*. 2000), suggests that patterns and causes of diversification vary along the entire Andean axis. Indeed, the south-central region of the Andes may more closely mirror patterns from temperate regions rather than lowland tropical ones. In the present study, we investigate timing and causes of diversification in a montane treefrog from southern Bolivia and northern Argentina.

Diversification of South American taxa has been influenced by uplift of the Andes and Pleistocene climatic cycling (e.g. Fjeldså 1994; Chesser 2000; Lougheed *et al*. 2000; Hubert & Renno 2006). The Andes began to rise in the late Cretaceous more than 60 million years ago (Ma), although it was not until the mid-Miocene (11–14 Ma) that central regions began to exceed 1000 m above sea level (a.s.l.) (Potts & Behrensmeyer 1992; Gregory-Wodzicki 2000). Major orogenesis occurred in the last 10 million years, resulting in the uplift to current elevations of greater than 4000 m a.s.l. (Gregory-Wodzicki 2000). Global cooling began 2.4 Ma, most notably with the advance of glaciers at high latitudes, with intense climatic cycling in the last 0.9 million years (Potts & Behrensmeyer 1992). Tropical regions also experienced variations in temperature and aridity associated with glacial and interglacial cycles, altering the distribution of various habitats (Vuilleumier 1971; Prance 1982; Potts & Behrensmeyer 1992; Hooghiemstra & van der Hammen 1998; Burnham & Graham 1999; Weng *et al*. 2007). The Andes and adjacent regions, including in southern South America, thus have a rich geological history of uplift and climate change, with necessarily pronounced effects on the distribution and diversification of flora and fauna (e.g. Muellner *et al*. 2005; Ruzzante *et al*. 2006; Yoke *et al*. 2006).

Contemporary northwestern Argentina possesses great diversity and complexity of habitats associated with the eastern ranges of the Andes reflecting spatial variation in precipitation and temperature (Handford 1988). The area is generally arid to semi-arid; however, heavy precipitation from humid summer air masses travelling from the northeast falls primarily on the eastern slopes of the Andean front ranges, resulting in longitudinal strips of forest stretching along the mountains south from Bolivia (an extension of the lowland Amazonian forests) to the province of Catamarca (~28 S). These montane forests are essentially continuous except for a narrow invasion of arid thorn scrub habitat in the province of Salta (~25 S, Fig. 1), which separates the southernmost tip of this forest peninsula (Brown *et al*. 2001). Away from these east-facing slopes, rainfall is much lower resulting in the semi-arid chaco on the flat terrain to the east, and the arid interior valleys and high plateau to the west (Handford 1988), both of which are possible dispersal barriers for taxa dependent on moist forests. Historical changes in elevation and climate likely resulted in changes in connectivity of habitats, possibly promoting diversification of taxa in this region.

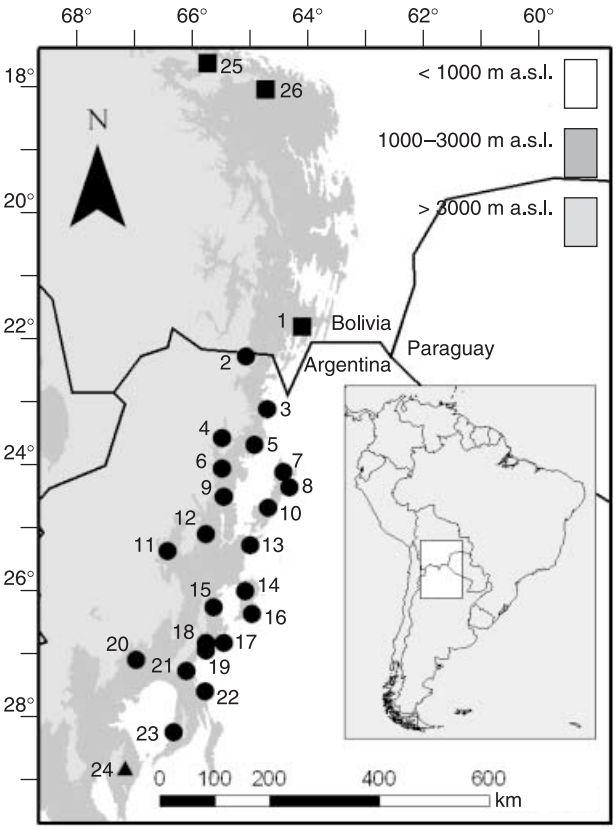

Fig. 1 Sampling localities of *Hypsiboas andinus* in northwestern Argentina (circles) and Bolivia (squares), and *Hypsiboas riojanus* (triangle). Site names and sample sizes are listed in Table 1.

Frogs are excellent taxa for studies of diversification and speciation as limited vagility tends to promote differentiation, and species vary markedly in fecundity and breeding habitats. Our study species, *Hypsiboas andinus*, is a moderate-sized treefrog (50–60 mm) in the large family Hylidae (Faivovich *et al*. 2005). *Hypsiboas andinus* is a member of the *Hypsiboas pulchellus* group, comprising 25 species distributed across southern South America, from the Brazilian lowlands in the state of São Paulo to the highlands of Peru, Bolivia and Argentina (Faivovich *et al*. 2004). Recent molecular phylogenies show that *H. andinus* is closely related to other Andean members of the group (*Hypsiboas balzani*, *H. marianitae*, *H. riojanus*) (Faivovich *et al*. 2004, 2005; Wiens *et al*. 2005). The relation between *H. andinus* and its putative sister species *H. riojanus* is unclear (Faivovich *et al*. 2004); therefore we included both *H. riojanus* and *H. balzani* in our phylogenetic analyses (detailed analyses of the four Andean taxa are underway, see discussion). *Hypsiboas andinus* occurs largely in deeply incised river valleys that penetrate into the Andes, from the province of Catamarca, Argentina (approximately 28 S) to north Bolivia (16 S), spanning

Table 1 Sources of tissues, sample sizes and distribution of haplotypes for mtDNA control region analyses for each population. Site numbers refer to Fig. 1 and haplotype codes refer to Figs 2 and 3. Samples were collected by D.K. and/or S.C.L. and/or P.H. in 1987, 2001, 2004, 2005 or 2006 unless otherwise noted. Haplotype diversity (h) and nucleotide diversity expressed as percent (π) are shown (standard deviations in parentheses) for all individuals in the population (values excluding lineage 2 individuals, where applicable, are italicized). Results from mismatch distributions for each population are as follows: NS, not significant (mismatch distribution is not significantly different from sudden expansion model); Unim, unimodal distribution; Bimod, bimodal distribution

