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Phylogenetic and phylogeographic analysis of the genus *Orestias* (Teleostei: Cyprinodontidae) in the southern Chilean Altiplano: the relevance of ancient and recent divergence processes in speciation

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This study presents phylogenetic molecular data of the Chilean species of *Orestias* to propose an allopatric divergence hypothesis and phylogeographic evidence that suggests the relevance of abiotic factors in promoting population divergence in this complex. The results reveal that diversification is still ongoing, *e.g.* in the Ascotán salt pan, where populations of *Orestias ascotanensis* restricted to individual freshwater springs exhibit strong genetic differentiation, reflecting putative independent evolutionary units. Diversification of *Orestias* in the southern Altiplano may be linked to historical vicariant events and contemporary variation in water level; these processes may have affected the populations from the Plio-Pleistocene until the present.

Key words: *agassii* complex; allopatry; paleolakes; speciation.

INTRODUCTION

The Andes are the world's longest continuous mountain range, and were formed through the subduction of the Nazca plate beneath the South American plate (Allmendinger *et al.,* 1997). During uplift, freshwater systems in the region underwent marked landscape modification, resulting in associated shifts in biodiversity following geological and climatological events that have resulted in both the extinction and formation of new species.

The Andes range is divided into the East and the West Mountains between latitudes 16° and 22° S. This results in a high-altitude (3600–4500 m a.s.l.) central plane known as the Altiplano. Freshwater systems located in this isolated area underwent marked geological and climatological fluctuations (Fornari *et al.,* 2001; Fritz *et al.,* 2004; Rigsby *et al.,* 2005). This complex geological scenario is thought to have

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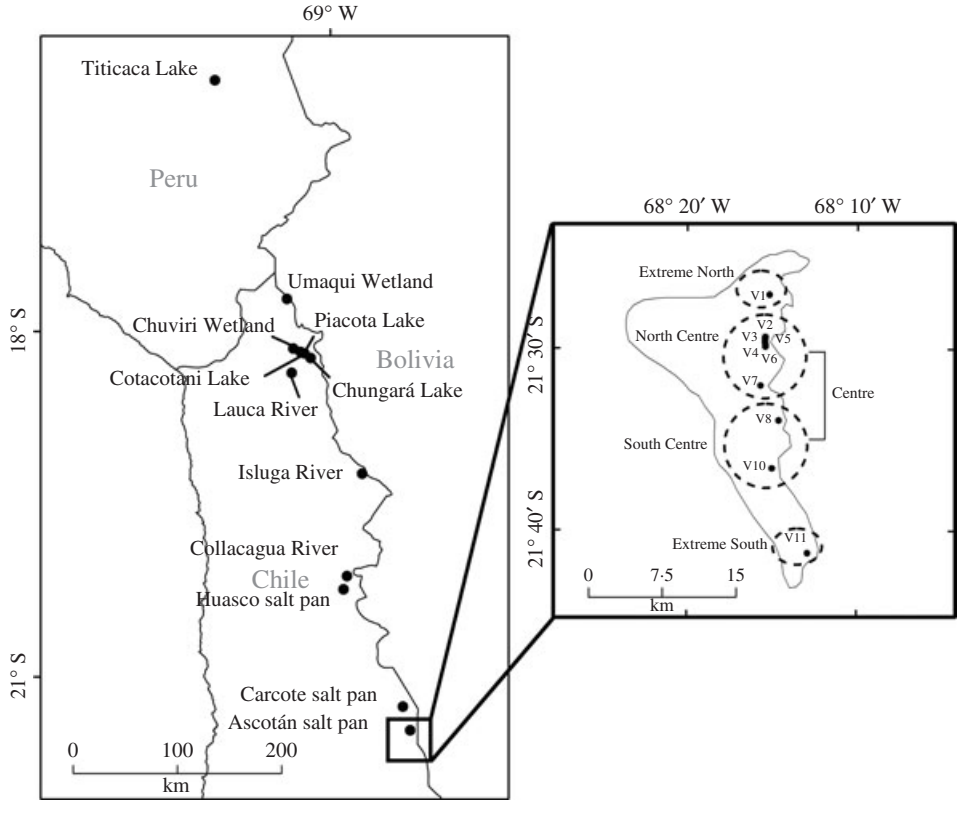

FIG. 1. Location of sites sampled for *Orestias* across the southern Altiplano in Chile. The box shows the detailed locations of the 10 springs in the Ascotán salt pan sampled for *Orestias ascotanensis*. The distribution of the four genetic groups detected through spatial analysis of molecular variance analysis is indicated (◌).

played a key role in promoting speciation in the region. Among the scenarios mentioned, allopatric speciation has been a mechanism frequently invoked (Guarnizo *et al.,* 2009).

In the southern Altiplano (between 17° and 22° S; Fig. 1), geological changes have resulted in the presence of large numbers of endorrheic salt pans, lakes and springs, where contemporary interconnections between freshwater ecosystems are entirely subterranean (Vila, 1975; Niemeyer & Cereceda, 1984, Chong, 1988). This area is now characterized by a negative hydrological balance, which coupled with increases in ambient temperatures, has led to elevated salinities in all aquatic systems (*i.e.* salt pans, lakes and springs), although these often have different physicochemical characteristics (Márquez-García *et al.,* 2009).

