

Across the southern Andes on fin: glacial refugia, drainage reversals and a secondary contact zone revealed by the phylogeographical signal of *Galaxias platei* in Patagonia

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Abstract

We employed DNA sequence variation at two mitochondrial (control region, COI) regions from 212 individuals of *Galaxias platei* (Pisces, Galaxiidae) collected throughout Patagonia (25 lakes/ivers) to examine how Andean orogeny and the climatic cycles throughout the Quaternary affected the genetic diversity and phylogeography of this species. Phylogenetic analyses revealed four deep genealogical lineages which likely represent the initial division of *G. platei* into eastern and western lineages by Andean uplift, followed by further subdivision of each lineage into separate glacial refugia by repeated Pleistocene glacial cycles. West of the Andes, refugia were likely restricted to the northern region of Patagonia with small relicts in the south, whereas eastern refugia appear to have been much larger and widespread, consisting of separate northern and southern regions that collectively spanned most of Argentinean Patagonia. The retreat of glacial ice following the last glacial maximum allowed re-colonization of central Chile from nonlocal refugia from the north and east, representing a region of secondary contact between all four glacial lineages. Northwestern glacial relicts likely followed pro-glacial lakes into central Chilean Patagonia, whereas catastrophic changes in drainage direction (Atlantic → Pacific) for several eastern palaeolakes were the likely avenues for invasions from the east. These mechanisms, combined with evidence for recent, rapid and widespread population growth could explain the extensive contemporary distribution of *G. platei* throughout Patagonia.

Keywords: drainage reversal, *Galaxias platei*, orogeny, phylogeography, Pleistocene glacial cycles, secondary contact

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Introduction

Compared with North America and Europe, little is known about the influence of Quaternary geological and climatic events on the evolutionary history of South American flora and fauna. This is particularly true for Patagonia, the southernmost region of Chile and Argentina, where the consequences of the Andean mountain uplift and of the Pleistocene climatic cycles for biodiversity are understood

only in broad general terms and only for a limited number of (mostly) terrestrial species. Patterns of postglacial dispersal have been described for some plants (Allnutt *et al.* 1999; Premoli *et al.* 2000; Pastorino & Gallo 2002; Muellner *et al.* 2005), lizards (Victoriano *et al.* 2008), rodents (Smith *et al.* 2001; Palma *et al.* 2005) and a relict marsupial (Himes *et al.* 2008); the results indicate the presence of terrestrial Pleistocene glacial refugia west and east of the Andes in northern Patagonia. Patterns of genetic diversity described for several terrestrial vertebrates inhabiting the Patagonian Steppe (lizards: Morando *et al.* 2004, 2007; Avila *et al.* 2006; rodents: Kim *et al.* 1998) have provided

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valuable insights regarding localized effects of late Quaternary processes on intraspecific diversity. Studies on freshwater crabs (Perez-Losada *et al.* 2004) have linked phylogenies to events such as drainage changes and marine incursions that occurred several to many millions of years ago, in northern Patagonia and regions to the northeast. Recent work on freshwater fish, however, suggests that events that have occurred during the past 1–3 million years may be most relevant to explaining current patterns of genetic diversity within Patagonian freshwater fauna (Ruzzante *et al.* 2006, 2008).

In the present study, we examine in detail the patterns of genetic diversity of one of the most widespread fish species endemic to Patagonia, *Galaxias platei*, in relation to the geological and climate changes of the Pleistocene. The contemporary distribution of *G. platei* is exclusively within postglacial lakes and rivers (Ruzzante *et al.* 2008). Tolerance of low temperatures and low oxygen environments, adaptations to low light (e.g. retinal modifications, cephalic lateral line), and highly protected gill epithelial tissues are characteristics that are thought to reflect adaptation to a benthic existence in the silty, dark environment of Patagonian lakes and rivers (see Cussac *et al.* 2004 and references therein), and likely influenced *G. platei* survival and dispersal during the repeated glacial cycles of the Quaternary.

The rise of the Andes and the glacial cycles of the Quaternary

In some cases, continental divides are formidable barriers to gene flow, not only for terrestrial species, but also for aquatic taxa, limiting exchange to valleys which transverse the range (e.g. Soltis *et al.* 2006). Deep and ancient genetic structuring might be expected for aquatic species east and west of the Andes, as the southern part of the range began to rise in the early Miocene [c. 23 million years ago (Ma) (Ramos 1989)].

Patterns of genetic diversity within Patagonian South America are also likely to reflect the climatic fluctuations of the Pleistocene (1.8 Ma–10 000 BP; Clapperton 1993). At the last glacial maximum (LGM), 23 000–25 000 BP, a continuous ice sheet existed along the Andes for over 1800 km, extending west to the Pacific Ocean south of 42°S [(Clapperton 1993; McCulloch *et al.* 2000) Fig. 1]. Glacial events influence the distribution of genetic diversity directly, through reduction of population size (producing genetic bottlenecks) and displacement of entire populations, and indirectly through their effects on landscapes, for example, the rearrangement of watersheds. The many glacial advances and retreats during the Pleistocene differed in extent and duration, and thus probably also in their effects on the abundance and distribution of local biota. The extent to which aquatic taxa survived the glacial cycles within watersheds is not known. Refugia certainly existed north of continental ice

(c. 43°S) west of the Andes, and probably also in some eastern watersheds (Ruzzante *et al.* 2006). Refugia may also have been present west of the ice sheet on areas of continental shelf exposed by lowered sea levels. Pleistocene watershed rearrangement and its effects on patterns of genetic diversity have been extensively documented for New Zealand systems (Waters & Wallis 2000; Waters *et al.* 2001, 2006; Burrridge *et al.* 2006, 2007; Craw & Waters 2007; Craw *et al.* 2007). Two effects of glacial cycles on Patagonian landscapes were likely important for aquatic organisms in southern South America. First, the large palaeolakes that formed east of the Andes during periods of glacial retreat probably allowed mixing of populations from adjacent watersheds (Ruzzante *et al.* 2008). Second, several drainages changed direction from the Atlantic to the Pacific during the melting of the ice following the LGM, including Lakes General Carrera/Buenos Aires and Cochrane/Pueyrredón, which began to drain west about 12 ka (Turner *et al.* 2005); reversals are likely responsible for all basins east of the Andes that currently drain into the Pacific.