	Site	n	Haplotypes	h	π	Mismatch distribution
1	Tarija, Bolivia*	1	28	—	—	—
2	Santa Victoria, Salta	2	20	0	0	—
3	San Andres, Salta	10	17, 18, 19, 21, 26, 27, 29	0.93 (0.06)	1.36 (0.82)	Unim, NS
4	Tilcara, Jujuy	11	1, 2, 3	0.65 (0.11)	0.26 (0.22)	Unim, NS
5	P. N. Calilegua, Jujuy	10	22, 23, 24, 25	0.53 (0.18)	0.24 (0.21)	Unim, NS
6	Lozano, Jujuy	19	1, 2, 10, 11, 23	0.57 (0.13)	0.37 (0.28)	Unim, NS
7	Villa Monte, Jujuy	6	1	0	0	—
8	Maiz Gordo, Jujuy	10	1, 10	0.36 (0.16)	0.10 (0.12)	Unim, NS
9	Ruta de Cornisa, Salta	7	1, 11, 23	0.52 (0.24)	0.60 (0.43)	—
10	P. N. El Rey, Salta	5	1, 10, 12	0.70 (0.22)	0.24 (0.23)	—
11	Molinos, Salta	3	14, 41, 42	1.00 (0.27)	2.00 (1.62)	—
12	S. F. de Escoipe, Salta	31	1, 4, 5, 13, 14, 15, 16	0.64 (0.09)	0.36 (0.26)	Unim, NS
13	Rio Piedras, Salta	5	1, 6	0.40 (0.24)	0.12 (0.15)	—
14	Rosario de la Frontera, Salta	7	1, 6	0.29 (0.20)	0.08 (0.11)	—
15	San Pedro, Tucumán	11	6, 30, 31, 37, 44, CF	0.85 (0.09) *0.82* (*0.10*)	2.44 (1.38) *1.17* (*0.73*)	Bimod, NS
16	Rio El Nio, Tucumán	20	6, 7, 8, 9, 15, 30, 31	0.81 (0.07)	0.92 (0.56)	Unim, NS
17	Villa Nogués, Tucumán	14	15, 31, 37, 39, 43, 50, CF	0.85 (0.07) *0.80* (*0.10*)	2.80 (1.54) *0.86* (*0.55*)	Bimod, NS
18	La Angostura, Tucumán	19	31, 37, 48, 49	0.52 (0.12)	0.44 (0.31)	Unim, NS
19	Rio Los Sosa, Tucumán	20	31, 33, 34, 37, 38, 39, 40, 43	0.85 (0.06)	0.78 (0.49)	Unim, NS
20	Haulfín, Catamarca	4	CB	0	0	—
21	Las Estancias, Catamarca	16	31, 32, 35, 36, 45, 47, CM, CN	0.81 (0.09) *0.75* (*0.11*)	2.40 (1.31) *0.48* (*0.34*)	Bimod, NS
22	Rio San Ignacio, Tucumán	21	31, 35, 43, 46, CA, CC, CD, CE, CG, CH	0.89 (0.04) *0.69* (*0.15*)	4.65 (2.41) *0.35* (*0.28*)	Bimod, $P = 0.022$
23	Pomán, Catamarca	6	CA, CI, CK, CL	0.80 (0.17)	1.06 (0.72)	—
24	Sanogasta, La Rioja (*H. riojanus*)	1	CJ	–	–	—
25	Cochabamba, Bolivia*	2	B1, B2	1.0 (0.5)	1.49 (1.62)	—
26	Santa Cruz, Bolivia*	2	B3	0	0	—

*Tissue loaned from the Museo Nacional de Ciencias Naturales, Madrid, Spain.

various habitats, from humid montane forests (500 m a.s.l.) to montane grasslands (> 1500 m a.s.l.) (Duellman *et al*. 1997).

The complex distribution of habitats across Northwestern Argentina and the complex tectonic and glacial history of the region suggest a range of possible historical and present-day connectivity scenarios that could have impacted patterns of genetic differentiation of populations. Our study seeks (i) to better understand the roles of aforementioned contemporary and historical factors in shaping the distribution of genetic diversity of *H. andinus* across its range in Northwestern Argentina, the southern edge of the 'tropical forest' ecosystem, and (ii) to compare the levels of divergence and underlying causes of diversification in this southern montane frog species to other, lower latitude, Neotropical taxa, and to Nearctic anurans.

Methods

Sampling

Samples included in this study were either (i) field collected by the authors ($n = 258$), or (ii) tissues loaned from the Museo Nacional de Ciencias Naturales (MNCN), Madrid, Spain ($n = 6$) (Table 1).

Field methods

A total of 257 individuals of *Hypsiboas andinus* from 23 sites across the Argentine portion of the species' range, approximately 700 km north to south, were collected under provincial and federal permits during several field expeditions (Fig. 1, Table 1). We also collected *Hypsiboas*

riojanus at one site for outgroup analyses. For each locale, streams and surrounding areas were typically searched between 21:00 and 03:00 hours (local time). Males were located by advertisement calls and captured by hand (females and tadpoles were sampled if encountered).

One or two frogs per site were killed by submersion in an anaesthetic (MS222) to be used to verify identification and build a permanent specimen reference collection. From these, liver tissue was removed and stored in 70% ethanol for subsequent molecular work. The frogs were then fixed in 10% formalin, stored in 70% ethanol, and deposited at Argentine Institutions (Appendix). For the remainder, one or two toes were clipped to provide tissue for genetic studies and the frogs released at the site of capture. Toes were stored in 70% ethanol until DNA extraction.

DNA extraction, polymerase chain reaction and sequencing

Samples collected in 1987 and 2001 were extracted using standard phenol–chloroform protocols (Sambrook & Russel 2001) and DNA was stored in 1× Tris EDTA buffer. Samples collected in 2004–2006 and those on loan from MNCN were extracted using the DNeasy Tissue Kit (QIAGEN).

A 663-bp fragment of mitochondrial control region was amplified using primers ControlP(H) and Wrev(L) from Goebel *et al*. (1999). Polymerase chain reaction (PCR) cocktails contained 10 ng genomic DNA, 2.5 mM $MgCl_2$, 4 g BSA, 0.2 M dNTP, 2 U *Taq* polymerase in a total volume of 20 L. For samples collected in 1987 and 2001, PCR products were electrophoresed in 1% agarose and purified by a 'freeze-squeeze' method (Thuring *et al*. 1975). Fragments were sequenced with the ControlP(H) primer using the Thermo Sequenase Radio-labelled terminator cycle sequencing kit according to manufacturer's protocols (Amersham Bio-Sciences), electrophoresed on vertical 6% polyacrylamide gels and exposed to autoradiography film for 24–36 h. All autoradiographs were scored by hand. For samples collected in 2004–2006 and those on loan from MNCN, PCR products were purified (Multiscreen; Millipore), and sequencing was performed using BigDye Terminator chemistry (version 3.1) and analysed on ABI 3730xl sequencers (Applied Biosystems). Bidirectional reads for a subset of 10 individuals resulted in identical sequences providing greater confidence that variation among haplotypes is not an artefact of PCR or sequencing error.

A 358-bp fragment of cytochrome *b* was amplified for a subset of samples representing the diversity of control region haplotypes (see below), all Bolivian *H. andinus* samples, one *H. riojanus*, and one *H. balzani* using primers MVZ15-L from Goebel *et al*. (1999) and a modified version of H15149 (Lougheed *et al*. 1999). PCR and sequencing protocols were the same as for the control region fragment for samples collected in 2004–2006.

Analyses

Sequence alignments were made in CLUSTAL_X, version 1.83 (Thompson *et al*. 1997) with subsequent visual verification. We used ARLEQUIN version 2.000 (Schneider *et al*. 2000) to calculate haplotype and nucleotide diversity indices for control region sequences for each population using Kimura 2-parameter (K2P) molecular distance. We categorized the results according to Grant & Bowen (1998) as reflecting (i) prolonged bottleneck ($h < 0.5$ and $\pi < 0.5\%$), (ii) rapid population growth from ancestral population with low effective population size ($h > 0.5$ and $\pi < 0.5\%$), (iii) a brief bottleneck ($h < 0.5$ and $\pi > 0.5\%$), or (iv) stable population with large historical effective population size, or secondary contact among differentiated lineages ($h > 0.5$ and $\pi > 0.5\%$). Although the boundaries appear sharp, Grant & Bowen (1998) have included nucleotide diversities up to 0.71% in category ii. We performed mismatch distributions for all populations with sample size greater than 10 and tested against a model of sudden expansion using the generalized nonlinear least-squares approach (Schneider & Excoffier 1999). Model validity was evaluated using 1000 parametric bootstraps as implemented in ARLEQUIN.

Phylogenetic analyses

For phylogenetic analysis, we used a combined data set of control region and cytochrome *b* for a subset of *H. andinus* samples collected in northwestern Argentina ($n = 33$, representing 18 of the 23 populations); one *H. riojanus* collected in northwestern Argentina; five *H. andinus* collected in Bolivia; and one *H. balzani* as an outgroup.

We used MODELTEST (version 3.7, Posada & Crandall 1998) and the Akaike information criterion to select the best model of evolution for subsequent maximum likelihood (ML) analysis. ML analyses were run using 10 random-addition replicates and we evaluated support of the resulting topologies using 100 nonparametric bootstraps with PAUP* (version 4.0b10, Swafford 2002). We also performed Bayesian analyses (MR.BAYES 3.1.2, Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) with two independent runs of 10^6 generations implementing Metropolis-coupled Markov chain Monte Carlo (MCMC), each with four incrementally heated Markov chains, sampling every 100 generations, and burn-in of 25%. Convergence of the two runs was assumed when the average standard deviation of the split frequencies was less than 0.01 and the potential scale reduction factor approached 1.00.