As both historical and contemporary process may have influenced the evolution of isolated aquatic populations in the southern Altiplano, at least since the most significant Andes elevation at the end of the Miocene (Wörner *et al.,* 2002), this zone provides a natural laboratory to evaluate different speciation hypotheses and the process of clade diversification both at historical and contemporary scales.

The ichthyofauna of the Altiplano is characterized by an endemic group of cyprinodontid killifish belonging to the genus *Orestias.* The species-rich Cyprinodontidae have probably populated this high altitude plateau since the Miocene. According to Parker & Kornfield (1995), the ancestor of the cyprinodonts colonized South America from the western part of the Tethys Ocean as a result of two dispersion events facilitated by tectonic plate movements. The first occurred *c.* 150 MYA during the opening of the northern part of the Tethys Ocean, giving rise to the Atlantic Ocean. Once this ocean was completely formed in the early Cretaceous, ancestors of the cyprinodonts remained along the coasts of North America and the Caribbean. A second phase would have occurred 80–100 MYA when cyprinodonts colonized new coastal areas. During this period, sea level reached its Cretaceous maximum, inundating a large part of northern South America up to the eastern side of the Andes range, as well as extensive areas of Africa and North America. Orogenic activity 15 MYA (Vandervoort *et al.,* 1995) produced changes in the existing rivers and watersheds, eventually isolating them. These changes would have affected the dispersal of an ancestral cyprinodont to the south of Lake Titicaca, generating the present distribution of the genus *Orestias.*

The genus *Orestias* is an endemic group inhabiting freshwater systems in the inter-Andean basin of Peru, Bolivia and Chile. They are distributed from 11° to 22° S, at altitudes from 2800 m a.s.l. (in the springs feeding the Ascotán salt pan, Chile) to 4600 m a.s.l. (Lauzanne, 1982; Parenti, 1984*a*; Vila *et al.,* 2010). This genus is composed of 45 species: 23 have been described as endemic to the Titicaca basin (Lauzanne, 1982; Parenti, 1984*a, b*), while the majority of the other species inhabit the southern Altiplano.

The current distribution of the different species of *Orestias* is thought to reflect the limits of water level fluctuations during the Plio-Pleistocene, which restricted their dispersion to the Altiplano region (Vila *et al.,* 2010). The historical changes in the size of these Plio-Pleistocene lakes following climatic and geological events produced a scenario in which populations of freshwater taxa (*e.g.* fishes inhabiting the once large lacustrine systems) became fragmented and divided into multiple populations. Such fragmentation would have generated a population mosaic with different degrees of divergence, and finally distinct species. It is likely that species inhabiting those systems that separated initially are more divergent (*e.g.* Lake Titicaca) than those from more recently formed systems, *e.g.* systems in the southern Altiplano such as Lakes Chungará and Cotacotani, and the Ascotán and Carcote salt pans. Lüssen *et al.* (2003), in a molecular study of species from the southern Altiplano, hypothesized that their divergence was associated with Pleistocene climatological events (*i.e.* the extension and recession of water bodies). Lüssen *et al.* (2003) did not recognize the systematics of *Orestias* proposed by Parenti (1984*a*) within the *agassii* complex. An unresolved nomenclatural problem of this species is whether this complex should be nominated as *agassii* or as *agassizii* (Eschmeyer, 2012). This study follows the recommendation of Parenti (1984*a*), who established that the correct spelling of this species is *O. agassii* and hence, the complex should be designated as *agassii.*

Here, phylogenetic and phylogeographic analyses are conducted for those species of the *agassii* complex that inhabit the southern Chilean Altiplano. The specific aims of this study are to (1) evaluate the systematics of this group using three mitochondrial markers: control region, cytochrome *b* (*cyt b*) and nicotinamide adenine

denucleotide dehydrogenase subunit 2 (*nd2*) and (2) assess the role of habitat fragmentation *via* water level fluctuations at the microevolutionary level using the control region. Population divergence was analysed from (1) a phylogenetic perspective using 12 localities of the Chilean southern Altiplano (17°–21° S) and (2) a detailed phylogeographic analysis using *Orestias ascotanensis* Parenti 1984 as a model. This is the southernmost taxon of *Orestias* in the Chilean Altiplano and inhabits several springs across the Ascotán salt pan, where it has historically experienced considerable water level fluctuations.

