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Comparative population genetics of *Basilichthys microlepidotus* (Atheriniformes: Atherinopsidae) and *Trichomycterus areolatus* (Siluriformes: Trichomycteridae) in north central Chile

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Abstract To describe comparative population genetic structure of the Chilean silverside *Basilichthys microlepidotus* and the catfish *Trichomycterus areolatus*, four rivers and three sites within each river were investigated by the analysis of haplotype polymorphisms of the mitochondrial Control Region. For both species, analyses revealed significant differentiation among rivers and low differences within rivers. However, the species differ in haplotype composition; individuals of *B. microlepidotus* shared some haplotypes in all four rivers, while individuals of *T. areolatus* showed a different haplotype composition in most rivers. This difference may be explained by the different ecological features of the species. Assuming that both silversides and catfish were present before the separation of the rivers, *B. microlepidotus* migrated after river isolation, probably using coastal water, while *T. areolatus* has probably never migrated between these rivers. The long times that the studied rivers have been separated should be taken into account in future conservation plans for the freshwater fish of Chile.

Keywords Silverside · Catfish · Andes mountains · Chilean rivers · Control Region

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Introduction

Understanding how geological processes have affected current population structure and genetic variability along the geographical range of a species is an important issue in biological conservation. In the case of freshwater fishes, it is known that physical barriers to dispersal tend to be stronger in freshwater habitats than their marine counterparts occupying otherwise comparable geographic ranges (Mank and Avise 2006). This physical subdivision limits gene flow, promotes population subdivision (Youngson et al. 2003) and ultimately reduces and fragments genetic variability. In this context, DeWoody and Avise (2000) demonstrated that freshwater fish exhibit lower genetic variability compared to anadromous and marine fish; explained by the differences in population size and potential for exchanges among populations.

Due to the species displacement and asymmetry of water flow, biological conservation may require protection of certain parts of river systems often geographically distant from the other biological features of interest (Moilanen et al. 2008). To attain this conservation expectation, immediate actions should include the investigation of the extent of genetic differentiation between populations, the establishment of backup populations and monitoring of new and existing populations (Harrod et al. 2001). However, the lack of knowledge of the structure, migration and even presence of freshwater species around the world (Lévêque et al. 2008) is a serious limitation.

In north central Chile, geological processes connected river formation with the final uplift of the Andes Range. Although the Andes uplift varied from north to south and from east to west (Gregory-Wodzicki 2000), for central Chile it has been estimated that modern orogeny began in the lower Miocene as a result of the inversion of the Abanico basin between 26° and 39° south latitude and between the current Trans/Cis drainage divides (Atlantic and Pacific waters) (Charrier et al. 2007; Farías et al. 2008; Fig. 2 in Charrier et al. 2009). Finally, surface uplift of the Andes and other morphostructural units in Chile were established between ~8.5 and ~4 Ma (Charrier et al. 2007; Farías et al. 2008).

Thus, considering the geological setting of the current drainage separations, it is likely that present rivers began to run from east to west, lost their palaeogeographical connections and became isolated basins starting at ~8.5 Ma at 30°–35° south latitude. These geological changes have shaped the ichthyological fauna inhabiting this area of Chile during the Neogene (c.a. ~23 Ma) affecting it to the point that the ichthyofauna is now characterized as being low in species richness, retaining primitive characters, exhibiting a high degree of endemism and frequently having restricted distribution of taxa (Vila et al. 1999). As well as geological events, climatic features appear to have shaped this unique fauna which is of small size and tends to be adapted to rivers with high slopes and variable stream flow (Vila et al. 1999; Dyer 2000b).

In this group of rivers, *Basilichthys microlepidotus* Jenyns and *Trichomycterus areolatus* Valenciennes are two of the most representative fish species (Vila et al. 2006). These species live mainly in the intermediate zone of the rivers where there are abundant macrophytes and ocean salinity does not intrude (Duarte et al. 1971); however, there is no information about possible migration or gene flow within and among rivers. These two species are indicated as vulnerable in the latest conservation list of the Chilean government (Ministerio Secretaría General de la Presidencia 2008), thus more information on their population structure is necessary for future conservation measures.

Our objective was to investigate the comparative population genetic structure of the silverside *B. microlepidotus* and the catfish *T. areolatus* inhabiting rivers in north central Chile. Using the variation of the mitochondrial Control Region we studied four rivers and three different sites along each to analyze the extent of structure at the population level. Considering that there is no prior information on the population structure of these species, we hypothesized that they should exhibit similar spatial patterns, determined by the geological processes that isolated the rivers.

Materials and methods

Sampling sites

Specimens of *B. microlepidotus* and *T. areolatus* were caught by electrofishing from four rivers (Limarí, Choapa, Aconcagua and Maipo) in north central Chile. To determine the relationships among and within these rivers, three sites from each river were sampled. Geographical locations sampled in this study and sample sizes are shown in Fig. 1 and Tables 1 and 2 respectively.

DNA extraction, amplification and $_{mt}$DNA sequencing

Total genomic DNA was extracted from ethanol-preserved fin clips using the salt-extraction method (Aljanabi and Martinez 1997). Purified DNA was stored at -20°C in 50 μL of water until analysis. Using the mitochondrial sequence of *Hypoatherina tsurugae* (GenBank AP004420; Miya et al. 2003) specific primers for the control region of *B. microlepidotus* were designed as follows: Forward (5'-CCT AAC TCC CAA AGC TAG GAT-3') and Reverse (5'-TGC GGT ACT TGC ATG TGT AA-3'). The mtDNA sequence of the *Pangasianodon gigas* (GenBank AY762971; Jondeung et al. 2007) was used to design the Reverse primer for *T. areolatus* 5' TGC GGA TAC TTG CAT GTA TAA 3'. The Forward primer developed for *B. microlepidotus* was used also for *T. areolatus*. For both species, amplification from the template DNA used the following conditions: 1x buffer (Invitrogen), 3.2 nM MgCl2, 0.2 U/μL dNTP, 5 pmol forward and reverse primers, and 0.1 U/μL Taq DNA polymerase (Invitrogen). The PCR reaction (in 25 μL final volume) involved a denaturing step of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 60°C for 90 s, and 72°C for 90 s with a final elongation step at 72°C for 10 min. PCR products were cleaned using QIAQuick columns