Phylogeographical analyses

We used only control region data for phylogeographical analyses. Based on the results of our phylogenetic analyses, we included all *H. andinus* from northwestern Argentina

and one individual from southern Bolivia, as well as one individual of *H. riojanus*. TCS version 1.21 (Clement *et al*. 2000) was used to generate a maximum parsimony network (MPN) representing the genealogical relationships among control region haplotypes. A nested clade analysis (NCA; Templeton *et al*. 1992; Templeton *et al*. 1995; Templeton 1998) was applied to integrate genealogical information with geographical distributions. Ambiguities were resolved following the rules of Templeton & Sing (1993). All distance measure calculations and permutations ($n = 1000$) were assessed using GEODIS version 2.5 (Posada *et al*. 2000) and the significant results were then interpreted using a published inference key (11 November 2005, available at http://darwin.uvigo.es/). Cognizant of criticisms of NCA due to the possibility of false–positives (Petit 2008), we performed additional analyses to aid us in interpreting patterns. These included the supplementary tests for secondary contact where appropriate (Templeton 2001), mismatch distribution analyses and comparisons of haplotype (h) and nucleotide diversity (π) for each of the main clades at the total cladogram level in ARLEQUIN.

Timing of divergence

To estimate the time of divergence among clades, we used only cytochrome *b* sequences because molecular evolution of this gene is better understood than that of control region. We calculated corrected average pairwise differences among the clades using the K2P corrected molecular distance using ARLEQUIN. We applied a slower poikilothermic molecular clock typically used for anuran cytochrome *b* (see Austin *et al*. 2004) with a rate of change between 0.5% and 1% per million years. We also used a coalescent approach as implemented in the program BEAST version 1.4.6 (Drummond & Rambaut 2007). We performed two independent runs of 20 million generations each with burn-ins of 2 000 000, which were then combined in TRACER version 1.4 (Rambaut & Drummond 2007). We employed a GTR + I + G model of evolution with six rate categories and assumed a relaxed lognormal clock (using rates of divergence of 0.5% and 1% per million years). Parameters were sampled every 1000 generations. All other initial parameters settings were the default provided by BEAST version 1.4.6.

Results

Molecular diversity

A 340-bp fragment of the mitochondrial control region was obtained for each of the 257 *Hypsiboas andinus* individuals sampled from across the entire Argentine range of the species, five individuals from Bolivia, and one individual of *Hypsiboas riojanus* (GenBank Accession nos EU403157–EU403420). Sixty-eight haplotypes were defined by 91 polymorphic sites (60 parsimony informative), differing by 1–41-bp substitutions. One shared deletion was observed in two individuals (representing two different haplotypes), and one insertion was detected in the outgroup. Gaps were treated in subsequent analyses as a fifth state (where possible). We found an overall AT nucleotide bias, as is characteristic of vertebrate mitochondrial DNA (mtDNA, Saccone *et al*. 1987). Haplotype and nucleotide diversity indices for each population are listed in Table 1.

A 358-bp fragment of cytochrome *b* was obtained for the 40 samples used for phylogenetic analyses (GenBank Accession nos EU403117–EU403156). A total of 22 haplotypes were defined by 59 polymorphic sites (33 parsimony informative), differing by 1–37-bp substitutions. As for control region, an overall AT nucleotide bias was evident. No stop codons were found.

Phylogenetic analyses on the combined data set

The combined data set of control region and cytochrome *b* comprised 699 bp for a subset of 33 individuals of *H. andinus* from northwestern Argentina and five from Bolivia, one individual of *H. riojanus*, and one individual of *Hypsiboas balzani* as an outgroup. The best-fit model of evolution was the general time reversible model with a set proportion of invariable sites (I = 0.3863), with gamma distributed rates (G = 0.3505) (GTR + I + G). Trees derived from both ML and Bayesian methods were similar, with generally stronger support for the clades obtained by Bayesian analyses (Fig. 2). Two main clades were evident. Clade no. 1 contained *H. andinus* from northwestern Argentina and one of five individuals of *H. andinus* from Bolivia (site no. 1). Clade no. 2 contained the single *H. riojanus* plus several *H. andinus* individuals from the southern portion of the Argentine range. The remaining four *H. andinus* samples from Bolivia (all in northern Bolivia) did not form a single clade but are basal to both Clade no. 1 and no. 2.

Phylogeographical patterns

We used all 257 individuals from Argentina as well as one sample from southern Bolivia and one sample of *H. riojanus* for phylogeographical analyses. Northern Bolivian samples were excluded due to small sample size and sparse coverage of that portion of the species range. The resulting two maximum parsimony networks could not be connected at the 95% parsimony level and were separated by a minimum of 21 mutational steps exceeding the parsimony limit of seven steps. We therefore analysed these independently (Fig. 3). Network no. 1 contained only individuals identified as *H. andinus*, and network no. 2 contained the single *H. riojanus* sample plus additional samples identified as *H. andinus* as found in the phylogenetic analyses.

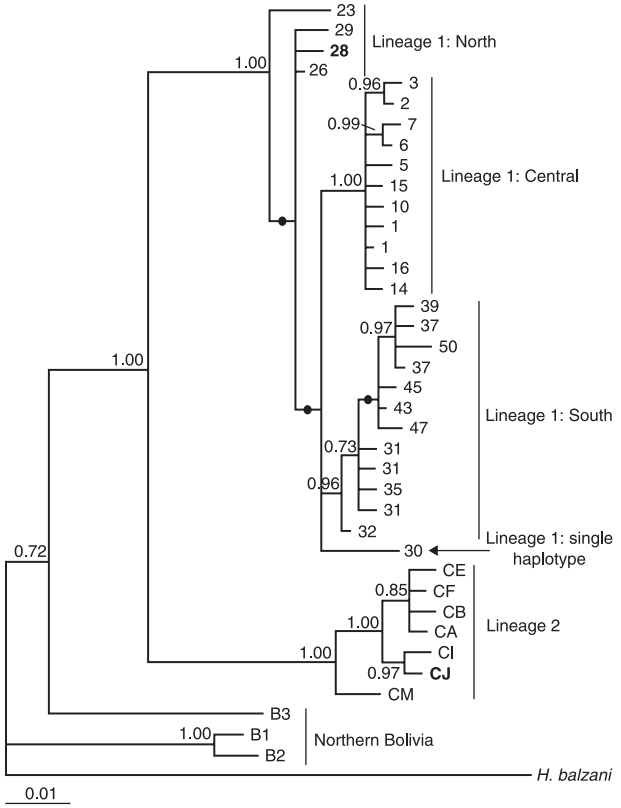

Fig. 2 Phylogenetic relationships of Argentine and Bolivian *Hypsiboas andinus*, and *Hypsiboas riojanus* based on Bayesian analyses of cytochrome *b* and control region sequences, using *Hypsiboas balzani* as an outgroup. Posterior probability values are indicated for each branch, filled circles indicate values less than 0.70. Haplotype codes correspond to those shown in Fig. 3 and listed in Table 1. Boldface haplotypes: no. 28, southern Bolivia, population no. 1; no. CJ, sample of *H. riojanus*, population no. 24.

Some ambiguous loops in network no. 1 could not be resolved following the rules of Templeton & Sing (1993) (Fig. 3); therefore, we used the combined data set tree as a guide for the nesting procedure. Hierarchical nesting resulted in four nested levels in network no. 1. At the total cladogram level, three distinct clades showed a north to south distribution with little geographical overlap: clade 4-2 (north), the large clade 4-1 (central) and clade 4-3 (south) (Fig. 4). We assigned clade 4-1 as interior and 4-2 and 4-3 as tips because the former contained haplotype no. 1, which had the highest outgroup probability and therefore is likely to be the oldest haplotype in the network (Castelloe & Templeton 1994). Inferences for lower nesting levels indicated a mixture of range expansion, restricted gene flow and past fragmentation (see Tables S1 and S2, Supplementary material).