MATERIALS AND METHODS

STUDY SITES

Specimens of *Orestias* were collected from 12 different sites located across the Chilean Altiplano (17°–21° S; Fig. 1 and Table I). A group of sites are located between latitudes 17° and 18° S: Lake Chungará, Parinacota Wetland, Cotacotani Lagoons, Lauca River, Lake Piacota, Umaqui Wetland and the Chuviri Wetland. Another group, located between 19° and 20° S, includes populations from the Isluga River, Collacagua River and the Huasco salt pan, while the Ascotán and Carcote salt pans are located at 21° S (Fig. 1). Specimens were captured by electrofishing, and a fin clip was preserved in 95% ethanol. Information on the sample size examined per location is provided in Table I. Individuals were identified to species using morphological descriptions from Parenti (1984*a*), Arratia (1982), Vila & Pinto (1986), Vila (2006) and Vila *et al.* (2010, 2011).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Methods used for DNA extraction, PCRs and sequencing are given in Table II. Fragments of the mitochondrial control region were amplified using the primers described by Morales *et al.* (2011). For mitochondrial coding, primers L14724 and H15915 were used for *cyt b* (Xiao *et al.,* 2001) and ND2B-L and ND2E-H (Broughton & Gold, 2000) for *nd2*. Primer sequences are given in Table III (details in Table SI, Supporting information). PCR products were purified and sequenced by Macrogen Inc. (www.macrogen.com). Sequences were edited and aligned by eye using the programme BioEdit 7.0.1 (Hall, 1999) or Proseq software (Filatov, 2002). The sequences were deposited in GenBank (Tables SII and SIII, Supporting information).

PHYLOGENETIC ANALYSES OF *ORESTIAS* FROM THE SOUTHERN CHILEAN ALTIPLANO

Maximum parsimony (MP) and maximum likelihood (ML) phylogenetic analyses were performed using PAUP* 4b10 software (Swofford, 2003). A Bayesian inference (BI) was also performed using the MrBayes v3.01 software (Huelsenbeck & Ronquist, 2001; Huelsenbeck *et al.,* 2002). MP analysis was performed with a heuristic search algorithm using TBR (tree bisection and reconnection) with the addition of random sequences. The statistical support of nodes was evaluated using bootstrapping (1000 pseudoreplicates) (Felsenstein, 1985). The appropriate substitution model for the ML and BI analyses was determined using the AIC implemented in Modeltest v 3.06 (Posada & Crandall, 1998). A summary of the models selected by each partition is shown in Table IV. For the ML analysis, tree topology was evaluated with bootstrapping (500 pseudoreplicates, Table V). For the BI, the phylogenetic reconstruction was performed using the same parameters as ML: 5×10^6 generations and one tree search for each 1000 generations. The first 500 trees were discarded as burn-in, resulting in a total of 4500 trees. Posterior probabilities were obtained with a consensus tree from the trees retained (Table V). This analysis was made twice. *Orestias puni* (Tchernavin 1944) from Lake Titicaca was used as an out-group for all analyses performed. This study adheres to the concept of a species as a lineage, proposed by Wiley (1981).

TABLE I. Analysed species, minimum and maximum standard length (L_S) of the analysed specimens, sample sites and number of specimens sequenced

Species	L_S range (mm)	Site	Geographical co-ordinates	Number of specimens
Orestias sp.	41·59–22·42	Umaqui wetland	17° 44′ 12·6″ S; 69° 23′ 26·2″ W	4
Orestias sp.	44·74–84·35	Chuviri wetland	18° 10′ 09·0″ S; 69° 20′ 05·0″ W	4
Orestias piacotensis	37·40–71·72	Piacota Lake	18° 12′ 01·5″ S; 69° 16′ 02·1″ W	5
Orestias parinacotensis	29·18–56·41	Parinacota wetland	18° 12′ 02·0″ S; 69° 16′ 04·0″ W	4
Orestias laucaensis	32·30–56·22	Cotacotani Lake	18° 12′ 38·9″ S; 69° 14′ 02·1″ W	4
Orestias chungarensis	39·53–82·10	Chungará Lake	18° 15′ 01·8″ S; 69° 10′ 34·6″ W	5
O. laucaensis	41·79–90·02	Lauca River	18° 22′ 50·4″ S; 69° 20′ 56·0″ W	4
Orestias agassii	41·48–69·23	Isluga River	19° 15′ 22·9″ S; 68° 42′ 22·7″ W	4
O. agassii	28·24–23·13	Collacagua River	20° 08′ 47·6″ S; 68° 50′ 36·8″ W	4
O. agassii	36·70–53·79	Huasco salt pan	20° 15′ 46·0″ S; 68° 52′ 31·6″ W	5
Orestias gloriae	21·90–58·92	Carcote salt pan	21° 16′ 46·3″ S; 68° 19′ 20·8″ W	5
Orestias ascotanensis	58·65–75·00	Ascotán salt pan	21° 29′ 07·7″ S; 68° 15′ 21·5″ W	4
Orestias puni	84·75–125·28	Titicaca Lake	15° 50′ 02·6″ S; 70° 01′ 56·5″ W	4

TABLE II. DNA extraction methods used for the phylogenetic and phylogeographic analyses and the annealing temperatures used to amplify each gene. The phylogeographic analysis was applied only for the control region

Analysis type	DNA extraction	Annealing temperatures		
		Control region (° C)	Cytochrome *b* (° C)	*nd2* (° C)
Phylogenetic	Phenol/chloroform method (Hillis *et al.,* 1990)	63	51	50
Phylogeographic	Salt-extraction method (Aljanabi & Martinez, 1997)	63	–	–