The Galaxiidae

Galaxias platei is one of six species of the family Galaxiidae in South America. Galaxiids are confined to the Southern Hemisphere with representatives on all major continents (and nearby islands), barring India and Antarctica (Croizat *et al.* 1974; Rosen 1978). Three genera are found in South America: *Galaxias* (three species — *G. platei*, *G. maculatus*, *G. globiceps*), *Aplochiton* (two species — *A. taeniatus*, *A. zebra*) and *Brachygalaxias* (*B. bullocki*) (McDowall 2006). All except *G. maculatus*, are endemic to South America and all are temperate species, restricted to latitudes south of 34°S. All six species are present in Chile, but only three species are found east of the Andean mountain range (*G. platei*, *G. maculatus* and *A. zebra*) (Ringuelet 1955; Cussac *et al.* 2004).

In the present study, we surveyed the genetic diversity [mitochondrial DNA (mtDNA) control region sequence and COI] of *G. platei* collected from across the species' latitudinal (39°S to 54°S) and altitudinal ranges (sea level to c. 1000 m) on both sides of the Andes. We tested for patterns of diversity that would reflect the impacts of the Andean orogeny, the influence of glacial cycles on population size (bottlenecks and population expansion) and the effects of landscape rearrangement during the Pleistocene. Our study provides genetic evidence consistent with (i) a relatively recent split (1.5 Ma) between eastern and western lineages of *G. platei*, (ii) the existence of Pleistocene glacial refugia west and east of the Andean mountains in both northern and southern Patagonia, and (iii) the existence of a zone of secondary contact between lineages from the eastern and western refugia, probably as a result of postglacial drainage reversals.



Fig. 1 Collection locations for *Galaxias platei* throughout Patagonia, South America. Sampled locations are represented by arrows that indicate contemporary drainage direction, either Atlantic or Pacific. Pacific (rev) denotes a drainage reversal, from Atlantic to Pacific, which likely occurred following the retreat of the Pleistocene glaciers (Turner *et al.* 2005). The extent of LGM and contour of the largest Patagonian glaciation were adapted from Clapperton (1993) and Turner *et al.* (2005).

Materials and methods

Samples

Samples were collected using gill nets and electrofishing between 1998 and 2007 from 25 lakes and rivers throughout Argentinean and Chilean Patagonia (Fig. 1). For the present study, a total of 212 individuals were examined for their

DNA composition; of these, 112 (53%) were from 13 Chilean lakes, 31 (15%) from 3 cross-border lakes, and 69 (32%) from 9 Argentinean lakes (Table 1). Whole samples were retained and stored at several institutions within Chile and Argentina (details available upon request). Subsamples (gill, muscle, fin, blood) for molecular analyses were fixed in 95% ethanol and transported to Dalhousie University for storage at -20°C .

Table 1 Collection site details for Patagonian lakes/ rivers, categorized according to relative position with the Andes, either west (Chile), east (Argentina) or spanning the range (cross-border), and therein ordered in a north/south orientation with respect to latitude. Cross-border lakes have two names (Chilean/Argentinean), the region of the lake sampled as indicated by underlined text

Collection site	No. of individuals	Basin	Latitude	Longitude	Ocean drainage		Sequence diversity	
					Current	Ancient	<i>h</i>	π
Chile								
L Rinihue	2	Valdivia	39°46'29"S	72°27'10"W	Pacific	Pacific	1.0000 ± 0.5000	0.006098 ± 0.006680
L Llanquihue	14	Maullin	41°15'44"S	72°59'40"W	Pacific	Pacific	0.7692 ± 0.0895	0.007719 ± 0.004372
R Cudil	14	Chiloe	42°22'29"S	73°48'22"W	Pacific	Pacific	0.2747 ± 0.1484	0.000348 ± 0.000441
L Risopatron	17	Palena	44°15'51"S	72°31'20"W	Pacific	Pacific	0.2279 ± 0.1295	0.000287 ± 0.000389
L Las Torres	9	Cisnes	44°48'05"S	72°12'23"W	Pacific	Pacific	0.6944 ± 0.1470	0.001355 ± 0.001103
L Los Palos	5	Aysen	45°19'00"S	72°43'00"W	Pacific	Pacific	0.9000 ± 0.1610	0.026309 ± 0.016426
L Riesco	8	Aysen	45°29'56"S	72°40'40"W	Pacific	Pacific	0.4286 ± 0.1687	0.006264 ± 0.003866
L Alta	1	Aysen	45°31'16"S	72°41'45"W	Pacific	Pacific	1.0000 ± 0.0000	0
L Escondida	9	Aysen	45°31'26"S	71°49'04"W	Pacific	Pacific	0.2222 ± 0.1662	0.000271 ± 0.000398
L Thompson	10	Aysen	45°38'18"S	71°47'15"W	Pacific	Pacific	0	0
L Pollux	10	Aysen	45°39'02"S	71°50'25"W	Pacific	Pacific	0	0
L Azul	10	Aysen	45°52'12"S	72°01'15"W	Pacific	Pacific	0.6444 ± 0.1012	0.000894 ± 0.000817
L Jeinimeni	3	Aysen	46°51'14"S	72°01'35"W	Pacific	Pacific	1.0000 ± 0.2722	0.012195 ± 0.009620
Cross-border								
L <u>General Carrera</u> /Buenos Aires	18	Aysen	46°17'52"S	71°56'14"W	Pacific	Pacific	0.8954 ± 0.0653	0.007118 ± 0.003991
L Cochrane/ <u>Pueyrredon</u>	12	Baker	47°18'00"S	71°55'00"W	Pacific	Atlantic	0.8182 ± 0.0957	0.006098 ± 0.003589
L O'Higgins/ <u>San Martin</u>	1	Chico	49°01'55"S	72°14'40"W	Pacific	Atlantic	1	0
Argentina								
L Espejo	10	Limay	40°41'00"S	71°40'00"W	Atlantic	Atlantic	0.3556 ± 0.1591	0.000434 ± 0.000518
L Puelo	10	Puelo	42°05'55"S	71°37'03"W	Pacific	Atlantic	0.2000 ± 0.1541	0.000488 ± 0.000556
L Rivadavia	10	Futalaufquen	42°37'00"S	71°40'28"W	Pacific	Atlantic	0.7333 ± 0.1005	0.001138 ± 0.000963
L La Plata	7	Senguerr	44°52'58"S	71°50'55"W	Atlantic	Atlantic	0	0
L Belgrano	10	Nansen/Bravo	47°52'10"S	72°08'46"W	Pacific	Atlantic	0.5111 ± 0.1643	0.000678 ± 0.000682
L Viedma	4	Santa Cruz	49°35'28"S	72°15'53"W	Atlantic	Atlantic	1.0000 ± 0.1768	0.004472 ± 0.003398
L Yehuin	7	Azopardo	54°24'00"S	67°44'00"W	Pacific	Atlantic	0.2857 ± 0.1964	0.000348 ± 0.000476
L Margarita	2	Azopardo	54°40'00"S	67°50'00"W	Pacific	Atlantic	0	0
L Escondido	9	Azopardo	54°40'24"S	67°44'31"W	Pacific	Atlantic	0.5556 ± 0.1653	0.000881 ± 0.000819
Haplotype Group								
1	40	—	—	—	—	—	0.7859 ± 0.0438	0.004138 ± 0.002403
2	20	—	—	—	—	—	0.1947 ± 0.1145	0.000244 ± 0.000352
3	101	—	—	—	—	—	0.6438 ± 0.0427	0.001132 ± 0.000872
4	51	—	—	—	—	—	0.8643 ± 0.0348	0.003024 ± 0.001843
All	212	—	—	—	—	—	0.8975 ± 0.0134	0.030968 ± 0.015093