Six populations (nos 6, 9, 11, 15, 16, and 17) showed patterns consistent with secondary contact among divergent lineages based on the supplemental tests from Templeton (2001) (data not shown). Populations 6 and 9 contain haplotypes from clades 4-1 and 4-2, whereas the remaining populations share haplotypes from clades 4-1 and 4-3. In all cases, the majority of individuals in a population contain haplotypes from their 'home' clade and only one or two individuals contain haplotypes from a geographically adjacent clade. Population nos 21 and 22 also contain haplotypes from network no. 1 (clade 4-3) and network no. 2.

Mismatch distributions for all populations (with $n > 10$) except Rio San Ignacio (no. 22) were consistent with population growth, as the distributions of pairwise differences did not differ significantly from the sudden expansion model ($P > 0.05$). Many populations showed high h and low π, also indicative of population growth according to Grant & Bowen (1998) (Table 1). Population nos 15, 17, and 21 showed bimodal distributions, although the deviation was not significant. Population no. 22 differed significantly from the sudden expansion model and showed a strongly bimodal distribution. Population nos 15, 17, 21, and 22 also showed both high h and π implying large stable populations or secondary contact between differentiated lineages (Grant & Bowen 1998). For populations containing haplotypes from network no. 1 and no. 2, we calculated h and π for all individuals and excluding those with network no. 2 haplotypes (Table 1).

For network no. 1, clade 4-1 (central) and clade 4-3 (south) exhibited patterns characteristic of rapid population expansion using both mismatch distributions (4-1: $\tau = 1.163$, $P = 0.67$, 4-3: $\tau = 2.441$, $P = 0.39$) and haplotype and nucleotide diversity patterns (4-1: $h = 0.69$ and $\pi = 0.355\%$, 4-3: $h = 0.82$ and $\pi = 0.588\%$). Clade 4-2 (north) had a multimodal mismatch distribution ($P = 0.07$), and both high h (0.87) and π (1.256%) suggesting either a stable population or secondary contact.

Three clades of network no. 2 showed significant geographical associations (Table S1) but inferences were not possible due to low sample size (tip/interior status could not be determined). All individuals in population nos 20 and 23 contained network no. 2 haplotypes, although some individuals from four other populations also contained network no. 2 haplotypes (nos 15, 17, 21, 22). For network no. 2, clade 3-1 showed high haplotype diversity (0.91) and low to moderate nucleotide diversity (0.63%) and a unimodal mismatch distribution ($\tau = 2.246$, $P = 0.11$) likely due to rapid population expansion. Given the three unique and divergent haplotypes found in clade 3-2 of network no. 2, the haplotype and nucleotide diversity were both high ($h = 1.0$, $\pi = 1.319\%$).

Timing of divergence

We estimated divergence times using cytochrome *b* at two distinct levels. At the deepest level, between network no. 1

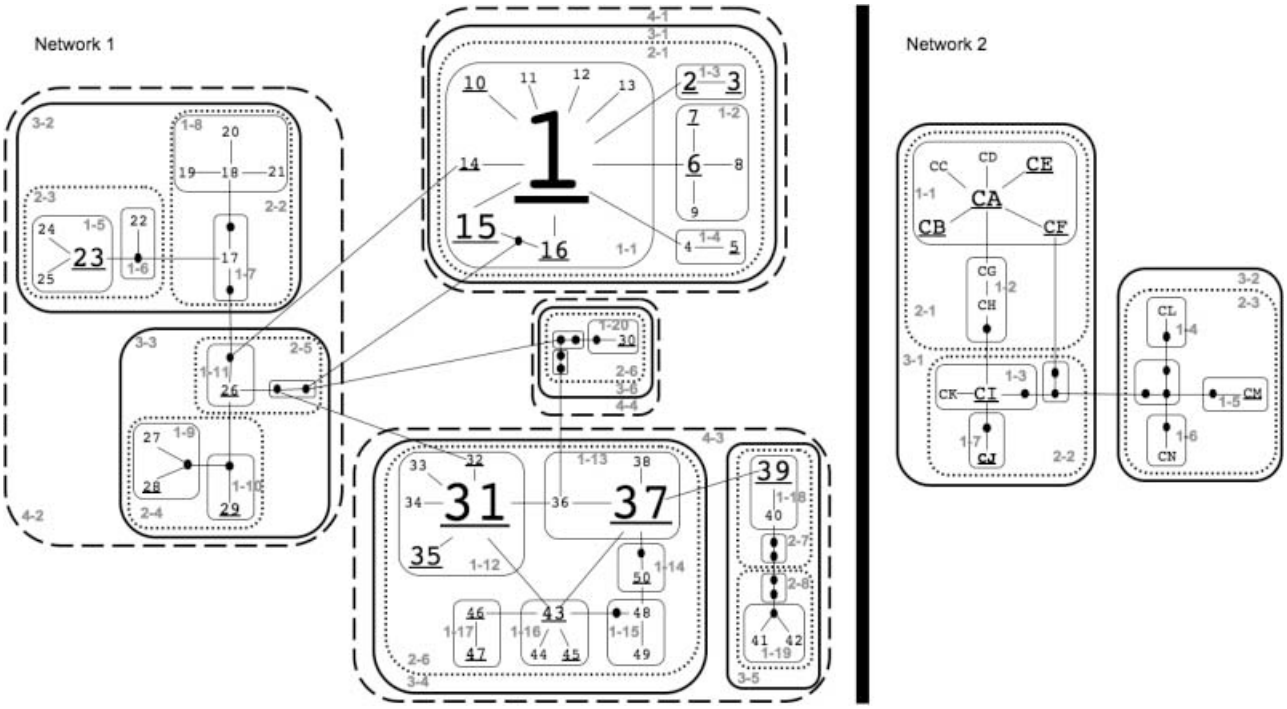

Fig. 3 Maximum parsimony networks using mtDNA control region from 259 individuals. Filled circles indicate missing or unsampled haplotypes and font size approximates relative sample size for each haplotype (distribution of haplotypes among populations listed in Table 1). Nested clades are indicated with rectangles: thin solid lines for 1-step clades, dotted for 2-step clades, thick solid for 3-step clades, and dashed for 4-step clades. The two networks could not be connected at the 95% parsimony level and were analysed separately. Underlined haplotypes are those included in the combined data set for phylogenetic analyses (haplotype CJ = *H. riojanus*).

Table 2 Estimates of divergence time based on analyses using BEAST (see text for details). The analyses were run using a molecular clock of 1% or 0.5% per million years. Clades are identified as for the phylogeographical analyses. TMRCA, time to most recent common ancestor; Ma, million years ago

	TMRCA (Ma)	
Clade	1%	0.5%
Networks 1 and 2	2.167	4.331
Network 1	0.920	1.846
Clade 4-1	0.144	0.286
Clade 4-2	0.528	1.048
Clade 4-3	0.840	1.684
Network 2	0.792	1.600

(*H. andinus* clade) and network no. 2 (*H. riojanus* clade), the corrected average pairwise K2P molecular distance was 3.1%. Using rates of 0.5% and 1% per million years suggests a divergence time between the *H. andinus* clade and *H. riojanus* clade of 3–6 Ma. Within network no. 1 only, the K2P distances of less than 1% (north-central = 0.84%, central-south = 0.56%, north–south = 0.27%) imply divergence times between 1 and 2 Ma. Divergence times calculated using a coalescent approach (Table 2) were similar. Again, the deepest divergences for network no. 1 were all less than 2 Ma, placing them within the Pleistocene (Table S3, Supplementary material).