PHYLOGEOGRAPHIC ANALYSIS IN ASCOTÁN SALT PAN

A total of 270 individuals of *O. ascotanensis* were sampled from 10 springs in the Ascotán salt pan (Fig. 1 and Table VI). The former names of these springs are composed of a 'V' (for 'vertiente', which means spring in Spanish) and a cardinal number (*e.g.* V1 and V2). PCR conditions and reactions to amplify the mtDNA control region are described above. Haplotypes were obtained using the DnaSP v 5 programme (Librado & Rozas, 2009). The number of polymorphic sites (S), haplotype number (K), haplotype diversity (H) and pair-wise differences (π) were calculated using ARLEQUIN v 3.1 (Excoffier *et al.,* 2005). To examine the effect of sample size on the number of haplotypes found, a rarefaction analysis was performed with the statistical package PAST (Hammer *et al.,* 2001). A minimum spanning tree (MST) was computed with ARLEQUIN v 3.1 (Excoffier *et al.,* 2005) based on the pair-wise differences between haplotypes. An unrooted tree was generated using HapStar (Teacher & Griffiths, 2011), and all alternative connections were considered in this analysis. A spatial analysis of molecular variance (SAMOVA, Dupanloup *et al.,* 2002) was performed to determine the spatial structure of populations of *O. ascotanensis.* SAMOVA defines groups of populations that are geographically homogeneous and maximally differentiated from each other. Significance tests of the fixation index Φ_{CT} were evaluated through a permutation test (1000 permutations) implemented in the same software.

Pair-wise Φ_{ST} were calculated with the software ARLEQUIN v 3.1 (Excoffier *et al.,* 2005) to compare the haplotype frequencies among the springs of Ascotán salt pan and among the genetic groups detected with SAMOVA. A Mantel test (Mantel, 1967) was performed to examine relationships between genetic (Φ_{ST}) and geographical distance. The significance of the Mantel correlation was estimated using 100 000 permutations (Smouse *et al.,* 1986). To detect past population expansion, Fu's F_s and Tajima's D (neutrality test) were calculated for these same genetic groups; their significance was estimated with 1000 replicates.

TABLE III. Primers utilized in this study

Gene	Fragment length (bp)	Primer name	Sequence 5′–3′	Reference
Control region	972	F-Control	ACC CCT AAC TCC CAA AGC T	Morales *et al.* (2011)
		R-Control	TGA TAG TAA AGT CAG GAC CAA	
Cytochrome *b*	1137	L14724	GAC TTG AAA AAC CAC CGT TG	Xiao *et al.* (2001)
		H15915	CTC CGA TCT CCG GAT TAC AAG AC	
nd2	1041	ND2B-L	TTC TAC TTA AAG CTT TGA AGG C	Broughton & Gold (2000)
		ND2E-H	TTC TAC TTA AAG CTT TGA AGG C	

TABLE IV. Substitution models which adjusted best to the data of each partition

	Nucleotide substitution models		
Partition	Model	α	π
Control region	TRN + I	0·6795	A = 0·31400, C = 0·15750, G = 0·29960, T = 0·22890
Cytochrome *b*	GTR + I	0·8356	A = 0·31830, C = 0·16190, G = 0·28220, T = 0·23760
nd2	GTR + I + G	0·6304	A = 0·30360, C = 0·15300, G = 0·31950, T = 0·22390
Total evidence	GTR + I + G	0·9037	A = 0·30280, C = 0·18470, G = 0·25670, T = 0·25580

RESULTS

PHYLOGENETIC ANALYSES OF *ORESTIAS* FROM THE SOUTHERN CHILEAN ALTIPLANO

All phylogenetic analyses performed here reveal the presence of four principal lineages (Fig. 2). One lineage (clade A) includes *O. agassii* from the Isluga River that appears as a sister group to the other lineages. This clade is consistent in all gene partitions and phylogenetic methods used, showing high node support values except for the *nd2* gene (Table V). The second lineage (clade B) includes the species inhabiting sites found in the Lauca National Park: *Orestias chungarensis* Vila & Pinto 1987, *Orestias laucaensis* Arratia 1982, *Orestias parinacotensis* Arratia 1982, *Orestias piacotensis* Vila 2006 and *Orestias* sp. from Chuviri. Although species are not clearly delineated with the phylogenetic analysis, clade B is recovered in all partitions and shows high node support except for the control region partition, in which MP and ML show values lower than 70 % (Table V).

Another lineage (clade C) is formed exclusively by *Orestias gloriae* Vila, Scott, Mendez, Valenzuela, Iturra & Poulin 2012 from the Carcote salt pan. All partitions and phylogenetic methods highlight the monophyletic origin of this clade, showing high values of nodal support (Table V). The position of this clade in the tree, however, varies depending on which partition is analysed: in the control region partition, clade C is the sister group of clade B, while in the *cyt b* partition it belongs to clade D, and in the *nd2* partition it is located as an independent group. Additionally, the

TABLE V. Bootstrap support for the nodes of each clade (Fig. 2) in each partition. The values shown are parsimony/maximum likelihood/Bayesian posterior probability, respectively. Values that are <70% for MP and ML or <0·95 for BI are indicated (*)

	Phylogenetic analysis (MP/ML/BI)			
Partition	Clade A Isluga	Clade B Lauca	Clade C Carcote	Clade D Ascotán/Huasco/Collacagua
Control region	92/78/1	*/*/1	98/98/1	79/*/1
Cytochrome *b*	73/*/0·97	93/93/1	96/95/1	73/81/0·99
nd2	*/*/*	91/91/1	96/93/1	86/78/0·88
Total evidence	95/93/1	99/99/1	100/100/1	99/98/1

MP, maximum parsimony; ML, maximum likelihood; BI, Bayesian inference.