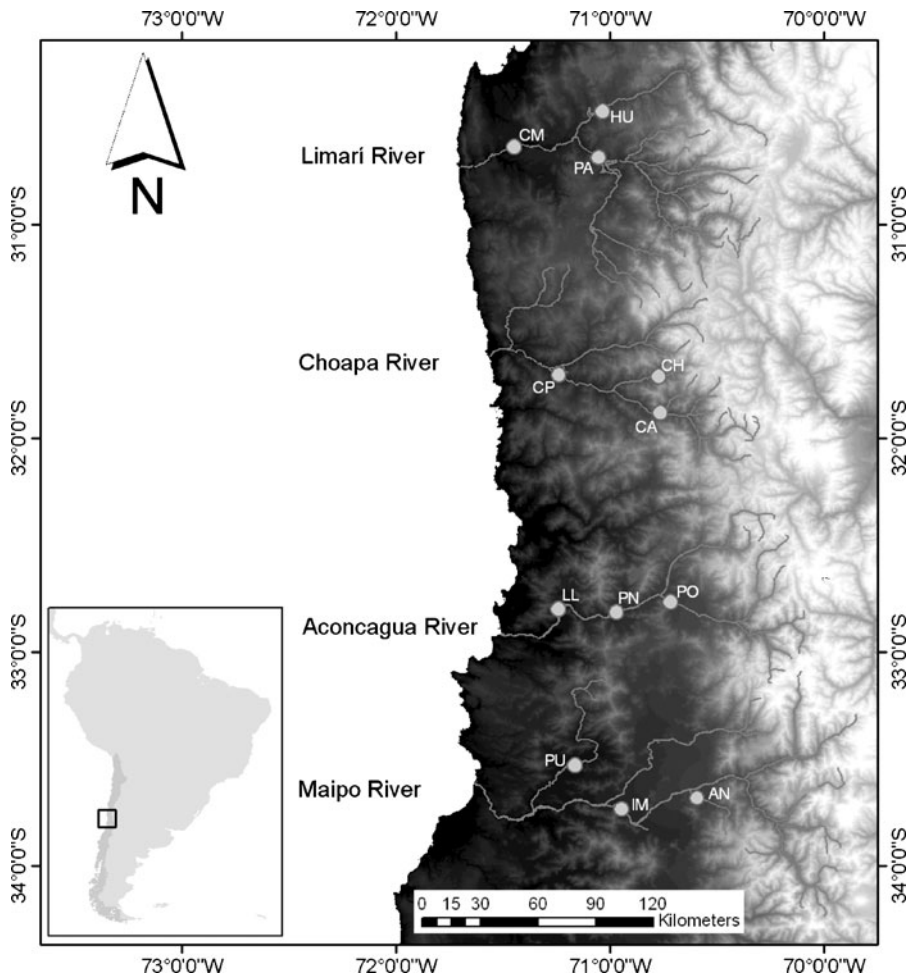

Fig. 1 Location of sample sites of *B. microlepidotus* and *T. areolatus* located in four rives in north central Chile. HU = Huampulla; PA: Paloma; CM: Caballo Muerto; CH: Chillepin; CA: Camisas; CP: Choapa Pueblo; PO: Pocuro; PN: Panuehue; LL: LlaLlay; AN: Angostura; IM: Isla de Maipo; PU: Puangue

(QIAGen, Mississauga, Ontario, Canada) and sequencing was performed in Macrogen Inc (www.macrogen.com). Sequences were aligned using ProSeq software (Filatov 2002) and checked using Multalign online software (Corpet 1988). Sequences were published in Genbank with the following accession numbers: *T. areolatus*: GQ178087 to GQ178156, *B. microlepidotus*: GQ178157 to GQ178214, FJ843108 to FJ843127 and FJ380093 to FJ380105.

Data analysis

Genetic variation within and among rivers was assessed by number of haplotypes, polymorphic sites and average number of pairwise differences using DnaSP 4.9 software (Rozas et al. 2003). To determine the partition of the molecular variance at the population level, within and among rivers, a hierarchical Analysis of Molecular Variance (AMOVA) was performed in Arlequin version 3.0 (Excoffier et al. 2005). Pairwise population structure was evaluated *a posteriori* by means of F_{ST}; significance was tested using 10 000 permutations with a level of significance of α=0.05. To visualize mutational steps and differences in haplotype composition we constructed a haplotype network using the median joining algorithm implemented in the Network software (Bandelt et al. 1999).

Considering that historical isolation and contemporary modifications of rivers should have affected the species equally, we tested for possible changes in demographic patterns. To quantify the significant departure from population equilibrium, we evaluated the coalescent-based neutrality estimators Tajima's D (Tajima 1989), Fu's F_S (Fu 1997) and the Raggedness index (Harpending et al. 1993, 1994) in Arlequin 3.0 (Excoffier et al. 2005). Using the Mantel test (Mantel 1967), we also tested the isolation by distance (IBD)

Table 1 Summary of genetic diversity indices and neutrality tests from each site and river for *B. microlepidotus* based on mtDNA (Control Region)

River	Site	n	k	s	Π	D	Fs	τ	θ	θ_1	Raggedness index
Limari	Huampulla (HU)	30	21	10	4.14±2.36	-0.66	-0.25	8.17	0.01	5.39	0.10
	Paloma (PA)	28	20	11	3.67±2.13	-0.92	-1.65	7.42	0.00	5.19	0.05
	Caballo Muerto (CM)	30	19	9	3.95±2.26	-0.62	0.29	8.50	0.01	5.25	0.06
Choapa	Chillepin (CH)	30	18	11	4.28±2.42	-0.19	-0.84	9.03	0.01	6.71	003
	Camisas (CA)	30	19	11	4.26±2.42	-0.43	-0.96	7.28	0.00	7.22	0.06
	Choapa Pueblo (CP)	28	20	13	4.72±2.65	-0.29	-2.21	8.07	0.01	8.79	0.02
Aconcagua	Pocuro (PO)	29	21	14	6.31±3.43	0.63	-1.55	9.39	0.00	39.28	0.05
	Panquehue (PN)	29	30	16	6.43±3.48	-0.53	-4.04	8.64	0.00	20.43	0.03
	LlayLlay (LL)	29	33	17	6.44±3.50	-0.79	-3.07	9.08	0.00	17.87	0.03
Maipo	Angostura (AN)	28	19	11	5.52±3.04	-0.78	-1.10	9.13	0.00	10.01	0.03
	Isla de Maipo (IM)	30	34	14	6.77±3.65	0.52	-0.10	9.45	0.00	14.42	0.03
	Puangue (PU)	30	23	10	3.84±2.21	-1.16	-0.53	9.32	0.01	12.02	0.06

n: sample sizes, k: number of haplotypes, s: polymorphic sites, Π: average number of pairwise differences, D: Tajima statistic, F_S: Fu statistic, mismatch parameters: τ, θ, θ_1 and the Raggedness index

* P≤0.05

by correlating site distances and pairwise F_{ST} values. We measured distances in GoogleEarth (www.google.earth.com) assuming a coastal route among rivers. The Mantel test was performed in the GENETIX software (Belkhir et al. 1996–2004) and the statistical significance of this correlation was estimated with 10 000 permutations.