Discussion

Both historical and contemporary connectivity among populations play important roles in shaping evolutionary trajectories of lineages. In highly heterogeneous regions like the Andes, there is high potential for isolation and for spatial habitat variation to affect the movement of individuals. Unsurprisingly, *Hypsiboas andinus* showed significant phylogeographical structuring across its Argentine range. The patterns likely represent an interaction between contemporary restriction of gene flow and historical barriers that caused range fragmentation with later secondary contact. Our study supports the growing body of research illustrating how historical events have impacted genetic diversity in the Andes, and especially the role more recent Pleistocene climate cycling may play in population differentiation.

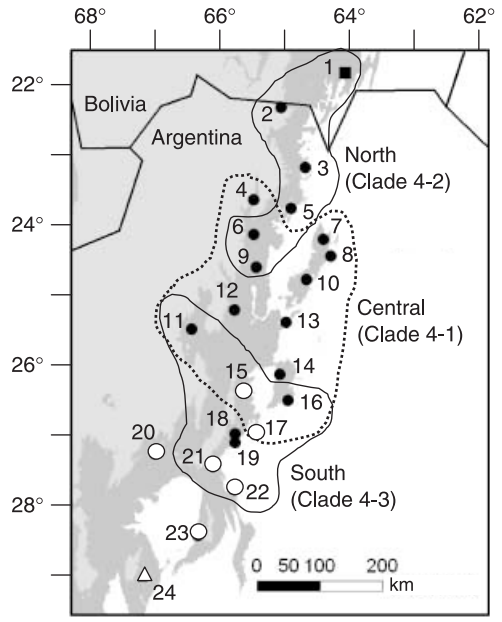

Fig. 4 Geographical distribution of 4-step clades from network no. 1. Populations within the three main groups of network no. 1 that also contain haplotypes from network no. 2 are indicated with unfilled circles. Population nos 20, 23 and 24 contain only network no. 2 haplotypes. Population nos 25 and 26 were not included in the analyses. Shading represents elevation as in Fig. 1.

Divergent lineages within the H. andinus/H. riojanus *complex*

While the relationships among *H. andinus* and other members of the *Hypsiboas pulchellus* group are uncertain (Barrio 1965; Duellman *et al*. 1997; Faivovich *et al*. 2004), recent work unambiguously shows Andean members of the group to be monophyletic within the broadly distributed (eastern Brazilian and Argentinian lowlands westward to the highlands of Peru, Bolivia and Argentina) *H. pulchellus* group (Faivovich *et al*. 2004, 2005; Wiens *et al*. 2005). Although *H. andinus* and *H. riojanus* together form a monophyletic group (Faivovich *et al*. 2004), our findings indicate that the relationship between these two taxa may be complicated.

A single individual of *H. andinus* from extreme southern Bolivia (population no. 1, haplotype no. 28) clustered with populations from northern Argentina (northern lineage, clade 4-2), but the four samples from northern Bolivia (population nos 25 and 26) are genealogically separate. The taxonomic status of Bolivian and Argentine populations of *H. andinus* is not well established (Faivovich *et al*. 2004 and Faivovich *et al*. 2005 employed no Bolivian samples). Our preliminary analyses imply that *H. riojanus* is nested within *H. andinus*, making *H. andinus* a paraphyletic taxon. We did find two distinct, divergent lineages, but 27 individuals from six populations, five of which were asserted to contain only *H. andinus* (Cei 1980; Duellman *et al*. 1997) cluster with *H. riojanus* (Figs 2 and 3). This may be due to a sympatric distribution of two morphologically similar taxa (i.e. cryptic species) in the provinces of Tucumán and Catamarca, or to secondary contact between divergent lineages. Nuclear loci are currently being developed to distinguish between these two scenarios.

All samples collected at our site nos 20 and 23 cluster with *H. riojanus*, and four other sites contain both mitochondrial lineages. The only morphological feature known to distinguish the two species is the presence of a dashed white or cream dorsolateral stripe that begins behind the eyes in *H. andinus* (Cei 1980; Faivovich *et al*. 2004), but the extensive colour and pattern variability seen in *H. andinus* shows that this is not a reliable diagnostic feature. Given that individuals representing both mitochondrial lineages can be found breeding in the same locale, only strong reproductive isolating mechanisms would prevent hybridization, if indeed the two are different species. Call differences, presumably the main cue for mate selection, are minimal between the taxa (Barrio 1965) although playback and female choice experiments have not been reported. Limited sonographic analyses of *H. andinus* from five of the populations sampled suggest that calls are highly variable within and among populations (Liadsky 2003).

The 3.1% divergence at cytochrome *b* between the lineage representing *H. andinus* alone (network no. 1) and that containing both *H. riojanus* + *H. andinus* (network no. 2) is lower than between-species divergences reported for many Neotropical frogs (Chek *et al*. 2001; Symula *et al*. 2003; Camargo *et al*. 2006). The two dating methods we used suggest a divergence time between 2 and 6 Ma between these networks, a time spanning both major uplift of the Andes Mountains, and the formation of arid high-altitude habitats (Gregory-Wodzicki 2000) that would have been significant barriers to movement. During uplift, populations would have become isolated, especially if drainage patterns, the presumed main paths of connectivity, changed. Following such isolation, subsequent range expansion, perhaps during warmer and moister interglacial periods, would have resulted in secondary contact of the *H. riojanus* lineage and southern populations of *H. andinus*. The current evidence — limited if any morphological and call differences; shallow genetic divergence — suggests that the two taxa are probably not reproductively isolated species.

Phylogeography of H. andinus

Our NCA and mismatch analyses implied that both demographic and historical processes affected the patterns of differentiation in *H. andinus*. Frogs generally have restricted movements (less than 10 km) due to their small size, biphasic life history, water dependence, and philopatry (reviewed

in Marsh & Trenham 2001). *Hypsiboas andinus* is a mountain stream breeder, and is never found far from water (Cei 1980, personal observation). Andean streams flow through various grassland, forest, and scrub habitats as they descend from the mountains and unite in the eastern semi-arid chaco, which is outside of the range of *H. andinus*. Connectivity of populations therefore would be constrained by the drainage patterns connecting streams, and/or by the probability of their crossing through moist habitats that might lie between water courses. Given that the streams flow through steep valleys and that connectivity of drainages is limited within the range of the species, and given its physiological limitations to movement, we expect that dispersal for this species is usually low except within the immediate local stream system. Limited dispersal in montane regions has been reported for several amphibians and topographical ridges have been implicated as strong barriers that promote marked differentiation among populations (Lougheed *et al*. 1999; Funk *et al*. 2005; Lowe *et al*. 2006). Similar to other montane frogs, *H. andinus* shows evidence of restricted gene flow resulting in the genetic structuring of populations.

At the total cladogram level for network no. 1, both NCA on the control region data and phylogenetic analysis of the combined data set support four lineages: clades 4-1, 4-2, and 4-3, plus one divergent haplotype (no. 30). The three main clades of network no. 1 form a north–south series with little geographical overlap between them (Fig. 4), and probably diverged less than 2 Ma. This span falls within the Pleistocene and corresponds to major climatic cycling and probable vegetational changes in the northern Argentine montane vegetation. Major global cooling and cycling between glacial vs. interglacial, reflecting cool and arid vs. warm and moist periods, respectively, was most pronounced in the last 0.9 million years (Potts & Behrensmeyer 1992; Hooghiemstra & van der Hammen 1998; Burnham & Graham 1999). Tropical regions experienced climate instability resulting in contraction and expansion of different habitat types, especially tropical forests (Vuilleumier 1971; Prance 1982; Potts & Behrensmeyer 1992; Hooghiemstra & van der Hammen 1998; Burnham & Graham 1999; Weng *et al*. 2007). Effects of climatic cycling on montane regions are particularly difficult to reconstruct given the rugged topography, the complexity of related changes in temperature and precipitation, and the interplay between the two factors, but there is little doubt that Pleistocene climatic cycling resulted in major shifts in Andean habitat. At glacial maxima, snow lines may have descended by as much as 1000 m, and global temperatures may have decreased by 6 C (Vuilleumier 1971; Potts & Behrensmeyer 1992). High altitude habitats expanded to lower altitudes (Vuilleumier 1971; Prance 1982; Weng *et al*. 2007) while in the lowlands, drier habitats also expanded in many areas (Vuilleumier 1971; Prance 1982; Burnham & Graham 1999), fragmenting moist forests. Vegetational distribution undoubtedly strongly influenced the movements and ranges of many animal taxa, likely isolating populations. *Hypsiboas andinus* currently does not inhabit the high-altitude puna nor the low-altitude seasonally dry chaco: it is restricted to the moister woodlands and grasslands typically found on mountain slopes between these two drier habitats. Thus, we propose that *H. andinus* populations became isolated during glacial maxima in the pockets of mesic forest that remained.