TABLE VI. Summary of genetic diversity indices by site for *Orestias ascotanensis* based on the mtDNA control region

Spring	*n*	*S*	*K*	Rarefaction	*H*	π
V1	35	9	9	6·78 ± 1·05	0·78	1·33
V2	29	21	15	12·35 ± 1·09	0·94	5·51
V3	24	17	9	8·14 ± 0·76	0·80	4·52
V4	26	20	11	9·69 ± 0·88	0·86	5·06
V5	21	15	8	7·81 ± 0·39	0·85	4·93
V6	21	19	15	14·38 ± 0·49	0·94	5·04
V7	34	26	16	11·57 ± 1·31	0·92	5·61
V8	30	19	14	10·46 ± 1·23	0·85	2·89
V10	30	13	10	9·02 ± 1·03	0·84	2·99
V11	20	7	8	8·00 ± 0·00	0·88	2·18
Total	270	53	71		0·95	5·83

n, sample size; *S,* polymorphic sites; *K,* number of haplotypes; rarefaction, including mean ± S.D. of the number of haplotypes; *H,* haplotype diversity; π, average number of nucleotide differences.

total evidence partition identifies this clade as a sister group of clade D. Finally, clade D contains *O. agassii* from the Collacagua River and the Huasco salt pan, *O. ascotanensis* from the Ascotán salt pan and *Orestias* sp. from the Umaqui wetland. In all partitions, phylogenetic analysis recovers this clade D with moderate and high nodal support (Table V). Within this clade, it is always possible to differentiate among *O. ascotanensis, O. agassii* from Huasco and *O. agassii* from Collacagua. Nodal support for these three lineages, however, is only found in the *nd2* and total evidence partitions. Individuals from Umaqui are separated from the other three lineages in all analyses, but without statistical nodal support.

PHYLOGEOGRAPHIC ANALYSIS OF THE ASCOTÁN SALT PAN

Within the Ascotán salt pan, diversity is lowest for the sample from spring V1 (6·78 haplotypes after rarefaction); fish from springs V2, V6 and V7 display the highest diversity, with haplotypic diversities near 1 (Table VI).

The MST analysis showed a large number of haplotypes, with many shared across several springs (Fig. 3). This analysis also identified a group of haplotypes that were largely characterized by individuals from spring V1 (coloured in dark grey in Fig. 3) and another haplogroup composed exclusively of individuals from spring V11 (coloured in light grey in Fig. 3).

The SAMOVA (Dupanloup *et al.,* 2002) indicated that variation among groups was maximized (44%) when the sample was divided into four groups ($\Phi_{CT} = 0{\cdot}441$, $P < 0{\cdot}001$; Fig. 1): the springs of the northern (V1) and southern (V11) extremes of the salt pan; and the springs located in the centre of the salt pan forming two groups, one included springs V2–V7, while the other group combined springs V8 and V10.

The pair-wise Φ_{ST} tests showed significant differences in haplotype frequencies in all comparisons performed, both among the springs of the salt pan and between the genetic groups detected by SAMOVA (Table VII). This includes springs V8 and V10, although SAMOVA assigned them in the same genetic group; however, their Φ_{ST} value was low (0·119).