To test for reciprocal migration of fishes among sites and rivers studied, we estimated migration rates using Markov chain Monte Carlo simulations as implemented in Migrate-n 3.0.3 (Beerli 2008). Due to the nature of data in this study (mitochondrial DNA) the analysis estimated the number of female migrants. In these analyses, different settings were

Table 2 Summary of genetic diversity indices and neutrality tests from each site and river for *T. areolatus* based on mtDNA (Control Region)

River	Site	n	*k*	*s*	Π	D	F_S	T	θ	θ_1	Raggedness index
Limari	Huampulla (HU)	27	2	1	0.07±0.17	-1.15	-1.12	3.00	0.00	0.08	0.73
	Paloma (PA)	16	1	0	0.00	0.00	NA	0.00	0.00	0.00	0.00*
	Caballo Muerto (CM)	20	1	0	0.00	0.00	NA	0.00	0.00	0.00	0.00*
Choapa	Chillepin (CH)	29	2	1	0.07±0.16	-1.14	-1.18	3.00	0.00	0.08	0.75
	Camisas (CA)	28	2	1	0.07±0.16	-1.15	-1.15	3.00	0.00	0.08	0.74
	Choapa Pueblo (CP)	30	2	1	0.07±0.15	-1.14*	-1.21	3.00	0.00	0.07	0.76
Aconcagua	Pocuro (PO)	30	12	17	4.92±2.74	0.49	-1.01	8.40	0.01	10.54	0.12*
	Panquehue (PN)	27	15	21	3.94±2.27	-1.20	-5.48*	1.55	3.26	14.79	0.23*
	Llayllay (LL)	25	16	35	6.09±3.34	-1.41	-4.48*	5.11	2.19	15.06	0.03
Maipo	Angostura (AN)	30	10	13	3.14±1.86	-0.13	-1.27	5.25	0.01	5.34	0.06
	Isla de Maipo (IM)	30	13	35	7.24±3.88	-0.65	-0.17	22.34	0.01	2.78	0.05
	Puangue (PA)	24	16	37	14.58±7.55	1.92	-0.70	30.23	0.00	22.53	0.03

n: sample sizes, k: number of haplotypes, s: polymorphic sites, Π: average number of pairwise differences, D: Tajima statistic, F_S: Fu statistic, mismatch parameters: τ, θ, θ_1 and the Raggedness index

* P≤0.05

used for each species to attain convergence of the Monte Carlo simulation. For *B. microlepidotus* we used 15 short chains with 22 500 sampled genealogies and four long chains with 300 000 genealogies, discarding the first 20 000 trees as burn-in (sampling every 40 generations). We also estimated initial parameters from an F_{ST} calculation, using a random tree as the first genealogy, averaging long chains over 4 replicates and using a static heating scheme of 6 chains with temperatures (1, 1.25, 1.67, 2.5, 5 and $1e^6$; the swapping interval was 1). For *T. areolatus* we used 20 short chains with 40 000 genealogies sampled and six long chains with 400 000 genealogies, discarding the first 20 000 trees as burn-in (sampling was every 40 genealogies). Initial parameters were estimated and replicated as with *B. microlepidotus*, but we used an adaptive heating scheme with 6 temperatures (1.00, 3226.77, 9678.32, 22581.42, 48387.61, $1e^5$; the swapping interval was 1). For each species we used the full migration matrix model with variable Theta and geographic distances between sampling sites as constraint, thus results were expressed as emigration and immigration rates among and within rivers scaled by geographic distances (Beerli 2008).

Finally, to estimate the age of most recent common ancestor (MRCA) among fish sequences and the relationship with historical river isolation according to geological data, we performed a Bayesian analysis using BEAST 1.5.3 software (Drummond and Rambaut 2007). For this analysis, sequences of *Basilichthys semotilus* (Cope) and *Trichomycterus laucaensis* Arratia were used as outgroup. The optimal model of nucleotide evolution was estimated with Modeltest 3.7 (Posada and Crandall 1998), selecting GTR + I + G according to Akaike's information criterion, for both silversides and catfish. For both species we used a relaxed molecular clock model (uncorrelated lognormal) with a mutation rate of 5% per million years (Bowen et al. 2006), a starting UPGMA tree with constant size population as tree priors and 10 gamma categories for the GTR + I + G model with empirical base frequencies. Convergence was screened in Tracer software (Rambaut and Drummond 2003), and several runs were necessary to achieve good posterior distributions of parameters and ESS values (Drummond and Rambaut 2007). For silversides we required 2 runs of 75×10^6 generations and for catfish we ran 2 analyses with 20×10^6 generations, sampling every 1,000 generations for both species. To facilitate convergence, we used preliminary runs of 50×10^6 generations for silverside and 20×10^6 generations for catfishes in order to set initial values for the parameters ag, at, cg, gt, alpha and plnv. Log and tree files were combined with LogCombiner 1.5.3 and phylogenetic trees were built in TreeAnotator 1.5.3 (Drummond and Rambaut 2007).

Results

Basilichthys microlepidotus

For the Control Region we sequenced 912 bp and observed 69 haplotypes in 350 individuals analyzed. No insertions or deletions were detected, so alignment was straightforward. Between 18 (Chillepin, Choapa River) and 34 haplotypes (Isla de Maipo, Maipo River) were observed and pairwise differences among sequences ranged from Π=3.67 (Paloma in Limarí River) to Π=6.77 (Isla de Maipo in Maipo River) (Table 1).