Details include the number of individuals sampled, local drainage basin membership (basin), GPS coordinates (latitude/longitude), and expected direction of ocean drainage before (historical) and after (current) the last glacial maximum. Haplotype (*h*) and nucleotide diversities (π) are listed for each location and post-hoc haplogroup cluster identified by phenetic and phylogenetic analyses of control region sequences (see Figs 2 and 3 and text for details).

Sequence data

Before DNA extraction, all tissue samples were dried of ethanol by exposure to ambient temperature for approximately 120 min. Total genomic DNA was isolated from each subsample (10 μ L of blood or 2×2 mm² tissue) using the glassmilk procedure described in Elphinstone *et al.* (2003) with slight modifications for execution using a Multi-PROBE® II HT PLUS EX robotic liquid handling system (PerkinElmer). Approximately 800 bp of the mitochondrial control region were amplified from each individual using the primer combination S-phe [(5'-GCTTTAGTTAAGCTA CG-3'; (Nielsen *et al.* 1994)] and P3 [5'-AACTTCCATCCTC AACTCCCAAAG-3'; (Sang *et al.* 1994)] with a Mastercycler EP Gradient (Eppendorf) thermal cycler. Mitochondrial cytochrome *c* oxidase subunit I (COI; ~640 bp) was also amplified for a subset of eight individuals, two from each of four post-hoc haplotype clusters (herein haplogroups 1–4) recovered by phenetic (minimum-spanning tree; Fig. 2) and phylogenetic (Bayesian inference; Fig. 3a) analyses of control region data using the following primer sequences from Ward *et al.* (2005): FishF1 (5'-TCAACCAA CCACAAAGACATTGGCAC-3') with FishR2 (5'-ACTTC AGGGTGACCGAAGAATCAG AA-3'). The thermal cycler was operated under the following conditions for control region: an initial denaturing temperature of 94 °C for five min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min 30 s, and 72 °C for 1 min 30 s, and a final extension at 72 °C for 5 min. The thermal regime used for COI was identical to that used for control region, excepting the use of a higher annealing temperature (i.e. 54 °C). All polymerase chain reactions (PCR) were 25 μ L: 2.5 μ L 10 \times reaction buffer [100 mM KCl, 100 mM (NH₄)₂SO₄, 200 mM Tris-HCl (pH 8.75, 22 °C), 1% Triton X-100, 1 mg/mL BSA], 2.5 μ L dNTPs (2 μ M each), 3.75 μ L MgSO₄ (20 mM), 13 μ L ddH₂O, 0.25 μ L of each 10 μ M primer, 1 U of Tsg Polymerase (BioBasic Inc.) and 2.0–2.5 μ L of DNA template. PCRs were visualized using 1.0% agarose gels and sent off-site for bidirectional DNA sequencing to Génome Québec Innovation Centre, Quebec, Canada or Macrogen Inc., Seoul, Korea.

Approximately 78% of the control region sequences and haplotypes used in this study were reported in an earlier study that examined the influence of climate on the demographic history of *Galaxias platei* and *Percichthys trucha* (Ruzzante *et al.* 2008); haplotype sequences are available in GenBank under the accession nos EU069832–EU069870. All newly collected control region and COI sequences were edited using Sequencher. Subsequently, BioEdit Sequence Alignment Editor version 7.0.5.3 (Hall 1999) was used to form contigs between bidirectional sequences, align contigs using the accessory application ClustalW (Thompson *et al.* 1994) and identify haplotypes. GenBank Accession numbers for control region haplotypes are EU673457–EU673467; COI sequences have accessions FJ178348–FJ178355.

