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Geographical isolation and genetic differentiation: the case of *Orestias ascotanensis* (Teleostei: Cyprinodontidae), an Andean killifish inhabiting a highland salt pan

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Orestias ascotanensis is a killifish endemic to the Ascotán salt pan in the Chilean Altiplano, where it inhabits 12 springs with different degrees of isolation. This species is a suitable model for studying the effect of serial geographical isolations on the differentiation process among populations. The present study examines the genetic variation and structure of the species using mitochondrial DNA control region sequences and eight microsatellite loci, analyzing populations across its distribution range. The evaluation of genetic variation revealed high levels of diversity within the species. The genetic structure analysis showed the existence of four differentiated groups: two groups were formed by the springs located in the northern and southern extremes of the salt pan and two groups were found in the centre of the salt pan. The latter two groups were formed by several springs, most likely as a consequence of the South American summer monsoon that could connect them and allow gene flow. The patterns of genetic differentiation appear to be determined based on the physical isolation of the populations. This isolation may be the result of a combination of factors, including geographical distance, a historical decrease in water levels and altitude differences in the springs of the salt pan. Therefore, this system is a rare example in which hydrological factors can explain genetic differentiation on a very small scale. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **117**, 747–759.

ADDITIONAL KEYWORDS: Altiplano – geographical distance – hydrological variation – microsatellites – mtDNA control region – Pleistocene – structure.

INTRODUCTION

Variations in lacustrine levels and geomorphological processes may cause fragmentation of the

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environment and the aquatic biota that inhabit these systems, favouring evolutionary processes (Kornfield & Smith, 2000; Waters *et al.*, 2001; Elmer *et al.*, 2010; Carrea *et al.*, 2012; Vogiatzi *et al.*, 2014). The tectonic activities and water level fluctuations in the African Great Lakes are a classic example of this phenomenon, which may have promoted the diversification in the cichlid species flock (Sturmbauer *et al.*, 2001; Verheyen *et al.*, 2003; Joyce *et al.*, 2005; Nevado *et al.*, 2013).

The Altiplano (South America; 14°–22°S) is a high plateau of approximately 200 000 km^2 between the eastern and western ranges of the central Andes, more than 3800 m a.s.l. It is currently characterized by very dry periods during most of the year. However, this climatic regime varied during the Quaternary, when glaciers (Smith *et al.*, 2005; Smith, Mark & Rodbell, 2008; Licciardi *et al.*, 2009) and extensive lakes were formed (Fornari, Risacher & Féraud, 2001; Fritz *et al.*, 2004; Rigsby *et al.*, 2005; Placzek, Quade & Patchett, 2006). These marked modifications of the landscape appear to have made the biodiversity in the Altiplano unique in the world. Examples of this biodiversity are the endemic lineages of snails of the genus *Biomphalaria* (Collado, Vila & Méndez, 2011; Collado & Méndez, 2013) and *Heleobia* (Collado, Valladares & Méndez, 2013), frogs of the genus *Telmatobius* (De La Riva, García-París & Parra-Olea, 2010; Sáez *et al.*, 2014), and fish of the genus *Orestias* (Parenti, 1984; Esquer-Garrigos, 2013; Esquer-Garrigos *et al.*, 2013; Vila *et al.*, 2013), all of which exhibit high species richness and high genetic diversity in this geographical region. Particularly, the high speciation rate in the genus *Orestias* has been associated with lacustrine level variations, which apparently facilitated the allopatric speciation process, especially in the southern part of the distributional range of this genus (Parker & Kornfield, 1995; Lüssen, Falk & Villwock, 2003). The southern extreme of the Altiplano is home to the species *Orestias ascotanensis* Parenti, 1984, which is endemic to the Ascotán salt pan in northern Chile (Fig. 1A, Table 1). This species is well characterized based on morphological, karyotypic, and genetic characters (Parenti, 1984; Vila *et al.*, 2010, 2013). It inhabits twelve isolated springs located in the eastern margin of the salt pan. These represent a fraction of the total bodies of water of the salt pan, whose total water area is 18 km^2 (Risacher, Alonso & Salazar, 2003). As a result of its very limited distribution range (extent of occurrence < 250 km^2 and area of occupancy < 18 km^2; Risacher *et al.*, 2003), the extreme fragmentation of its populations and the important fluctuations of its habitat, *O. ascotanensis* has been recognized as an endangered species by Vila *et al.* (2007) and by the Lista de Especies Nativas según Estado de Conservación (Ministerio del Medio Ambiente, Chile) (http://www.mma.gob.cl/clasificacionespecies/listado-especies-nativas-segun-estado-2014.htm).