Analysis of Molecular Variance revealed significant differences in haplotype frequencies at both levels (Table 3). F_{ST} pairwise analysis indicated that sites within rivers (Limari, Choapa and Aconcagua Rivers) are part of single populations (Table 4). Within the Maipo River, all pairwise comparisons showed significant differences among sites ($P<0.05$). Interestingly, Angostura (Maipo River) did not show differences with several sites in the Aconcagua and the Choapa Rivers; Pocuro and Llayllay also showed absence of population structure with two sites of the Choapa River (Table 4). Genealogical relationships observed in the network suggest also differences in haplotype frequencies but not in haplotype composition among rivers. Three haplotypes were shared in all rivers and four haplotypes were common to three rivers (Fig. 2).

Demographic analyses showed no reliable evidence of population expansion for every site. Whereas Tajima'D and Fu'F_S statistics were consistent with population equilibrium, the Raggedness index showed low values ($P>0.05$) (Table 1) which suggests a no rejection of the null hypothesis of population expansion. The IBD analysis showed a nonsignificant relationship between paired F_{ST} values and distances among sites (Fig. 3a).

Table 3 Analysis of molecular variance (AMOVA) among 12 samples of *Basilichthys microlepidotus* separated in four rivers (Limarí, Choapa, Aconcagua and Maipo) using sequences of the Control Region

Sources of variation	df	Sum of squares	Variance components	Percentage variation	Fixation índices
All sites					
Among groups	3	140.205	0.470	15.19	F_{CT}=0.152*
Among samples within groups	8	45.160	0.107	3.47	F_{SC}=0.041*
Within samples	338	849.809	2.514	81.34	F_{ST}=0.190*
Total	349	1,035.174	3.091		

*P<0.05

In *B. microlepidotus*, the pattern of migration revealed a high number of emigrants/immigrants, with asymmetric exchange within sampling sites of each river and asymmetry in female movements among rivers (Table 5). Also it is interesting to note that the results showed a gradient of connections between sampling sites; i.e., Paloma and Caballo Muerto (Limarí River) exhibited links with five other sites belonging to all 4 rivers; whereas other populations such as Huampulla (Limarí River) and Puangue (Maipo River) only had one link (as immigrants), and two links (as both immigrants and emigrants), respectively. Overall, a high number of connections between sampling sites and a significant number of historical emigrants/immigrants among rivers and within sampling sites were detected.

Phylogenetic analyses of *B. microlepidotus* estimated that the time of MRCA of the haplotypes sampled was 310 Kyr, with a 95% highest posterior density (HPD) of 489 Kyr to 169 Kyr (Fig. 5). As was observed in the haplotype network, a high mixture of haplotypes among rivers was also found in the phylogenetic reconstruction (Fig. 5), and interestingly, more derived haplotypes were shared in 3 (haplotypes 6, 10, 19 and 20) and all 4 rivers (haplotypes 2, 4 and 7).

Trichomycterus areolatus

The 755 bp sequences for the Control Region of *T. areolatus* revealed the presence of 70 haplotypes in the 316 individuals sampled. The analyzed part of the mtDNA did not present alignment problems; only

Table 4 Pairwise F_{ST} (above the diagonal) and the associated P-values (below diagonal) among sites of *Basilichthys microlepidotus*

		Limarí			Choapa			Aconcagua			Maipo		
River	Site	HU	PA	CM	CH	CA	CP	PO	PN	LL	AN	IM	PU
	HU		-0.017	-0.007	0.260	0.266	0.247	0.225	0.207	0.2192	0.217	0.311	0.311
Limarí	PA	0.715		-0.016	0.294	0.286	0.270	0.258	0.237	0.2483	0.249	0.334	0.345
	CM	0.469	0.705		0.319	0.318	0.301	0. 279	0. 262	0. 2764	0. 268	0. 352	0. 366
Choapa	CH	0.000*	0.000*	0.000*		-0.005	-0.019	0.048	0.065	0.0370	0.001	0.173	0.082
	CA	0.000*	0.000*	0.000*	0.460		-0.018	0.089	0.095	0.0692	0.019	0.209	0.105
	CP	0.000*	0.000*	0.000*	0.776	0.783		0.037	0.059	0.0231	0.007	0.144	0.086
Aconcagua	PO	0.000*	0.000*	0.000*	0.050*	0.006*	0.070		0.000	-0.0159	0.040	0.065	0.133
	PN	0.000*	0.000*	0.000*	0.013*	0.001*	0.021*	0.397		0.0162	0.063	0.150	0.153
	LL	0.000*	0.000*	0.000*	0.064	0.010*	0.126	0.750	0.153		0.027	0.050	0.106
Maipo	AN	0.000*	0.000*	0.000*	0.339	0.148	0.237	0.072	0.018*	0.104		0.130	0.089
	IM	0.000*	0.000*	0.000*	0.000*	0.000*	0.001*	0.024*	0.000*	0.036*	0.003*		0.221
	PU	0.000*	0.000*	0.000*	0.004*	0.000*	0.003*	0.000*	0.000*	0.000*	0.007*	0.000*	

*P≤0.05

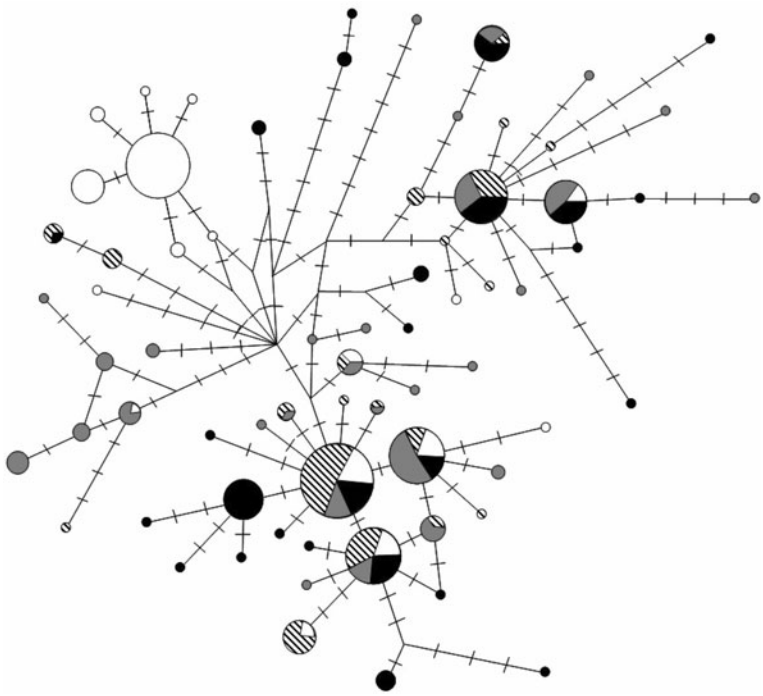

Fig. 2 Unrooted haplotype network of *B. microlepidotus* Control Region. The area of each circle is proportional to the number of individuals. Each perpendicular line between haplotypes indicates a single mutational step. White circle: Limarí River; white circle with black lines: Choapa River; grey circle: Aconcagua River; black circles: Maipo River