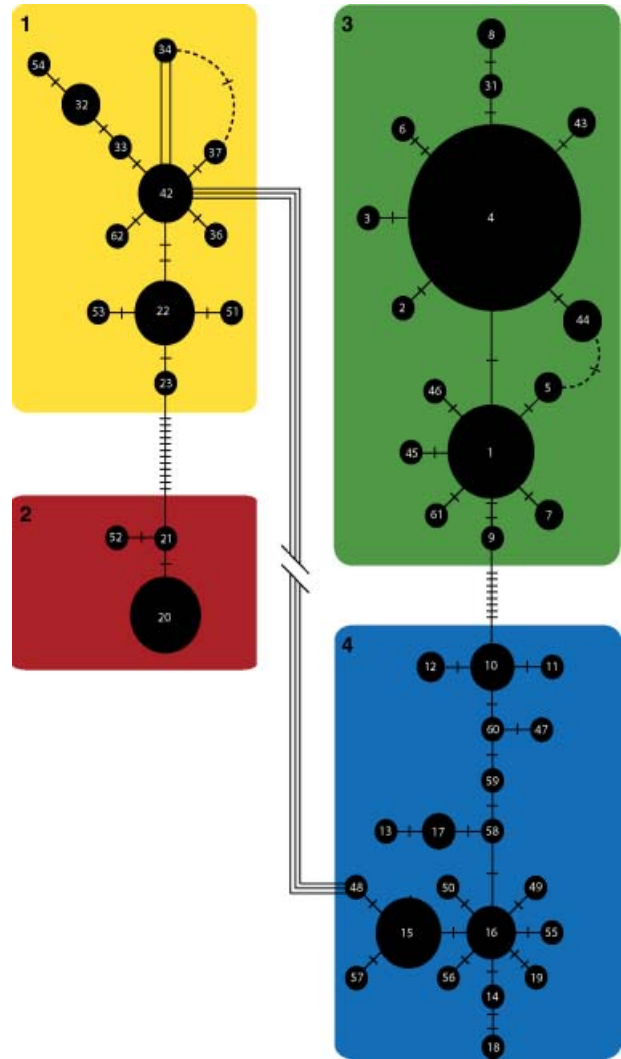


Fig. 2 Minimum-spanning tree of mitochondrial control region haplotypes ($n = 50$) for 212 individuals of *Galaxias platei*. Circles represent haplotypes (Table S1) and are scaled proportionally to the frequency of each haplotype in the sample. Single mutational steps between haplotypes are represented by a perpendicular line. The triple line connecting haplotype 42 with 48, and double line connecting 34 with 42, represent 49 and 33 mutational steps, respectively. Groups 1–4 are clusters of haplotypes, or haplogroups, separated by several mutational steps.

Data analysis

Standard molecular diversity indices [haplotype diversity (h), nucleotide diversity (π), nucleotide frequencies, transition/transversion ratio, number of polymorphic sites] were calculated using Arlequin version 3.1 (Excoffier *et al.* 2005). Estimates of haplotype and nucleotide diversities were first obtained for each lake/river individually, and then pooled to obtain an overall estimate of sequence diversity in *G. platei* over the entire region. Additional *post-hoc* estimates of molecular diversity were also generated for each haplogroup.

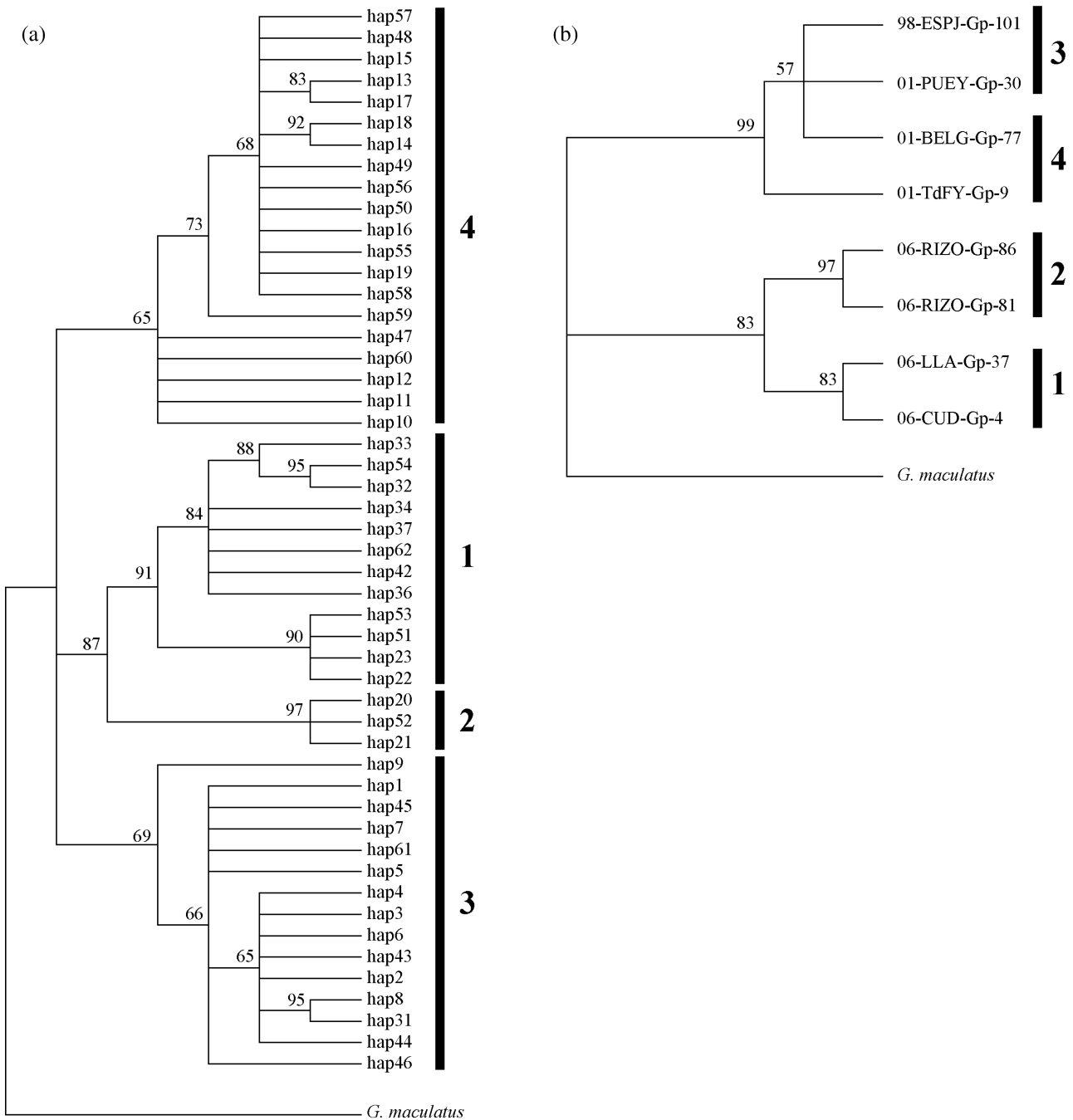


Fig. 3 Phylogenetic majority consensus trees constructed using Bayesian inference employing (a) 50 control region haplotypes assuming an HKY + G + I model of molecular evolution, (b) eight COI sequences selected to represent the four post-hoc haplogroups (1–4) recovered by control region data. Analysis of COI data employed an HKY + G model of molecular evolution. Branch support for each analysis is based on Bayesian posterior probability densities.