Morales, Vila & Poulin (2011) described the genetic diversity and structure of *O. ascotanensis*, analyzing a fragment of the control region of mitochondrial (mt)DNA. It was shown that the geographically most

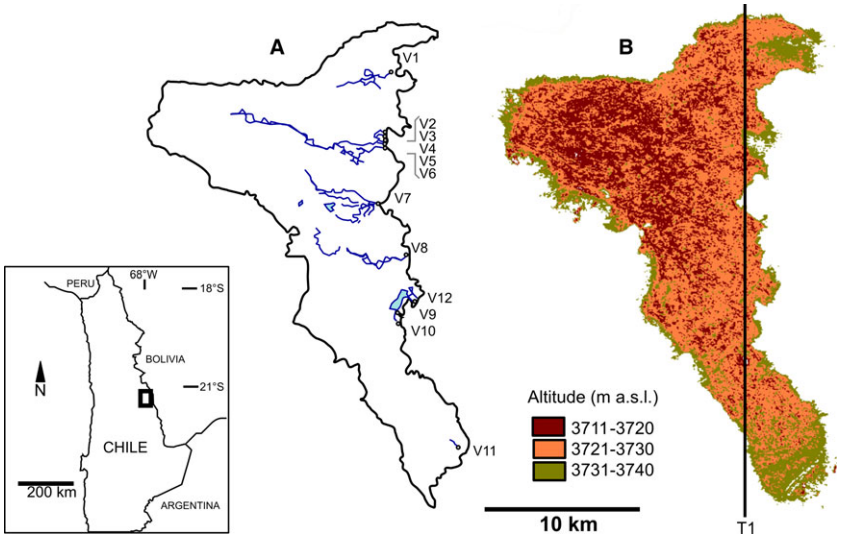

Figure 1. Distribution of *Orestias ascotanensis*. A, map of the Ascotán salt pan. The twelve sampled localities (springs) are indicated. B, digital elevation model of the salt pan. The three altitude ranges in which the zones were classified are indicated (dark brown, low areas; light brown, medium areas; green, high areas). T1: transect chosen to show the altitudinal profile of the salt pan (Fig. 2B).

Table 1. Genetic diversity in *Orestias ascotanensis* based on the analysis of mitochodrial (mt)DNA control region and microsatellites

			mtDNA control region						Microsatellites				
Sampling site	Geographical coordinates	Altitude (m)	*N*	Number of polymorphic sites	Number of haplotypes	Haplotype diversity	Nucleotide diversity	Mean number of pairwise differences	*N*	Number of alleles (range)	H_O	H_E	F_{IS}
V1	21°27′02.0″S, 68°15′09.8″W	3733	35	9	9	0.78	0.0015	1.33 ± 0.85	17	6.13 (2–13)	0.4692	0.5134	0.11873
V2	21°29′21.2″S, 68°15′24.8″W	3732	29	23	19	0.965	0.0067	6.20 ± 3.03	13	6.38 (2–13)	0.6988	0.677	0.01103
V3	21°29′27.8″S, 68°15′25.6″W	3734	24	19	10	0.859	0.0054	4.99 ± 2.51	23	7.38 (3–17)	0.6496	0.6334	0.00082
V4	21°29′39.8″S, 68°15′25.6″W	3730	26	21	14	0.914	0.0058	5.33 ± 2.66	19	6.63 (2–16)	0.5866	0.6434	0.11607*
V5	21°29′47.2″S, 68°15′24.5″W	3729	21	17	10	0.905	0.0059	5.37 ± 2.70	20	7.38 (2–17)	0.6104	0.6566	0.09775
V6	21°29′52.8″S, 68°15′24.4″W	3732	21	21	16	0.967	0.0061	5.60 ± 2.80	19	6.63 (2–12)	0.5640	0.6256	0.14019*
V7	21°32′02.7″S, 68°15′40.7″W	3731	34	27	19	0.946	0.0066	6.10 ± 2.97	12	6.50 (2–15)	0.6989	0.5981	−0.12586
V8	21°33′57.2″S, 68°14′36.8″W	3738	30	21	16	0.878	0.0035	3.22 ± 1.71	11	6.00 (1–16)	0.5477	0.5831	0.10942
V9	21°36′11.7″S, 68°15′01.4″W	3735	14	9	8	0.857	0.0031	2.82 ± 1.58	14	5.38 (2–11)	0.5089	0.552	0.11982
V10	21°36′35.0″S, 68°15′00.7″W	3738	30	15	13	0.906	0.0039	3.61 ± 1.88	29	7.38 (2–15)	0.5535	0.5697	0.0476
V11	21°41′13.9″S, 68°12′54.0″W	3740	20	7	8	0.879	0.0024	2.18 ± 1.26	18	4.75 (1–11)	0.4469	0.4448	0.02501
V12	21°35′13.9″S, 68°14′32.3″W	3735	7	15	5	0.857	0.0075	6.86 ± 3.67	7	4.13 (1–6)	0.5536	0.557	0.0851
Total			291	59	94	0.973	0.0071	6.53 ± 3.10	202		0.5729	0.6567	0.13021