three insertions were observed in individuals sampled from the Aconcagua River. Between 1 (two sites in the Limari River) and 16 haplotypes (one site each in the Aconcagua and Maipo Rivers) were observed and pairwise differences among sequences ranged from Π=3.67 (Paloma from Limarí River) to Π=6.77 (Isla de Maipo in Maipo River) (Table 2).

AMOVA showed differences at both levels of variation (Table 6). Pairwise analyses did not show differences between and within the Limarí and Choapa Rivers (Table 7). All other comparisons among rivers showed highly significant differences ($P<0.001$); however within the Aconcagua and Maipo rivers, the analysis detected population structure in some sites (Table 7). Genealogical relationships observed in the network suggest slight differences in haplotype frequencies and haplotype composition between the Limarí and Choapa Rivers, while other rivers presented strong differences (Fig. 4).

Demographic analysis suggested population expansion for some indexes in some sampling sites; nevertheless none of three indexes agreed (Table 2). The weak signal of statistical significance of tests presumes population equilibrium as was observed for *B. microlepidotus*. In contrast to the silverside, the catfish *T. areolatus* showed a significant relation between geographic distance and population structure (Fig. 3a, b), which means that isolation by distance may be an important process that has shaped current gene diversity.

Gene flow estimates of *T. areolatus* indicated a low number of emigrants/immigrants within sampling sites of each river and zero exchange among rivers, except for one sampling site of the Limarí and Choapa Rivers (Table 8).

Phylogenetic analyses of catfish showed no shared haplotypes among rivers (except for the Limarí and Choapa Rivers) and an ordered arrangement of these haplotypes from south to north (Fig. 5). Haplotypes of the Limarí and Choapa rivers appear to be more recently derived, and lineages of Maipo River appear to be more basal within the phylogenetic framework. The age of MRCA of the Control Region for *T. areolatus* was estimated to be 737 Kyr (95% HPD: 378 to 1,224 Kyr) and the sequential break of some haplotypes of Maipo and northern rivers was estimated to be 440 Kyr (95% HPD: 261 to 580 Kyr). An intermediate break and MRCA of the Aconcagua, Limarí and Choapa rivers was estimated to be 308 Kyr (95% HPD: 151 to 515 Kyr), and MRCA of Limarí and Choapa rivers was 34 Kyr (95% HPD: 8 to 68 Kyr).

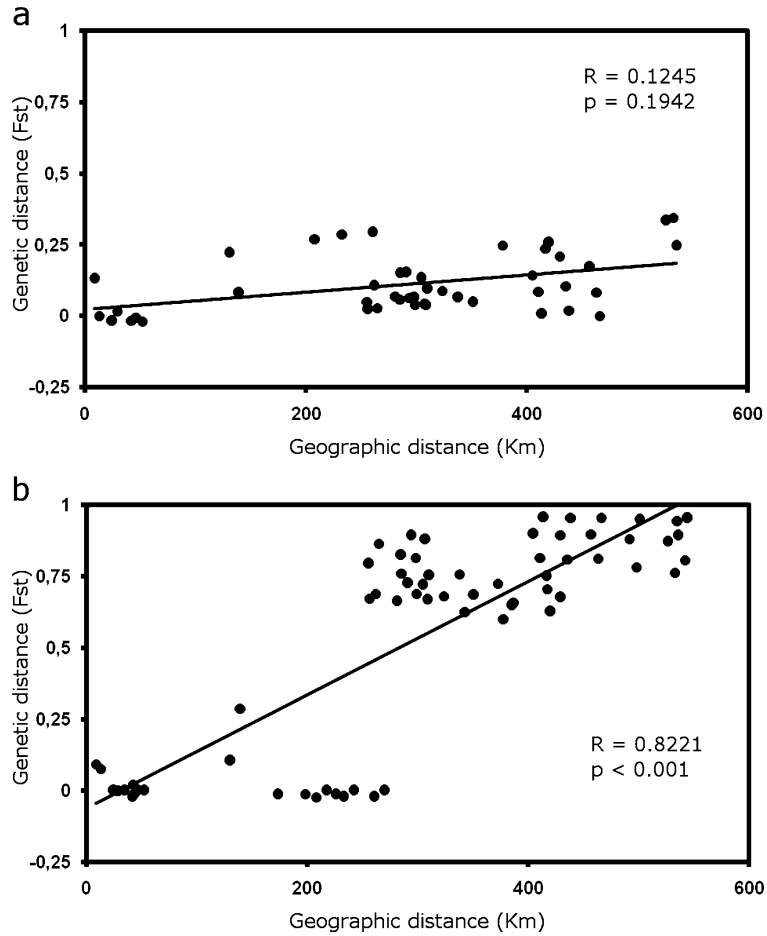

Fig. 3 Isolation by distance plots using paired F_{ST} values and distance among sites. a) *Basilichthys microlepidotus* and b) *Trichomycterus areolatus*