Palaeoclimatic data from northwestern Argentina are too limited to identify unambiguously potential mesic refugia that correspond to each of the three clades identified here. However, two contemporary well-defined areas, at ~22 S and ~27 S, of especially moist habitat could have retained their mesic character during glacial dry periods and thus supported montane forests and dependent populations of taxa such as *H. andinus*. These two areas also contain high numbers of endemic taxa and have been suggested as potential refugia during glacial intervals (Brown *et al*. 2001; Quiroga & Premoli 2007). Wet sites in the northern area (~22 S) around the Argentine/Bolivian border in the Upper Bermejo River Basin currently receive more than 2300 mm of rain annually (Grau & Brown 2000). The southern mesic region, west of Concepción, Tucumán (~27 S), receives more than 1400 mm precipitation annually (Grau & Veblen 2000). The high elevation cordilleras (> 5000 m a.s.l.) to the west of these regions trap much of the precipitation from the humid summer air masses moving southwestward. Additionally, the concave shape of the Cordillera Aconquija to the west of Concepción contributes to the trapping of rainfall. On a larger scale, the similar concave topography in the northern Andes (between 5 and 15 south) has been implicated in maintaining moist Amazon rainforest during the same glacial periods (Hooghiemstra & van der Hammen 1998). The area between these two sites (~24 to 26 south) currently contains narrow bands of rainforest (Sierras de Metán, Lumbrera, Santa Barbara) that are separated by chacoan arid thorn scrub, effectively isolating the southernmost extension of moist Andean forest. During dry glacial periods, the arid thorn scrub almost certainly extended its range, probably completely severing the connection between the southern (26 to 27 S) and the northern (< 24 S) moist forests. The three network no. 1 clades described here correspond closely with these three areas: northern moist, central dryer, southern moist. Clade 4-3, found in the southern moist region, and clade 4-1, found in the central dry region, show evidence of secondary contact of divergent lineages, as well as contiguous range expansion based on the NCA, mismatch distributions and haplotype and nucleotide diversity patterns, lending further support to range contraction during dry glacials and subsequent range expansion during moister interglacial periods. Further work in northwestern Argentina will clarify the role of historical climatic fluctuation, and the resultant habitat changes, in isolation and diversification of *H. andinus* and other taxa.

Diversification in the Andes

Depths of divergence among populations have been shown to differ across latitudes (as does species richness), both being greater at lower latitudes (Martin & McKay 2004). The differentiation events inferred for the whole *H. andinus* and *H. riojanus* complex (2–6 Ma), as well as for clades within the northern network no. 1 (less than 2 Ma), are more recent than those found for many other Neotropical taxa (birds: Voelker 1999; Burns & Naoki 2004; Cheviron *et al*. 2005; mammals: Patton *et al*. 2000; Costa 2003; frogs: Chek *et al*. 2001; Symula *et al*. 2003; Camargo *et al*. 2006; Lougheed *et al*. 2006). These studies suggest that much of the diversification in the Andean region occurred during the Pliocene, whereas few taxa have been shown to have levels of divergence shallow enough to coincide with Pleistocene climatic fluctuation (García-Moreno *et al*. 1999; Chesser 2000; Noonan & Gaucher 2005; Rull 2006). These latter levels of divergence are more typical of divergence times of temperate anurans (Green *et al*. 1996; Masta *et al*. 2002; Martínez-Solano 2004; Nielson *et al*. 2006). Although relatively few studies exist for the Neotropics, especially prominent are the increasing number of data sets, including ours, supporting the much shallower divergences of vertebrate taxa in the Andes as compared to the Amazon Basin (reviewed in Moritz *et al*. 2000) suggesting that the Andes may not mirror patterns typically associated with lowland low-latitude South America. Factors that affect diversification rates in high latitude temperate regions such as seasonality, climatic cycling, range shifting and recolonization (Martin & McKay 2004), may also contribute to shallower divergences for higher altitude regions as well as high latitude regions in southern South America.

Faivovich *et al*. (2004) suggested that the *H. pulchellus* group originated in the Atlantic Forest of southeastern Brazil. The four taxa discussed above are the only Andean representatives in this clade — likely a result of a recent dispersal event of the ancestor into the Andes. Since the late Tertiary, the Atlantic Forest was probably intermittently connected by two or three pathways with the lowland Amazon forests (Costa 2003), and this may have been the route taken by the ancestor of the Andean members of the *H. pulchellus* group to reach the Andes. The low level of differentiation and distinct geographical distribution from other members of the *H. pulchellus* group suggests a radiation of the Andean taxa perhaps less than 10 Ma. However, future work with more extensive sampling and additional markers will clarify evolutionary affinities and refine estimates of the timing of divergence, particularly of the Bolivian populations of *H. andinus*.

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References

Austin JD, Lougheed SC, Boag PT (2004) Discordant temporal and geographic patterns in maternal lineages of eastern north American frogs, *Rana catesbeiana* (Ranidae) and *Pseudacris crucifer* (Hylidae). *Molecular Phylogenetics and Evolution*, **32**, 799–816.

Barrio A (1965) Las subespecias de *Hyla pulchella* Duméril y Bibron (Anura, Hylidae). *Physis*, **69**, 115–128.

Brown AD, Grau HR, Malizia LR, Grau A (2001) Argentina. In: *Bosques Nublados del Neotrópico* (eds Kappelle M, Brown AD). Instituto Nacional de Biodiversidad, Costa Rica.

Burnham RJ, Graham A (1999) The history of Neotropical vegetation: new developments and status. *Annals of the Missouri Botanical Garden*, **86**, 546–589.

Burns KJ, Naoki K (2004) Molecular phylogenetics and biogeography of Neotropical tanagers in the genus *Tangara*. *Molecular Phylogenetics and Evolution*, **32**, 838–854.

Camargo A, De Sá RO, Heyer WR (2006) Phylogenetic analyses of mtDNA sequences reveal three cryptic lineages in the widespread neotropical frog *Leptodactylus fuscus* (Schneider, 1799) (Anura, Leptodactylidae). *Biological Journal of the Linnean Society*, **87**, 325–341.

Castelloe J, Templeton AR (1994) Root probabilities for intraspecific gene trees under neutral coalescent theory. *Molecular Phylogenetics and Evolution*, **3**, 102–113.

Cei JM (1980) Amphibians of Argentina. *Monitore Zoologico Italiano N. S Monografia 2*, **2**, 1–609.

Chek AA, Lougheed SC, Bogart JP, Boag PT (2001) Perception and history: molecular phylogeny of a diverse group of Neotropical frogs, the 30-chromosome *Hyla* (Anura: Hylidae). *Molecular Phylogenetics and Evolution*, **18**, 370–385.

Chek AA, Austin JD, Lougheed SC (2003) Why is there a tropical-temperate disparity in the genetic diversity and taxonomy of species? *Evolutionary Ecology Research*, **5**, 69–77.

Chesser RT (2000) Evolution in the High Andes: the phylogenetics of *Muscisaxicola* ground-tyrants. *Molecular Phylogenetics and Evolution*, **15**, 369–380.

Cheviron ZA, Hackett SJ, Capparella AP (2005) Complex evolutionary history of a Neotropical lowland forest bird (*Lepidothrix coronata*) and its implications for historical hypotheses of the origin of Neotropical avian diversity. *Molecular Phylogenetics and Evolution*, **36**, 338–357.