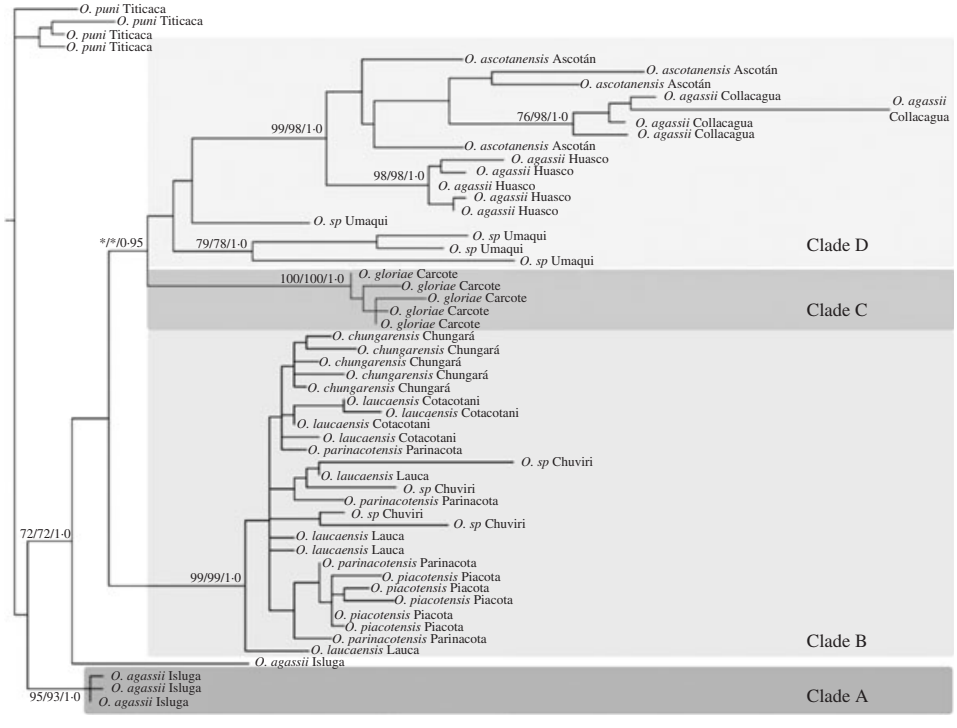

FIG. 2. Maximum likelihood (ML) phylogenetic tree of three mtDNA genes with the total evidence matrix (*nd2*; control region; cytochrome *b*). Bootstrap support values in the nodes are parsimony, ML and Bayesian posterior probability, respectively. Values that are not shown or are shown with an * were <70%.

The results of the neutrality tests (Tajima's D and Fu's F_s) are shown in Table VIII, complete with corresponding P-values. All values of Tajima's D are negative except for V11; only the value of V8–V10 was significant. All Fu's F_s values were negative, and only V11 was not significant.

The Mantel test was significant ($r = 0{\cdot}6427$, $P < 0{\cdot}001$) using the data from all springs. The test was not significant, however, among springs for comparisons within or between different genetic groups (both $P > 0{\cdot}05$). In summary, fish collected from the springs located in the northern (V1) and southern (V11) extremes of the salt pan were well differentiated genetically, while fish from the remaining two groups, (1) V2–V7 and (2) V8 and V10, were undifferentiated, but displayed a greater genetic diversity than the more differentiated groups inhabiting springs in the north and south of the Ascotán salt pan.

DISCUSSION

It has been proposed that the presence of *Orestias* in the freshwater systems of the southern Altiplano reflects their distribution during an earlier period, when paleolakes dominated the landscape (Keller & Soto, 1998), and that the species present