Table 5 Gene-flow estimates for *B. microlepidotus*

			Limarí			Choapa			Aconcagua			Maipo		
River	Site	Theta [2 N mu]	HU	PA	CM	CH	CA	CP	PO	PN	LL	AN	IM	PU
	HU	5.8 e^{-4}		9.5 e^{5}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Limarí	PA	4.6 e^{-3}	0.00		5.4 e^{4}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	CM	5.0 e^{-3}	0.00	0.00		0.00	0.00	1.5 e^{4}	0.00	0.00	1.5 e^{4}	0.00	0.00	0.00
	CH	5.3 e^{-3}	0.00	5.7 e^{5}	1.1 e^{5}		7.3 e^{5}	2.8 e^{5}	0.00	0.00	0.00	0.00	0.00	0.00
Choapa	CA	2.8 e^{-3}	0.00	1.3 e^{6}	0.00	3.8 e^{5}		1.9 e^{5}	0.00	4.7 e^{5}	0.00	0.00	0.00	0.00
	CP	3.8 e^{-3}	0.00	7.0 e^{5}	0.00	1.1 e^{6}	5.9 e^{4}		0.00	0.00	2.0 e^{5}	0.00	0.00	0.00
	PO	3.3 e^{-3}	0.00	0.00	0.00	0.00	0.00	0.00		3.1 e^{5}	0.00	0.00	0.00	0.00
Aconcagua	PN	1.1 e^{-3}	0.00	0.00	5.3 e^{5}	0.00	0.00	0.00	0.00		4.8 e^{4}	0.00	4.3 e^{4}	0.00
	LL	1.1 e^{-3}	0.00	6.3 e^{5}	1.7 e^{5}	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00
	AN	1.3 e^{-3}	0.00	0.00	0.00	0.00	4.9 e^{5}	0.00	0.00	0.00	0.00		6.1 e^{4}	7.8 e^{4}
Maipo	IM	1.0 e^{-3}	0.00	0.00	5.7 e^{5}	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00
	PU	3.8 e^{-3}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.7 e^{5}	0.00	0.00	0.00	

Columns are donor populations, whereas rows are receiving populations

All values within bounds of 95% confidence limit

Table 6 Analysis of molecular variance (AMOVA) among 12 samples of *Trichomycterus areolatus* separated in four rivers (Limarí, Choapa, Aconcagua and Maipo) using sequences of Control Region

Source of Variation	df	Sum of squares	Variance components	Percentage variation	Fixation indices
Among groups	3	2,073.834	8.692	81.79	F_{CT}=0.818*
Among samples within groups	8	62.062	0.231	2.18	F_{SC}=0.120*
Within samples	304	518.085	1.704	16.04	F_{ST}=0.840*
Total	315	2,653.981	10.628		

*P<0.05

Discussion

We hypothesized that geological processes were determinants of population structure of *B. microlepidotus* and *T. areolatus* in north central Chile. According to geological data, the Abanico basin, a late Eocene-Oligocene extensional environment (Charrier et al. 2007, 2009) should have connected and allowed biotic homogenization among fluvial and lacustrine palaeo-environments in central Chile. The subsequent elevation of the Andes (Ramos et al. 2002; Giambiagi et al. 2003) has obliged an east–west drainage since 8.5—4 Ma (Farías et al. 2008). This elevation established the current separation of the Trans/Cis Andean drainage divides and originated the geographic isolation observed currently in rivers of north central Chile. Even though Miocene fossil records of the fishes *Nematogenys cuivi* Azpelicueta and Rubilar, *Basilichthys sp.* and *Percilia? sp.*—genus currently present only in Trans Andean drainages—(Rubilar 1994; Azpelicueta and Rubilar 1998; Dyer 1998, 2000b) suggest that the Abanico basin was an important stage in diversification of fishes in Chile, the pattern observed in this study is not as ancient as geological evidence suggests. Even though the extensive use of different mutation rates for the Control Region (Donaldson and Wilson 1999; Bowen et al. 2006; Koblmuller et al. 2006) and the lack of estimates explicitly determined for our focal species, Bayesian reconstruction of MRCA indicated important differences between silversides and catfish. While *B. microlepidotus* showed no geographic structure and recent ancestors about 300 Kyr, *T. areolatus* more than doubles the age of MRCA to over 700 Kyr and shows a pattern of genetic diversification from south to north. An interesting finding is the divergence among haplotypes of the Maipo River, which suggests more complex processes than river isolation by

Table 7 Pairwise F_{ST} (above the diagonal) and the associated P-values (below diagonal) among sites of *Trichomycterus areolatus*

		Limarí			Choapa			Aconcagua			Maipo		
River	Site	HU	PA	CM	CH	CA	CP	PO	PN	LL	AN	IM	PU
	HU		-0.0210	-0.0116	0.0001	0.0000	0.0002	0.6772	0.7503	0.6588	0.9547	0.8943	0.8067
Limarí	PA	0.999		0.0000	-0.0225	-0.0218	-0.0232	0.6271	0.7053	0.6008	0.9451	0.8719	0.7638
	CM	0.999	0.999		-0.0135	-0.0126	-0.0143	0.6485	0.7251	0.6256	0.9494	0.8815	0.7819
Choapa	CH	0.745	0.999	0.999		0.0000	0.0000	0.6852	0.7574	0.6679	0.9562	0.8976	0.8130
	CA	0.730	0.999	0.999	0.753		0.0001	0.6813	0.7539	0.6634	0.9554	0.8958	0.8096
	CP	0.727	0.999	0.999	0.752	0.761		0.6891	0.7608	0.6722	0.9569	0.8991	0.8158
Aconcagua	PO	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*		0.0759	0.0200	0.8801	0.8159	0.7210
	PN	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.023*		-0.0030	0.8933	0.8257	0.7267
	LL	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.150	0.392		0.8647	0.7944	0.6891
Maipo	AN	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*		0.0917	0.2880
	IM	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.002*		0.1050
	PU	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.032*	