Clapperton CC (1993) *Quaternary Geology and Geomorphology of South America*. Elsevier, Amsterdam, The Netherlands.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Costa LP (2003) The historical bridge between the Amazon and Atlantic Forest of Brazil: a study of molecular phylogeography with small mammals. *Journal of Biogeography*, **30**, 71–86.

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.

Duellman WE, de la Riva I, Wild ER (1997) Frogs of the *Hyla armata* and *Hyla pulchella* groups in the Andes of South America, with definitions and analyses of phylogenetic relationships of Andean groups of *Hyla*. *Scientific Papers of the Natural History Museum of the University of Kansas*, **3**, 1–41.

Faivovich J, García PCA, Ananias F *et al*. (2004) A molecular perspective on the phylogeny of the *Hyla pulchella* species group (Anura, Hylidae). *Molecular Phylogenetics and Evolution*, **32**, 938–950.

Faivovich J, Haddad CFB, García PCA *et al*. (2005) Systematic review of the frog family Hylidae, with special reference to Hylinae: phylogenetic analysis and taxonomic revision. *Bulletin of the American Museum of Natural History*, **294**, 1–240.

Fjeldså J (1994) Geographical patterns for relict and young species of birds in Africa and South America and implications for conservation priorities. *Biodiversity and Conservation*, **3**, 207–226.

Funk WC, Blouin MS, Corn PS *et al*. (2005) Population structure of Columbia spotted frogs (*Rana luteiventris*) is strongly affected by the landscape. *Molecular Ecology*, **14**, 483–496.

García-Moreno J, Arctander P, Fjeldså J (1999) A case of rapid diversification in the Neotropics: phylogenetic relationships among *Cranioleuca* spinetails (Aves, Furnariidae). *Molecular Phylogenetics and Evolution*, **12**, 273–281.

Goebel AM, Donnelly JM, Atz ME (1999) PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome *b* in bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Molecular Phylogenetics and Evolution*, **11**, 163–199.

Grant WS, Bowen BW (1998) Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. *Journal of Heredity*, **89**, 415–426.

Grau A, Brown AD (2000) Development threats to biodiversity and opportunities for conservation in the mountain ranges of the Upper Bermejo River Basin, NW Argentina and SW Bolivia. *Ambio*, **29**, 445–450.

Grau HR, Veblen TT (2000) Rainfall variability, fire and vegetation dynamics in Neotropical montane ecosystems in north-western Argentina. *Journal of Biogeography*, **27**, 1107–1121.

Green DM, Sharbel TF, Kearsley J, Kaiser H (1996) Postglacial range fluctuations, genetic subdivision and speciation in the Western North American spotted frog complex, *Rana pretiosa*. *Evolution*, **50**, 374–390.

Gregory-Wodzicki KM (2000) Uplift history of the Central and Northern Andes: a review. *Geological Society of America Bulletin*, **112**, 1091–1105.

Hackett SJ, Rosenberg KV (1990) Comparison of phenotypic and genetic differentiation in South American antwrens Formocariidae. *Auk*, **107**, 473–489.

Handford P (1988) Trill rate dialects in the rufous-collared sparrow, *Zonotrichia capensis*, in northwestern Argentina. *Canadian Journal of Zoology*, **66**, 2658–2670.

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.

Hooghiemstra H, van der Hammen T (1998) Neogene and Quaternary development of the Neotropical rain forest: the forest refugia hypothesis, and a literature overview. *Earth-Science Reviews*, **44**, 147–183.

Hubert N, Renno J-F (2006) Historical biogeography of South American freshwater fishes. *Journal of Biogeography*, **33**, 1414–1436.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, **17**, 754–755.

Liadsky D (2003) *Call variation in the Andean treefrog,* Hyla andina Bachelor of Science (Honours) Thesis, Queen's University, Ontario, Canada

Lougheed SC, Gascon C, Jones DA, Bogart JP, Boag PT (1999) Ridges and rivers: a test of competing hypotheses of Amazonian diversification using a dart-poison frog (*Epipedobates femoralis*). *Proceedings of the Royal Society B: Biological Sciences*, **266**, 1829–1835.

Lougheed SC, Freeland JR, Handford P, Boag PT (2000) A molecular phylogeny of warbling-finches (*Poospiza*): paraphyly in a Neotropical emberizid genus. *Molecular Phylogenetics and Evolution*, **17**, 367–378.

Lougheed SC, Austin JD, Bogart JP, Boag PT, Chek AA (2006) Multi-character perspectives on the evolution of intraspecific differentiation in a Neotropical hylid frog. *BMC Evolutionary Biology*, **6**, 16.

Lowe WH, Likens GE, McPeek MA, Buso DC (2006) Linking direct and indirect data on dispersal: isolation by slope in a headwater stream salamander. *Ecology*, **87**, 334–339.

Marsh DM, Trenham PC (2001) Metapopulation dynamics and amphibian conservation. *Conservation Biology*, **15**, 40–49.

Martin PR, McKay JK (2004) Latitudinal variation in genetic divergence of populations and the potential for future speciation. *Evolution*, **58**, 938–945.

Martínez-Solano I (2004) Phylogeography of Iberian *Discoglossus* (Anura: Discoglossidae). *Journal of Zoological Systematics and Evolutionary Research*, **42**, 298–305.

Masta SE, Sullivan BK, Lamb T, Routman EJ (2002) Molecular systematics, hybridization, and phylogeography of the *Bufo americanus* complex in Eastern North America. *Molecular Phylogenetics and Evolution*, **24**, 302–314.

McCulloch RD, Bentley MJ, Purves RS *et al*. (2000) Climatic inferences from glacial and palaeoecological evidence at the last glacial termination, southern South America. *Journal of Quaternary Science*, **15**, 409–417.

Moritz C, Patton JL, Schneider CJ, Smith TB (2000) Diversification of rainforest faunas: an integrated molecular approach. *Annual Review of Ecology and Systematics*, **31**, 533–563.

Muellner AN, Tremetsberger K, Stuessy T, Baeza CM (2005) Pleistocene refugia and recolonization routes in the southern Andes: insights from *Hypochaeris palustris* (Asteraceae, Lactuceae). *Molecular Ecology*, **14**, 203–212.

Nielson M, Lohman K, Daugherty CH *et al*. (2006) Allozyme and mitochondrial DNA variation in the tailed frog (Anura: *Ascaphus*): the influence of geography and gene flow. *Herpetologica*, **62**, 235–258.

Noonan BP, Gaucher P (2005) Phylogeography and demography of Guianan harlequin toads (*Atelopus*): diversification within a refuge. *Molecular Ecology*, **14**, 3017–3031.

Patton JL, Dasilva MNF, Malcolm JR (1994) Gene genealogy and differentiation among arboreal spiny rats (Rodentia, Echimyidae) of the Amazon basin – a test of the riverine barrier hypothesis. *Evolution*, **48**, 1314–1323.

Patton JL, Da Silva MNF, Malcolm JR (2000) Mammals of the Rio Jurua and the evolutionary and ecological diversification of Amazonia. *Bulletin of the American Museum of Natural History*, **244**, 1–306.

Petit RJ (2008) The coup de grâce for the nested clade phylogeographic analysis? *Molecular Ecology*, **17**, 516–518.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Potts R, Behrensmeyer AK (1992) Late Cenozoic terrestrial ecosystems. In: *Terrestrial Ecosystems Through Time – Evolutionary Paleoecology of Terrestrial Plants and Animals* (eds Behrensmeyer AK, Damuth JD, DiMichele WA *et al*.), pp. 419–541. Chicago University Press, Chicago.

Prance GT (1982) A review of the phytogeographic evidences for Pleistocene climate changes in the Neotropics. *Annals of the Missouri Botanical Garden*, **69**, 594–624.

Quiroga MP, Premoli AC (2007) Genetic patterns in *Podocarpus parlatorei* reveal the long-term persistence of cold-tolerant elements in the southern Yungas. *Journal of Biogeography*, **34**, 447–455.

Rambaut A, Drummond AJ (2007) *TRACER v1.4*. Available from http://tree.bio.ed.ac.uk/software/tracer

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Rull V (2006) Quaternary speciation in the Neotropics. *Molecular Ecology*, **15**, 4257–4259.