Relationships among control region haplotypes were first explored using a minimum-spanning tree based on uncorrected ' p ' distances by *MINSPNET*, as implemented in *Arlequin* version 3.1 (Excoffier *et al.* 2005). Subsequently, Bayesian inference was used to estimate phylogenetic relationships between haplotypes using *MrBayes* version 3.1

(Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003), based on the HKY + G + I model of molecular evolution which was determined by *ModelTest* (Posada & Crandall 1998) as the most appropriate model of DNA substitution. Parameter space was searched for 3 000 000 generations using two independent runs based on different

random number seeds that each employed four heated chains with temperatures of 0.2 and swap rates of 1. Branch support and order were based on Bayesian posterior probability densities after discarding the first 750 000 generations as burn-in and confirming convergence among independent runs by visual inspection of resultant tree topologies. The tree was rooted with *Galaxias maculatus*. Phylogenetic analysis of COI data was conducted using identical parameter settings, but using the HKY + G model of sequence evolution, also determined by ModelTest (Posada & Crandall 1998).

Corrected average pairwise divergences (controlling for within-cluster variation) between control region haplotype groups was calculated using Arlequin version 3.1 (Excoffier *et al.* 2005) based on the Kimura 2-Parameter (K2P) model of molecular evolution to correct for multiple substitutions (Kimura 1980). Rough estimates of population divergence were first calculated for each haplogroup independently and then pooled into eastern and western groups to estimate Andean-induced divergence. The split between east and west was also estimated using COI sequence data in an attempt to account for the possible underestimation of ancient divergence because of multiple unaccounted hits (saturation) based on control region sequences. Similar calculations of within-lineage divergence using COI data seemed inappropriate considering sample sizes, and the paraphyly of eastern haplotypes. Thus, we only report divergence estimates for the deepest split in the tree that was recovered by COI data. Rough estimates of time since divergence were subsequently calculated for both control region and COI using the 'asymptotic' galaxiid mtDNA mutation rates (cyt *b*, control region) of 0.01876 changes/site/MY recently estimated by BurrIDGE *et al.* (2008).

Hierarchical analysis of molecular variance (AMOVA), executed in Arlequin version 3.1, was used to determine how contemporary genetic variation is partitioned over the range of *G. platei*. Hierarchical groupings of samples included: (i) the lake/river from which individuals were collected; (ii) the freshwater drainage that each lake or river belongs to; (iii) the present-day ocean drainage (Pacific or Atlantic); and (iv) the historical or ancient ocean drainage [i.e. ancient refers to conditions before the LGM approximately 23 000–25 000 BP (Clapperton 1993; McCulloch *et al.* 2000; Tatur *et al.* 2002; Turner *et al.* 2005)]. Ancient drainage patterns are expected to have been very similar to contemporary drainage patterns with the exception of those post-glacial lakes located east of the Andes that currently drain into the Pacific. At the height of the LGM, ice was present to the west of these lakes. This ice disappeared during the melting of the glaciers that followed the LGM leading to catastrophic drainage reversals from the Atlantic to the Pacific Ocean. Although this phenomenon is well documented for only some of the systems located east of the Andes and currently draining into the Pacific (Tatur *et al.*

2002; Turner *et al.* 2005), here we treat all sample locations east of the Andean mountain range currently draining into the Pacific as historically Atlantic draining. See Table 1 for details regarding hierarchical groupings.

Limitations

While the present case is an important first approach to understanding the neutral genetic structure and diversity of freshwater fishes in Patagonian lakes and rivers, we do realize the limitations of our approach and suggest caution when interpreting results. Inferences about populations are based solely on the analysis of organellar genes (i.e. control region, COI), which despite offering accurate depictions of mitochondrial history may not necessarily be representative of the overall genetic aspects of the organisms themselves. Several studies now recognize the importance of including at least one, or as many as several unlinked nuclear loci in order to resolve sex-specific patterns and maximize the statistical power of divergence estimates by controlling for influences of ancestral polymorphisms and the stochastic nature of lineage sorting process (Wakely & Hey 1997; Edwards & Beerli 2000; Hare 2001; Nichols 2001). Uncertainties about an appropriate molecular clock for control sequences further confound lineage divergence estimates and the timings of important demographic events. We employed the 'asymptotic' rate of 1.88%/million years reported in BurrIDGE *et al.* (2008) for galaxiid fishes, but consider it a very conservative estimate for the control region based on the range of rates reviewed by Bowen *et al.* (2006) for bony fishes. Considering the inverse relationship between substitution rate and divergence time, our estimates likely represent the upper bounds of time since lineage divergence, particularly considering that our single-gene based approach is particularly amenable to overestimating population divergence (Edwards & Beerli 2000). Despite the above uncertainties, we are confident that the mtDNA analyses presented here are appropriate for the questions presented and provide important new insights and hypotheses regarding the diversification and biogeography of freshwater fishes in the understudied region of Patagonian South America. Efforts involving multigene approaches for South American galaxiid fishes are encouraged to further test and refine hypotheses set forth in the present case and are currently underway in our laboratory.

Results

Intraspecific population structure

A total of 50 mtDNA control region haplotypes were identified from 212 individuals of *Galaxias platei* collected from throughout the species distribution (Table S1, Supporting information). Overall nucleotide frequencies were: C (22.34%),

T (28.48%), A (29.78%), G (19.41%). The total number of polymorphic sites was 103, with a transition/transversion ratio of 2.15, and 37 observed indels.

A minimum-spanning tree (MST) of 50 control region haplotypes recognized four major clusters (Fig. 2), or haplogroups 1 ($N = 12$), 2 ($N = 3$), 3 ($N = 15$), and 4 ($N = 20$), each identified as a single entity by several mutational steps: $1 \leftrightarrow 4 = 49$, $1 \leftrightarrow 2 = 12$ and $3 \leftrightarrow 4 = 8$. Phylogenetic reconstruction of those same haplotypes using Bayesian inference also recognized the four haplogroups, 1 and 2 receiving much greater branch support than groups 3 and 4 (Fig. 3a). Additional strongly supported clusters were evident within each of the haplogroups, but were separated from other potential groups by only one or two mutational steps. Phylogenetic analysis of COI data provided further support for a deep split between eastern and western groups ($1,2 \leftrightarrow 3,4$) and recognized the distinct division between western haplogroups 1 and 2, but revealed a paraphyletic relationship between eastern haplogroups 3 and 4 (Fig. 3b). Of the 212 individuals sequenced, haplogroups 1, 2, 3, and 4 had 40, 20, 101, and 51 individuals, respectively.