*P≤0.05

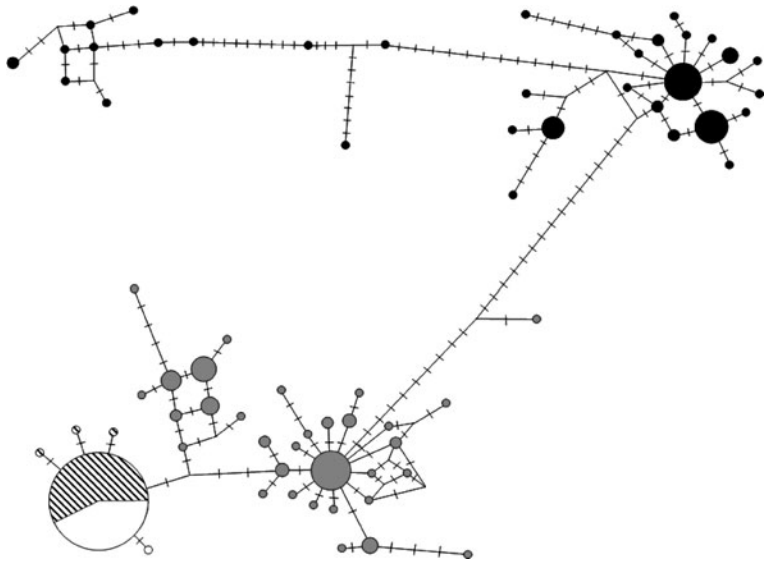

Fig. 4 Unrooted haplotype network of *T. areolatus* Control Region. The area of each circle is proportional to the number of individuals. Each perpendicular line between haplotypes indicates a single mutational step. White circle: Limarí River; white circle with black lines: Choapa River; grey circle: Aconcagua River; black circles: Maipo River

itself. This issue is being analyzed in a phylogenetic context including fish morphology and other molecular markers to estimate more precisely divergence times and age of MRCA.

Studies conducted on different taxa have reinforced the impact of the Andes Cordillera on the isolation of species between the western vs. eastern areas (rodents: Smith et al. 2001; freshwater crabs: Perez-Losada et al. 2004; freshwater fishes: Ruzzante et al. 2006; lizards: Victoriano et al. 2008), which have left a genetic signature of stable habitats and limited dispersal between watersheds on the west side of the Andes (Ruzzante et al. 2006). Our findings on the western side of the Andes Range appear to be comparatively less complex than those described for species of Atherinopsidae in the Atlantic coast of Argentina. The most recent processes on the east side of the Andes occurred about 120 Kyr ago during the last sea-level change, the coastal topography changing so drastically that contemporary drainage structure does not provide a good explanation for the diversification of *Odontesthes* species (Beheregaray et al. 2002).

Table 8 Gene-flow estimates for *T. areolatus*

			Limarí		Choapa			Aconcagua			Maipo			
River	Site	Theta [2 N mu]	HU	PA	CM	CH	CA	CP	PO	PN	LL	AN	IM	PU
	HU	7.0 e^{-3}		0.00	0.00	0.00	4.5 e^{6}	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Limarí	PA	5.0 e^{-3}	9.4 e^{5}		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	CM	1.3e^{-8}	1.4 e^{6}	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	CH	5.1e^{-9}	0.00	1.0 e^{7}	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Choapa	CA	7.0 e^{-3}	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00
	CP	7.9e^{-12}	0.00	0.00	0.00	0.00	1.8 e^{6}		0.00	0.00	0.00	0.00	0.00	0.00
	PO	9.1 e^{-3}	0.00	0.00	0.00	0.00	0.00	0.00		0.00	6.6 e^{4}	0.00	0.00	0.00
Aconcagua	PN	2.5 e^{-2}	0.00	0.00	0.00	0.00	0.00	0.00	5.4 e^{3}		1.1 e^{5}	0.00	0.00	0.00
	LL	8.8 e^{-3}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.3 e^{3}		0.00	0.00	0.00
	AN	7.0 e^{-3}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		2.1 e^{4}	0.00
Maipo	IM	1.2 e^{-2}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		6.1 e^{3}
	PU	9.4 $^{e-3}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.1 e^{4}	

Columns are donor populations, whereas rows are receiving populations. All values within bounds of 95% confidence limit

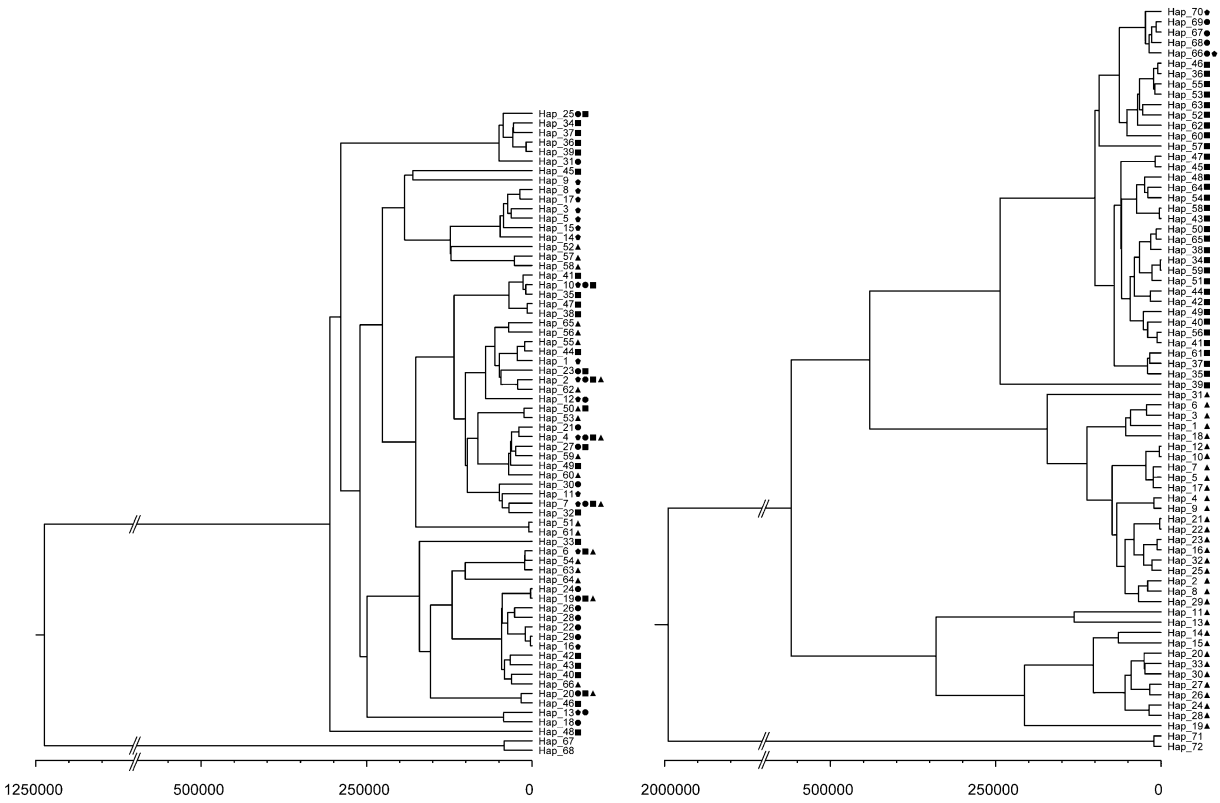

Fig. 5 Bayesian trees inferred in BEAST software with sequences of Control Region. On the left is shown phylogenetic hypothesis of haplotypes of *B. microlepidotus* and on the right is shown tree of *T. areolatus*. Next to each haplotype label was drawn a symbol for each river: Limarí River, pentagon; Choapa River, circle; Aconcagua River, square; and Maipo River, triangle