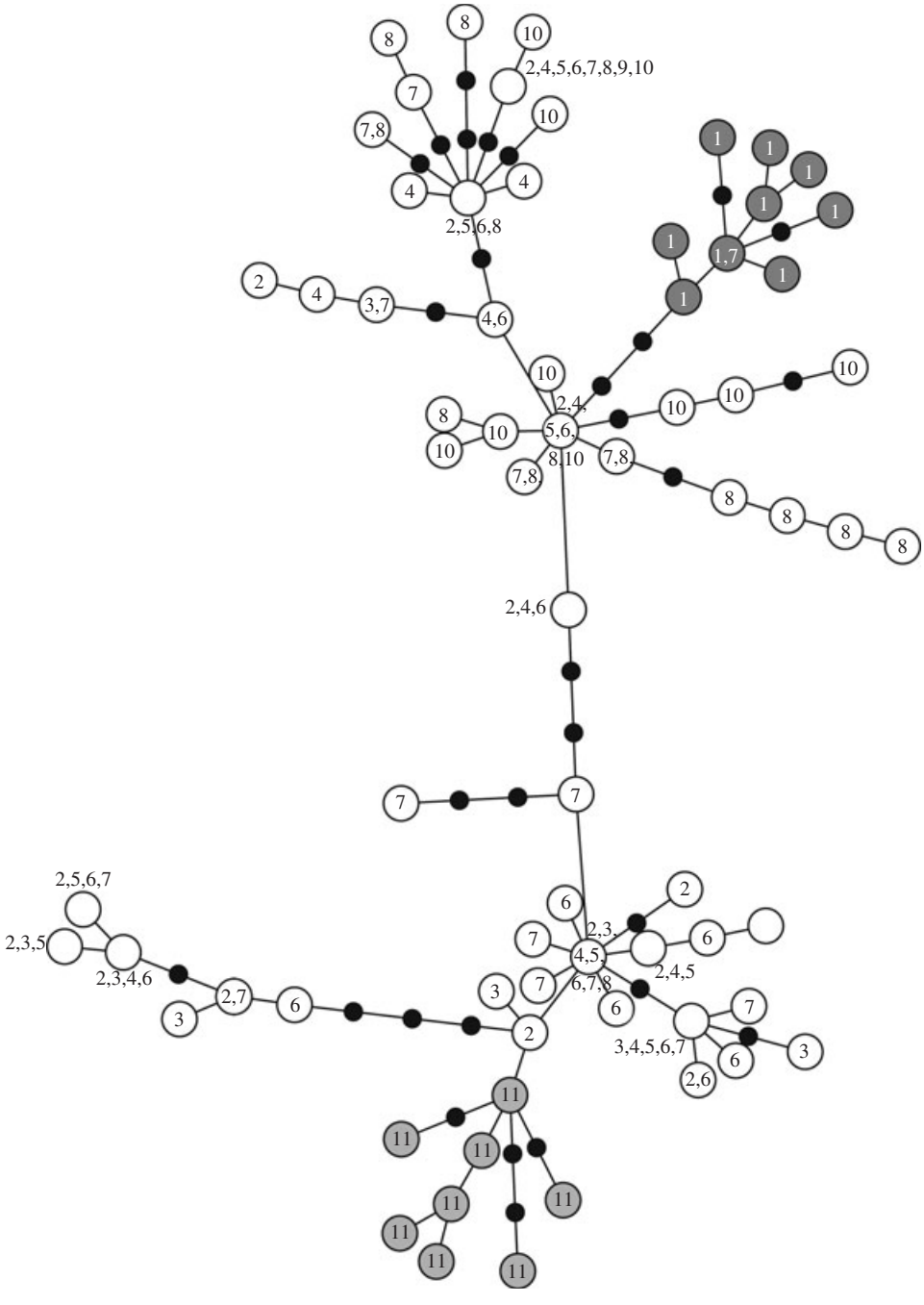

FIG. 3. Minimum spanning tree of haplotypes of *Orestias ascotanensis* obtained from mitochondrial control region analysis. ○, represent each haplotype and the numbers on them indicate the number of the spring where the haplotypes were found. ●, represent intermediate haplotypes. The group of haplotypes found in V1 is shown in dark grey (this also includes one individual from V7). The haplotypes from V11 are indicated in light grey. ◌, the haplotypes from the centre salt pan springs (V2–V10).

TABLE VII. Pair-wise Φ_{ST} among springs of the Ascotán salt pan (above the main diagonal) and among the genetic groups detected by spatial analysis of molecular variance (below the main diagonal) (Extreme North: only V1; North Centre: V2–V7; South Centre: V8 and V10; Extreme South: only V11). Significant values are shown in bold

		Extreme North	North Centre						South Centre		Extreme South
		V1	V2	V3	V4	V5	V6	V7	V8	V10	V11
V1	Extreme North		**0·473**	**0·581**	**0·501**	**0·526**	**0·545**	**0·449**	**0·707**	**0·662**	**0·764**
V2	North Centre	**0·400**		0·046	−0·001	−0·007	0·006	−0·004	**0·393**	**0·355**	**0·413**
V3					**0·089**	0·006	−0·004	0·037	**0·569**	**0·531**	**0·396**
V4						−0·002	0·006	−0·016	**0·341**	**0·316**	**0·457**
V5							−0·042	−0·028	**0·458**	**0·421**	**0·442**
V6								−0·021	**0·476**	**0·442**	**0·417**
V7									**0·367**	**0·347**	**0·405**
V8	South Centre	**0·642**				**0·368**				**0·119**	**0·774**
V10											**0·750**
V11	Extreme South	**0·764**				**0·364**				**0·744**	

TABLE VIII. Neutrality tests, Tajima's D and Fu's F_s for the genetic groups detected by spatial analysis of molecular variance. The P-value of each test is shown in parentheses; significant values are shown in bold

Genetic group	Tajima's D	Fu's F_s
V1	−1·170 (0·105)	**−3·537 (0·015)**
V11	0·352 (0·686)	−1·891 (0·139)
V2–V7	−0·582 (0·325)	**−16·990 (0·001)**
V8 and V10	**−1·993 (0·009)**	**−13·468 (0·001)**

in the region today originated through a process of divergence *via* fragmentation of one or more ancestral populations (Northcote, 2000; Vila *et al.,* 2010). In this model, *Orestias* diversified through allopatric speciation driven by habitat instability and heterogeneous environmental conditions (Northcote, 2000). The phylogenetic analyses presented here indicate that different clades are spatially separated, suggesting a pattern consistent with a differentiation process mediated by vicariance. Clade A includes specimens of *O. agassii* from Isluga: this species is the most widely distributed species of *Orestias,* which has been described from Lake Titicaca to the Collacagua River (Parenti, 1984*a*). Clade D includes the southernmost species, including *O. agassii* from the Collacagua River and Huasco salt pan, and *O. ascotanensis* from the Ascotán salt pan. This clade also includes *Orestias* sp. from the Umaqui wetland (a recently discovered site for *Orestias*).

The current data suggest that *O. agassii* is a polyphyletic group. The three populations constituting this species belong to three different lineages distributed in two different clades: *O. agassii* from the Isluga River and in another clade, *O. agassii* from the Huasco salt pan and Collacagua River. These results suggest the existence of three independent lineages (species) that are currently grouped within *O. agassii.* This evidence is supported by chromosomal information, which shows differences in the distribution and location of constitutive heterochromatin and the nucleolar organizer region among individuals of each lineage (C. Araya, pers. comm.).

The species in clade B inhabit systems located within the Lauca National Park. *Orestias chungarensis, O. laucaensis, O. parinacotensis, O. piacotensis* and *Orestias* sp. from Chuviri are all grouped together, in a clearly differentiated clade. Species from this clade also show a close relationship with *O. gloriae* from the Carcote salt pan (clade C) and lineages of clade D. This result is consistent with geological evidence that suggests that both the Ascotán salt pan and the Paleo-Lauca River were originally part of the Tauca paleolake (Placzek *et al.,* 2006).