Haplotypes from groups 1 and 2 were found exclusively in locations west of the Andes, and were most abundant in northern drainages (Fig. 4). Haplotypes from groups 3 and 4 were the most widely distributed, and were found in lakes and rivers on both sides of the Andes (Fig. 4). Haplogroup 3 dominated northern drainages east of the Andes, while haplogroup 4 dominated southern drainages. All

haplotype groups were present in the central-west drainages (see Table S1 for haplotype frequency by collection site).

The average pairwise divergence between haplotypes belonging to haplogroups 1 and 2 was 1.31%, similar to average divergence between 3 and 4 (1.26%) for control region sequence data. If haplogroups are pooled into the two major lineages (western: 1–2, eastern: 3–4), average pairwise divergence increases to 5.70%. For COI data, divergence between the eastern and western lineages was estimated to be 5.64%. Applying these divergence values to approximate per site mutation rates of approximately 1.88%/million years for control region and COI data (Burrige *et al.* 2008), we obtained the following rough estimates of times since divergence: 1–2 \leftrightarrow 3–4 (1.5 Ma), $1 \leftrightarrow 2$ (349 000 years ago) and $3 \leftrightarrow 4$ (337 000 years ago).

Most of the contemporary genetic variation could be explained by grouping individuals by lake/river (91%), with very little variation associated with individual collection sites (8.98%) (1-way AMOVA, Table 2). Analysis of sequence variation of the control region using a hierarchical AMOVA showed that nearly half (45%) of the variation was associated with basin or watershed, while a similar amount of variation was associated with different locations within a basin (Table 2). We then nested collection sites within drainage direction (Pacific vs. Atlantic). Current drainage direction explained none of the variance (–0.49%). However, historical drainage direction (before the LGM) explained 25.23% of the total variance.

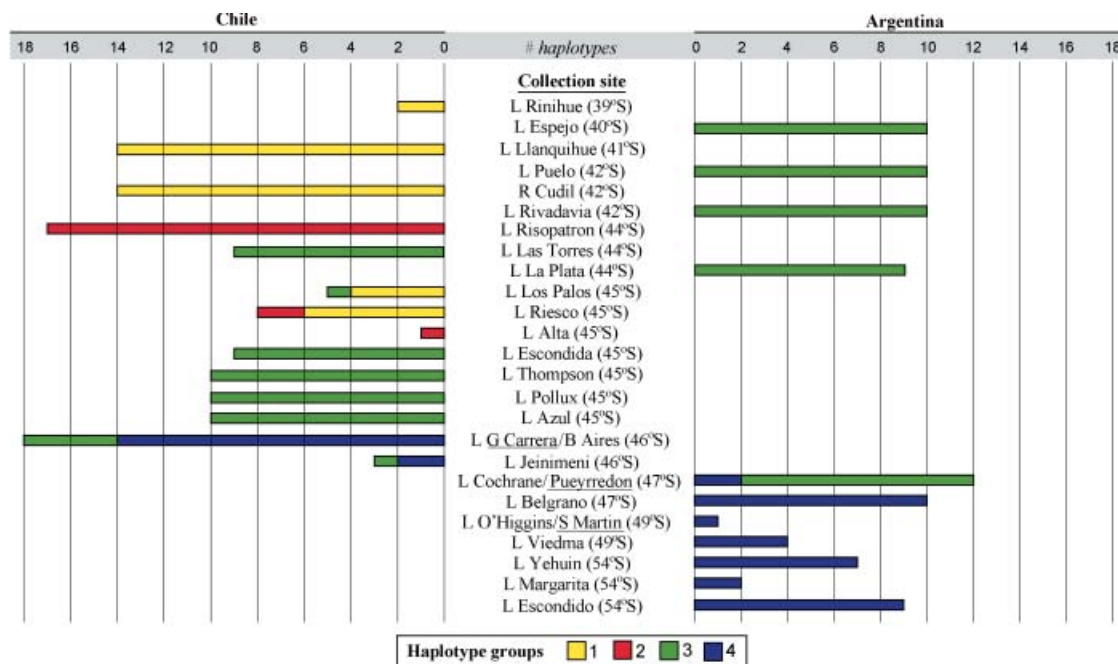


Fig. 4 Control region haplotype frequency distribution based on haplogroup membership (number of individuals carrying a haplotype from a given haplogroup) for 212 individuals of *Galaxias platei* throughout the sample region. Lakes/ rivers are organized according to latitude and therein categorized as Chilean or Argentinean. Cross-border lakes were categorized by the site in which samples were collected (Chile/Argentina), as indicated by underlined text.

Table 2 Analysis of molecular variance (AMOVA) using mitochondrial control region sequences for 212 individuals of *Galaxias platei* collected over the study area. Sequences were grouped according to (A) lake/river from which individuals were collected, (B) the freshwater basin containing each lake/river, and ocean (Pacific or Atlantic) into which each basin, (C) currently drains, or (D) historically drained [i.e. ancient refers to conditions before last glacial maximum (LGM) approximately 10 000–20 000 BP]. Variation is reported as a percentage of the total with degrees of freedom (d.f.)

Grouping	Percentage of variation (d.f.)		
	Among groups	Among populations within groups	Within populations
A. Lake/river	91.02 (24)	—	8.98 (187)
B. Current basin	44.87 (13)	46.5 (11)	8.68 (187)
C. Current basin drainage	–0.49 (1)	91.47 (23)	9.02 (187)
D. Ancient basin drainage	25.23 (1)	66.92 (23)	7.85 (187)

Demography

Haplotype diversity was similar for populations east (Argentina) and west (Chile) of the Andes, but eastern populations had lower nucleotide diversity (Table 1). Measures of haplotype and nucleotide diversity covary for most locations (i.e. lakes/ rivers showing high haplotype diversity also show high nucleotide diversity and vice versa), but display no clear latitudinal clines (Table 1). The highest levels of haplotype and nucleotide diversity were seen in groups 1 ($h = 0.7859$, $\pi = 0.0041$) and 4 ($h = 0.8643$, $\pi = 0.0018$), followed by groups 3 ($h = 0.6438$, $\pi = 0.0009$) and then 2 ($h = 0.1947$, $\pi = 0.0004$) (Table 1). According to classifications by Grant & Bowen (1998), haplogroup 2 qualified as category I (recent population bottleneck or founder event by single or a few mtDNA lineages; $h < 0.5$, $\pi < 0.5\%$), while haplogroups 1, 3 and 4 all placed within category II (population bottleneck followed by rapid population growth and accumulation of mutations; $h > 0.5$, $\pi < 0.5\%$).