Ruzzante DE, Walde SJ, Cussac VE *et al*. (2006) Phylogeography of the Percichthyidae (Pisces) in Patagonia: roles of orogeny, glaciation, and volcanism. *Molecular Ecology*, **15**, 2949–2968.

Saccone C, Attimonelli M, Sbisà E (1987) Structural elements highly preserved during the evolution of the D-loop-containing region in vertebrate mitochondrial DNA. *Journal of Molecular Evolution*, **26**, 205–211.

Sambrook J, Russel DW (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York.

Schneider S, Excoffier L (1999) Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics*, **152**, 1079–1089.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN ver. 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Simpson BB (1979) Quaternary biogeography of the high montane regions of South America. In: *The South American Herpetofauna: its Origin, Evolution, and Dispersal* (ed. Duellman WE), pp. 157–188. The University of Kansas, Lawrence, Kansas.

Soltis DE, Morris AB, McLachlan JS, Manos PS, Soltis PS (2006) Comparative phylogeography of unglaciated eastern North America. *Molecular Ecology*, **15**, 4261–4293.

Swafford DL (2002) *PAUP**. Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland.

Symula R, Schulte R, Summers K (2003) Molecular systematics and phylogeography of Amazonian poison frogs of the genus *Dendrobates*. *Molecular Phylogenetics and Evolution*, **26**, 452–475.

Templeton AR (1998) Nested clade analysis of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381–397.

Templeton AR (2001) Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology*, **10**, 779–791.

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics*, **134**, 659–669.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, **132**, 619–633.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767–782.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24**, 4876–4882.

Thuring RWJ, Sanders JPM, Borst P (1975) A freeze-squeeze method for recovering long DNA from agarose gels. *Analytical Biochemistry*, **66**, 213–220.

Voelker G (1999) Dispersal, vicariance, and clocks: historical biogeography and speciation in a cosmopolitan passerine genus (*Anthus*: Motacillidae). *Evolution*, **53**, 1536–1552.

Vuilleumier BS (1971) Pleistocene changes in the fauna and flora of South America. *Science*, **173**, 771–780.

Weng C, Hooghiemstra H, Duivenvoorden J (2007) Response of pollen diversity to the climate-driven altitudinal shift of vegetation in the Colombian Andes. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **362**, 253–262.

Wiens JJ, Fetzner JW, Parkinson CL, Reeder TW (2005) Hylid frog phylogeny and sampling strategies for speciose clades. *Systematic Biology*, **54**, 719–748.

World Conservation Monitoring Centre (1992) *Global biodiversity: Status of the Earth's Living Resources*. Chapman & Hall, London.

Yoke MM, Morando M, Avila LJ, Sites JW Jr (2006) Phylogeography and genetic structure in the *Cnemidophorus longicauda* complex (Squamata, Teiidae). *Herpetologica*, **62**, 420–434.

Daria Koscinski studies the patterns of genetic and biological diversity, particularly spatial patterns in heterogeneous and fragmented landscapes, and this research forms part of her PhD thesis with Handford. Paul Handford studies the characterization and interpretation of patterns of behavioural (mainly song), morphological and genetic variation, among individuals, among populations or among species. Pablo Luis Tubaro is Curator of Ornithology and Vice Director of the Argentine Museum of Natural Sciences. He has broad interests in ornithology, focusing particularly on the adaptive character of morphology and vocalizations, and is deeply involved with the All Birds Barcoding Initiative. Sarah Sharp did her undergraduate thesis in Biology with Lougheed and is now in the midst of completing her law degree at the University of Victoria with a focus on health and the environment. Stephen C. Lougheed's research focuses on understanding the origins of biodiversity from the level of local adaptation and limiting gene flow in single landscapes, through the genetics of entire species' ranges, to understanding the causes of diversification of entire clades.

Supplementary material

The following supplementary material is available for this article:

Table S1 Nested clade analysis results for (a) network no. 1 and (b) network no. 2 clades tested using 1000 permutations. Significant geographical associations from permutation tests indicated in boldface.

Table S2 Inferences made for each clade in network no. 1 found to have significant geographical associations.

Table S3 Details of analyses of divergence time from the program BEAST using a molecular clock of (a) 1% per million years, and (b) 0.5% per million years.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-294X.2008.03733.x
(This link will take you to the article abstract).

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Appendix

Voucher specimens collected during the study. Some specimens have not been assigned voucher numbers yet and are listed as pending. GenBank Accession numbers for mtDNA control region are listed for each specimen. Cytochrome *b* accession numbers listed in parentheses where applicable

Collector number	Locality no.	Museum*	Voucher no.	GenBank Accession no.
JMP856	1	MNCN	5947	EU403414 (cyt. *b* EU403150)
DK-04-784	2	MCN	pending	EU403412
DK-04-786	2	MCN	pending	EU403413
DK-04-343	3	MCN	1203	EU403276
DK-04-344	3	MCN	1203	EU403277 (cyt. *b* EU403129)
DK-01-166†	4	MACN	39037	
DK-04-382	5	MCN	1204	EU403294
DK-04-383	5	MCN	1204	EU403295
DK-01-167†	6	MACN	39038	
DK-04-151	7	MCN	1200	EU403254 (cyt. *b* EU403126)
DK-04-152	7	MCN	1200	EU403255
DK-04-321	8	MCN	1202	EU403274 (cyt. *b* EU403128)
DK-04-322	8	MCN	1202	EU403275
DK-04-232	9	MCN	1201	EU403264
DK-04-233	9	MCN	1201	EU403265
DK-04-758	11	MCN	pending	EU403402
DK-04-759	11	MCN	pending	EU403403
DK-04-760	11	MCN	1209	EU403404
DK-01-125	12	MACN	39035	EU403173
DK-01-126	12	MACN	39036	EU403174
DK-04-763	14	MCN	pending	EU403405
DK-04-764	14	MCN	pending	EU403406
DK-04-765	14	MCN	pending	EU403407
DK-04-766	14	MCN	pending	EU403408
DK-04-767	14	MCN	pending	EU403409
DK-04-768	14	MCN	pending	EU403410
DK-04-780	14	MCN	pending	EU403411
DK-04-547	16	MCN	1208	EU403384 (cyt. *b* EU403144)
DK-04-548	16	MCN	1208	EU403385
DK-04-478	17	MCN	1207	EU403341
DK-04-479	17	MCN	1207	EU403342
JPB14516	18	CMNAR	33140	EU403189
JPB14519	18	CMNAR	33140	EU403190
JPB14523	18	CMNAR	33140	EU403191
JPB14524	18	CMNAR	33140	EU403192
DK-04-438	18	MCN	1205	EU403308
DK-04-439	18	MCN	1205	EU403309
DK-01-188	19	FML	16112	EU403226
DK-04-465	20	MCN	pending	EU403333 (cyt. *b* EU403139)
DK-01-097	21	MACN	39031	EU403157
DK-04-503	22	MCN	1206	EU403364
DK-04-504	22	MCN	1206	EU403365
DK-04-459	23	MCN	pending	EU403327
DK-04-460	23	MCN	pending	EU403328
DK-04–461	23	MCN	pending	EU403329 (cyt. *b* EU403137)
DK-01-091	24	MACN	39028	EU403415 (cyt. *b* EU403155)
JMP297	25	MNCN	4086	EU403416 (cyt. *b* EU403151)
JMP298	25	MNCN	4087	EU403417 (cyt. *b* EU403152)
JMP395	26	MNCN	6083	EU403419 (cyt. *b* EU403154)
JMP396	26	MNCN	6069	EU403418 (cyt. *b* EU403153)

*Museum codes:
CMNAR, Canadian Museum of Nature, Ottawa, Canada.
FML, Fundación Miguel Lillo, Tucumán, Argentina.
MACN, Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina.
MCN, Museo de Ciencias Naturales, Universidad de Salta, Salta, Argentina.
MNCN, Museo Nacional de Ciencias Naturales, Madrid, Spain.
†Samples in process, not included in this study.