The clade composition highlighted in these phylogenetic analyses suggests that speciation in southern Altiplano species of *Orestias* has followed an allopatric mode. Lüssen *et al.* (2003), using a mitochondrial marker (control region), found that the species of *Orestias* examined across the distribution of the genus formed two groups, one including sites from Lake Titicaca, and the other, localities from the southern Altiplano. The tree presented for the *Orestias* examined here clearly identifies the species from the Lauca National Park as a monophyletic group. Within the clade, however, species described were not clearly identified by the phylogenetic analysis. The low phylogenetic resolution observed in this clade contrasts with

the evidence reported by Vila *et al.* (2010) who, using morphological and karyological analyses, suggested that these species show clear differences. This suggests that in this clade at least, the mitochondrial markers used are not suitable to adequately reflect this speciation process, probably reflecting a recent divergence of these species. This idea is concordant with Scott (2010), who proposed that speciation in this clade started *c.* 300 000 years before present. Geological evidence supports a recent origin of the Lauca system: Lake Chungará was formed following the collapse of the cone of the Parinacota volcano, which subsequently blocked the passage of the Paleo-Lauca River causing the isolation of this sub-watershed (Wörner *et al.,* 2002). Hydrological reconstructions for this sub-watershed reveal fluctuations in water levels through the Pleistocene–Holocene, and the water levels of Lake Chungará were greatest in the late Holocene (Sáez *et al.,* 2007). This evidence, together with the tree topology reported here, suggests that the divergence of the species in the clade associated with the Lauca National Park could be recent and still in process.

Consequently, by extending the current results from the Ascotán salt pan to the situation in water bodies located over the wider area of the southern Altiplano including the Lauca National Park, it is possible to identify another divergence mechanism to explain population diversification and speciation of *Orestias* in the region. It is likely that successive and marked climatic changes through the Pleistocene period led to extreme variation in the water level in the area, which in turn isolated, and subsequently reconnected distant basins. During wet periods, connectivity permitted genetic mixing, while dry periods resulted in isolation and genetic differentiation. The dry periods were longer (Placzek *et al.,* 2006), resulting in an accumulation of genetic differences, probably including adaptation to different local environments. This would have led to divergence, and where this led to genetic or ecological differences, genetic separation would be maintained, even with subsequent recontact following raised water levels.

Presently, freshwater taxa are recognized as among the most threatened (Ricciardi & Rasmussen, 1999; Saunders *et al.,* 2002); as such, their conservation is of the utmost importance. In the Altiplano, such measures are largely driven by the need to conserve and maintain water levels in the systems supporting *Orestias* and the taxa that co-occur with them. This is not a trivial matter in an area with a negative water balance, where water itself represents a limiting resource for aquatic conservation management. *Orestias* is an extremely diverse taxon: initial work has described an amazing diversity of species, and it is certain that many species remain undescribed. This endemic genus requires realistic and robust conservation plans that account for the fact that many species are endemic to individual sites, thus representing different and important evolutionarily significant units (Ryder, 1986). The more southerly populations of *Orestias* inhabit geographically extreme springs in the Ascotán salt pan. These populations are worthy of elevated conservation concern not only due to their genetic divergence and unique diversity but also because they are most at risk of desiccation due to future climate change and water extraction to support mineral extraction in the region (Bradley *et al.,* 2006; Vila *et al.,* 2007). Thus, the conservation of *Orestias* represents a significant challenge, as populations show both large and fine scale genetic differentiation. This not only raises the potential for site-specific adaptation, but also limits the capacity for future translocation of threatened endemic *Orestias* populations to new habitats.

In conclusion, diversification of *Orestias* in the southern Altiplano could be linked with historical (vicariant) and contemporary variation in water levels. Three main historical events were detected: (1) the ancient northern-southern division, (2) diversification within Lauca National Park and (3) recent population isolation within the Ascotán salt pan. The relevance of this process to speciation of fishes in the region should be evaluated through analyses of other co-distributed species such as snails of genus *Biomphalaria,* among others. Recently, Collado *et al.* (2011) found that populations of *Biomphalaria* from the Chilean Altiplano form a monophyletic group. Additionally, they detected a similar pattern of an ancient divergence among northern and southern populations and that populations from the Lauca National Park represented a monophyletic group. Interestingly, some *Biomphalaria* populations of the Ascotán and Carcote salt pans, geographically close, appear in different clades in the phylogenetic tree, as observed here for *Orestias.*

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Supporting Information

Supporting Information may be found in the online version of this paper:

TABLE SI. Components and cycling reaction for the PCR of the three mitochondrial genes, control region, *cyt b* and *nd2.* Concentration of each PCR component is indicated. The cycle number, time (min) and temperature (° C) are indicated for each step of the cycling reaction. All cycling reactions involved an initial denaturation at 94° C for 3 min for *cyt b* and *nd2* and for 2 min for the control region, and a final extension step at 72° C for 10 min. The final volume of the reaction was 30 μl and contains 50–200 ng of DNA. *Taq* DNA polymerase, PCR buffer, $MgCl_2$ and deoxynucleotide triphosphates were from Invitrogen

TABLE SII. GenBank accession numbers of the sequences used in the phylogenetic analysis of Chilean *Orestias*

TABLE SIII. GenBank accession numbers of the haplotypes used in the phylogeographic analysis of *Orestias ascotanensis*

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