Discussion

Of the various historical factors that have influenced the diversity, abundance and distribution of native temperate South America flora and fauna, both the orogeny of the southern Andes and the glacial cycles of the Pleistocene appear to have played important roles in shaping the contemporary distribution and intraspecific genetic diversity of *Galaxias platei*. First, the Andes have separated *G. platei* into eastern and western lineages for at least 1.5 Ma. The deep genetic structure detected within each of the eastern and western lineages was dated to approximately 330 000–350 000 years ago, the timing of which suggests that glacial cycles may have been responsible, probably by displacement into separate refugia. Furthermore, the distribution of genetic variance reflects patterns of drainage evolution following the LGM, especially the catastrophic drainage shifts (from the Atlantic to the Pacific Ocean) in the north-central part of the latitudinal range of *G. platei*. We identify

a zone of secondary contact between eastern and western lineages in western drainages (44–45°S) and a zone of contact between the northern and southern clades of the eastern lineage at ~47°S.

The Andean divide

Mountain build-up is well supported as an important mechanism of genetic isolation; evidence is present at both the inter- and intraspecific levels for diverse assemblages of taxa from regions all over the world (see Soltis *et al.* 2006). Here we found evidence that the Andean divide has played an important historical role in creating deep intraspecific structure within *G. platei*, effectively sundering its historical range into eastern (combined haplogroups 1 and 2) and western (combined haplogroups 3 and 4) lineages. Similar phylogeographical breaks are associated with the Andes for another Patagonian freshwater fish, *Percichthys trucha* (Ruzzante *et al.* 2006), as well as a long-lived conifer (Allnutt *et al.* 1999). Biogeographical patterns at the species level coincide with intraspecific discontinuities, with numerous aquatic species found to the west but not east of the Andes (Dyer 2000). For example, only three of the six putative species of galaxiids currently recognized in South America have distributions which extend east of the Andes, two of which have a substantial presence (*G. platei* and *G. maculatus*) and one (*Aplochiton zebra*) is restricted to Pacific draining systems (Ringuelet 1955). These concordant patterns strongly indicate that the southern Andean uplift has historically presented an important barrier to dispersal for species like *G. platei* with distributions that span both sides of the Andes.

Molecular-clock estimates suggest that the split between eastern and western lineages of *G. platei* occurred roughly 1.5 Ma. As the southern Andes began their uplift much earlier, c. 23 Ma (Ramos 1989), the data imply gene flow across the divide. Trans-Andean gene flow has been indicated for terrestrial organisms, including two widespread species of Patagonian rodents *Oligoryzomys longicaudatus* (Palma *et al.* 2005) and *Abrothrix olivaceus* (Smith *et al.* 2001), and

the marsupial *Dromiciops gliroides* (Himes *et al.* 2008). These mammals are thought to have utilized the continuous forest habitat which exists in low-lying passes, which effectively served as corridors for dispersal in the past and likely continue to do so in the present. Gene flow for aquatic taxa may have occurred through drainages that currently or previously traversed the Andes from the Argentinean side. The timing of the split coincides roughly with the first evidence that the Patagonian ice sheet was fully developed (Rabassa 2008). At this time, *G. platei* may have been reduced to populations occupying northern drainages on both sides of the Andes, or the current split between the eastern and western lineages formed during re-colonization when the climate warmed.

Pleistocene glaciations

Superimposed on the Andean-linked structure, a further subdivision was detected within each of the eastern and western lineages. Molecular-clock estimates using control region data suggest the gene splits occurred in the east at ~337 000 years ago and in the west at ~349 000 years ago. While long-term palaeoclimatic data are not available for this time period in southern South America, Ruzzante *et al.* (2008) report on the likelihood of several glacial advances and retreats between 0.2 Ma and 1.0 Ma using ocean marine sediment records as proxies for glacial fluctuations on land. It is likely that the lineage splitting detected within eastern and western groups is the product of survival in separate refugia on both sides of the Andes during a similar glacial event in this period.

The number, size and location of potential Pleistocene refugia suitable for *G. platei* and/or other freshwater taxa in Patagonia are not known. West of the Andes, the northernmost region of Patagonia, north of 35°S, is likely to have contained continuous aquatic habitats, since the region remained ice free during glacial maxima, and the climate remained humid, at least during the LGM (Heusser *et al.* 2000). This region has likely been key to the preservation of biodiversity in general through numerous glacial cycles, as indicated by the relatively deep intraspecific genetic diversity, as well as species-level biodiversity that it supports for various taxa (Dyer 2000; Ruzzante *et al.* 2006). The same general area has been suggested as the most likely western refuge for other Patagonian taxa, including: plants (*Fitzroya cupressoides*, Premoli *et al.* 2000; *Hypochaeris palustris*, Muellner *et al.* 2005), rodents (*Abrothrix olivaceus*, Smith *et al.* 2001) and fish (*P. trucha*, Ruzzante *et al.* 2006). However, our data suggest that *G. platei* also survived in a second western refuge. It is certainly conceivable that areas to the south could have been glacial refugia, perhaps within discontinuities of the ice field or on exposed portions of the Pacific continental shelf revealed by lowered sea levels. It seems likely that one group (haplogroup 1) of the western

lineage utilized the ice-free region of the Patagonian northwest, whereas the other (haplogroup 2) found refuge further south. The low frequency, diversity and restricted distribution of haplogroup 2 suggests that this refuge population was initially very small, and/or that it endured repeated population bottlenecks in subsequent glacial cycles.

East of the Andes lies the Patagonian Steppe, a large flatland characterized by an arid environment with limited annual rainfall and limited suitable habitat for *G. platei*. During the glacial maxima, this aridness increased, presumably reducing or eliminating available habitat for *G. platei* populations. However, our data suggest that the eastern lineage has been present for at least 3 million years, and that the northern and southern lineages were formed during a subsequent glacial period approximately 330 000 years ago. Populations of *G. platei* must have survived in northern drainages, and there must also have been a southern refuge (haplogroup 4 is not found north of 46°S). Pastorino & Gallo (2002) suggest that a native conifer, the cypress (*Austrocedrus chilensis*), survived the LGM in several small terrestrial refugia directly east of the glaciers, where conditions were locally cold but moist in a narrow strip between the ice and the dry steppe. The steppe itself may have contained refugial populations of cypress (Pastorino & Gallo 2002), as well as two rodents (Kim *et al.* 1998; Palma *et al.* 2005). The close association between present-day cypress and lakes in Argentina suggests that at least some of the refugia may have been moist enough to contain water bodies with relict populations of *G. platei*.