For *T. areolatus* we found no evidence of gene flow among populations of rivers (except for the Limarí and Choapa rivers), but high levels of historical migrants within each basin, with the exception of some localities in the Aconcagua and Maipo Rivers. These findings coupled with a pattern of isolation by distance suggest that catfish are effectively being isolated by terrestrial barriers and coastal routes probably were not used. Contrary to our predictions, the silverside *B. microlepidotus* exhibited high levels of gene flow, haplotypes shared among rivers and absence of correlation between distance and genetic differentiation. The same geological processes appear to have shaped the two species in different ways in the area we studied. It is also important to note the differences observed in haplotype composition. While *T. areolatus* has different haplotypes in the Maipo, Aconcagua and Limarí-Choapa rivers, *B. microlepidotus* has haplotypes common to all four rivers and those shared were comparatively derived. Considering that there is no clear geological evidence of secondary contact between river basins after their formation, and the area was not affected by the last glaciations (Clapperton 1994), this difference in haplotype composition might be explained by the different ecological features of the species. These different ecological attributes and habitat requirements determine how and when fish may have moved among basins during lower sea levels (Thacker et al. 2007; Burridge et al. 2008) or taken advantage of coastal routes (see discussion below).

Catfish of the genus *Trichomycterus* are related to strictly freshwater species of tropical and template environments (Arratia et al. 1983, 1990). This genus is found only in the Americas, from Costa Rica (Arratia 1990) to Patagonia (de Pinna and Wosiacki 2003), with a great diversity in the Amazon basin (Arratia 1997). Given that there are a number of species of *Trichomycterus* both on the Trans and Cis sides of the Andes, even in the absence of paleontological evidence it is probable that catfish were present in the Abanico basin during the Miocene, losing contact due to the tectonic inversion and rise of

the Andes Mountains. We do not yet have a concrete explanation for the similarity of haplotypes found in Limarí and Choapa rivers, but as suggested by Bayesian phylogenetic reconstruction, it is possible that rivers were isolated sequentially from south to north.

The South American silversides endemic to Argentina, Peru and Chile (Dyer 1998) have a phylogenetic history related to both freshwater and marine environments. Species of the genus *Odontesthes* (which belongs to the tribe Sorgentinini as does *Basilichthys*) are marine, estuarine and freshwater (Dyer 1998, 2000a), and phylogenetic evidence indicates multiple transitions from ocean to freshwater and *vice versa* (Bamber and Henderson 1988; Beheregaray et al. 2002; Mank and Avise 2006). The presence of fossils of the genus *Basilichthys* in the Cura-Mallín formation (Rubilar 1994) deposited in fluvial and lacustrine environments during the existence of the Abanico basin shows that a) South American silversides were primitively freshwater inhabitants (Dyer 1998), and b) they were present in the Abanico basin and could disperse in the area, to be then separated during the rise of the Andes and subsequent division of drainage systems.

Given the geological and phylogeographic evidence, it is plausible that *B. microlepidotus* had a secondary contact among river basins via the coast. Urzúa et al. (1977) stated that individuals of this species were found living in marine areas and laboratory experiments indicate that individuals of *Basilichthys sp.* could survive for short periods of time –although not able to reproduce– in these environments (B. Dyer pres. comm.). There is currently no other evidence that demonstrates the presence of this species in marine ecosystems, but an interesting possibility is that during El Niño years more precipitations generate elevated water discharges in central Chile (Aceituno 1988) and decreased salinity (Sievers and Vega 2000) allowing putative coastal routes for migration without the need of a lowered sea level.

Conservation concerns

It is known that freshwater fauna are among the most threatened taxa on the planet (Ricciardi and Rasmussen 1999; Saunders et al. 2002). The particular vulnerability of freshwater fishes to flow modification, destruction of habitats, invasion of exotic species, pollution and eutrophication reflects the fact that both fish and freshwater are resources that humans need and they have been heavily impacted by human usage and regulation (Lévêque et al. 2008).

In Chile, 64% of the species of Chilean freshwater fishes have been reported to be in danger of extinction and 29% are vulnerable or insufficiently known (Habit et al. 2006; Vila et al. 2006); there are currently few effective protection measures for these organisms. At our knowledge, the present study provides some of the first information about genetic structure of freshwater fish in the north central zone of Chile, which will be very important for protecting these species.

The results of this study provide several important points for future conservation plans. First, if there is environmental contamination which eliminates the biota, it is highly unlikely that they would be naturally re-colonized by fish from nearby rivers in the case of catfish, except for the Limarí and Choapa rivers. These rivers require additional studies to quantify the extent of differentiation and actual migration with other techniques. Our data indicate a very low probability of contact among the basins, and that in the case of *T. areolatus* the populations of the Maipo, Aconcagua and Limarí-Choapa rivers each represent a non-replaceable, elemental conservation unit (*sensu* Wood and Gross 2008). On the other hand, for silversides, all evidence suggests that migrations were or are frequently enough to prevent strong differentiation between rivers. Although we did not test whether there is current movement of organisms between watersheds, due to their genetic similarity it is possible to translocate individuals from one basin to another in case of contamination event. Second, conservation plans must be accommodated to the characteristics of each drainage system. While there was evidence of considerable gene flow within the Limarí, Choapa and Aconcagua rivers, this was not the case in the Maipo River (and for *T. areolatus* in one locality of the Aconcagua River). Protection zones for these sites should be established in such a way that they conserve part of each of the populations detected, as well as the processes which sustain them. Naturally it will be necessary to perform additional studies with direct (capture-recapture, telemetry) and indirect methods (molecular nuclear markers) to determine the number of separate populations present in rivers of north-central Chile.

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