Although populations in individual lakes and rivers differ genetically, and significant structure was detected within each of the four haplogroups, the amount of divergence was small, typically one or two mutation steps. In part, the low intralinesage structure can likely be explained by one or more founder-flush cycles during Pleistocene glacial cycles that effectively purged much of the intraspecific variation. Evidence from molecular diversity indices in the present study, as well as coalescent approaches used by Ruzzante *et al.* (2008) suggest that a recent and widespread bottleneck for *G. platei* was likely. The large palaeolakes that formed during glacial melt were also likely contributors to the low genetic structure observed. Geological evidence indicates the presence of a number of large palaeolakes east of the Andes following the LGM, including *Elpalafquen*, *Cari Lafquen*, *Caldenius*, *Fuegian* and an unnamed lake which joined present-day lakes General Carrera/Argentino, Pueyrredon/Cochrane (Clapperton 1993; Tatur *et al.* 2002; Turner *et al.* 2005). Palaeolake sizes dwarf those of present-day lakes, and the large increase in habitat availability probably led to rapid population growth and/or increased the potential for gene flow among watersheds, resulting in the low genetic diversity and structure observed within present lineages (haplogroups 3, 4). Similar mechanisms have been

suggested to explain low levels of genetic structure east of the Andes for another Patagonian fish, the perch, *P. trucha* (Ruzzante *et al.* 2006). The presence of palaeolakes west of the Andes is less certain. Western slopes are much steeper than that of the very flat eastern steppe, reducing the opportunity for meltwater to accumulate. If proglacial lakes formed, they likely remained small.

The highest levels of genetic diversity within *G. platei* were found in central Chilean Patagonia; this is the only region where all four haplogroups were found. Glaciers are believed to have reached the Pacific Ocean 42°S, and current populations are thus likely the result of colonization. The southern haplogroup of the western lineage (2) may have persisted in refugia near or within the region. The northern haplogroup (1) probably expanded south, perhaps utilizing proglacial lakes, as has been suggested for the colonization of postglacial water bodies by North American freshwater fish (Bernatchez & Wilson 1998). Eastern groups, however, likely crossed the Andean divide with the extreme fluxes of water that accompanied catastrophic drainage shifts during glacial retreat. The Rio Baker which currently drains Lakes General Carrera/Buenos Aires and Pueyrredon/Cochrane (46–47°S) formed after the LGM when discharge for these two lakes abruptly changed direction and began to flow west when the ice barrier that had formed the western margin of a large palaeolake broke *c.* 12 000 BP (Turner *et al.* 2005). Our genetic data strongly suggest that drainage reversals brought eastern lineages into central Chilean Patagonia. Historic drainage patterns better explain geographical patterns of genetic variation than do current drainage patterns, and central Chilean Patagonia is the only region where eastern lineages are found west of the Andes. Less information is available for other Pacific-draining watersheds with headwater lakes situated east of the divide, but our hierarchical analysis of molecular variance (AMOVA) suggests it is likely that they share a similar history of drainage reversal. Such widespread changes in drainage patterns would have created an efficient mechanism for asymmetrical gene flow across the Andes, increasing genetic diversity west of the Andes. Consequences of drainage rearrangements for patterns of genetic diversity have been particularly well documented for New Zealand galaxiids (Waters & Wallis 2000; Waters *et al.* 2001, 2006; BurrIDGE *et al.* 2006, 2007; Craw & Waters 2007; Craw *et al.* 2007). Such an approach might eventually help attain more accurate substitution rates for South American galaxiids and effectively reduce uncertainties for applications that employ these metrics (e.g. timing of lineage splitting).

Taxonomy and conservation

Species-level taxonomy for the Galaxiidae in South America has a tangled past that extends from the early parts of the

20th century until present. Most controversy concerns the genus *Galaxias* to which as many as 16 nominal taxa have been assigned, only three of which are currently recognized as good species (McDowall 1971, 2006). The source of this inflation is mainly attributed to poor records and the description of distinct juveniles as new species, but can also be blamed on the extreme variation in morphology observed in some widespread species, particularly *G. platei* (Milano *et al.* 2002, 2006).

The phylogeographical evidence presented here suggests *G. platei* constitutes one species over its entire range supporting the original conclusions of McDowall (1971). Future studies employing nuclear markers in central Chile (area of proposed secondary contact) are being planned to further test the species-level status of *G. platei*.

Below the species level, four distinct evolutionary lineages were detected by phylogeographical analyses. Each of these units likely represents an independent evolutionary trajectory, each contributing an important source of genetic variation to the species recognized as *G. platei*. Secondary contact between each of these lineages in central Chile has resulted in this region currently hosting the greatest genetic diversity of *G. platei* throughout its range in Patagonia. This has important implications for conservation and management of the species, particularly considering the hydroelectric dams sited for construction in this area for the near future. We suggest that, unless additional genetic analysis indicates otherwise, future conservation strategies consider *G. platei* as a single species, but recognize the deep genealogical lineages that are distributed throughout Patagonia which meet in central Chile to form what is potentially the richest source of historical neutral genetic variation.

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This paper is part of TSZ's PhD research on the phylogeography and conservation genetics of the *Galaxiidae* in Patagonia (with DER). TSZ is interested in applying genomic approaches to phylogenetics and phylogeography in aquatic organisms. The work is a result of an ongoing collaboration between DER and SJW of Dalhousie University (Canada), EH of Universidad de Concepción (Chile) and MAB of Universidad del Comahue (Argentina) to study the interplay between the evolution and phylogeography of the native fish fauna of Patagonia. DER is interested in conservation genetics, biodiversity and phylogeography of aquatic organisms, SJW works in freshwater ecology, and MAB and EH conduct research on the native and introduced fishes of Argentina and Chile, respectively. EDMA conducted her honours research on *G. platei* (with DER).

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 Haplotype frequencies by collection site (lake/river)

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