m, metres above sea level; *N*, sample size per location; number of alleles, mean number of alleles across loci (range across loci in parentheses); H_E, expected heterozygosity; H_O, observed heterozygosity.

*F_{IS} values significant for two loci ($P < 0.01$).

isolated springs V1 and V11 ('V' for 'vertiente', which means spring in Spanish) represent evolutionarily significant units (Waples, 1991) that are characterized by local genetic endemism. Morales *et al.* (2011) proposed that these springs may have been interconnected for the last time during the last humid period, 11 kyr BP (Fornari *et al.*, 2001; Fritz *et al.*, 2004; Placzek *et al.*, 2006). This geographical isolation, in addition to processes of genetic drift associated with small populations, would have generated a loss of genetic diversity and fixed unique haplotypes in the springs. By contrast, springs that were close to each other had high genetic diversity, which was shared among them, indicating possible gene flow and metapopulation-type dynamics (Morales *et al.*, 2011). Gene flow could be possible during the austral summer in the Altiplano, when the South American summer monsoons (Zhou & Lau, 1998) occur from December to March. During this rainy period, water levels increase, which, in some years, could generate corridors that allow dispersion between different sectors and gene flow among some of the populations that are otherwise isolated (Morales *et al.*, 2011). This would imply that hydrological mechanisms that are generally considered to explain genetic differentiation patterns at large scales (Kornfield & Smith, 2000; Hodges, Donnellan & Georges, 2014) may also apply on a much smaller scale. However, a limitation of the study by Morales *et al.* (2011) is the coarse estimation of the genetic variation as a result of the use of a single marker: the mtDNA control region. The present study seeks to evaluate the diversity and genetic structure of *O. ascotanensis* across the entire distribution range of the species, using both the mtDNA control region (complementing the study of Morales *et al.*, 2011) and microsatellite loci. The results are discussed in relation to the distribution patterns and the geography of the salt pan.

MATERIAL AND METHODS

Description of the study system and sample collection

The Ascotán salt pan (21°33′S, 68°15′W) is located in an endorheic basin in the southern Altiplano, in the Antofagasta Region of Chile (Fig. 1). This area has little annual precipitation, approximately 150 mm per year (Risacher *et al.*, 2003), mainly from the Amazon basin in a period of intense rainfall during the austral summer known as the South American summer monsoon (Zhou & Lau, 1998). The salt pan extends for approximately 30 km; it has twelve springs on its eastern side that originate from subterranean water and are separated by evaporative surfaces. The populations of *O. ascotanensis* were sampled from each of these springs (Table 1). Individuals were collected between the years 2005 and 2013. The fish were captured using scoop nets. They were euthanized with an overdose of tricaine methylsulphonate (MS-222), in accordance with the suggestions of the American Veterinary Medical Association (AVMA, 2013). Fin clips were then preserved in 96% ethanol for genetic analyses.

DNA extraction and amplification of the molecular markers

High molecular weight genomic DNA was obtained using the salt extraction method, modified from Aljanabi & Martinez (1997). The mtDNA control region data set comprised 291 sequenced individuals (Table 1), which included the 273 sequences (Genbank accession numbers: JN543271–JN543361) obtained in Morales *et al.* (2011) in addition to 14 individuals from spring V9 and four from spring V12 sequenced in the present study, representing four new haplotypes (Genbank accession numbers: KR605135–KR605138). The 18 new sequences were obtained using the primers described in Morales *et al.* (2011). The polymerase chain reaction (PCR) was performed in a total volume of 25 μL containing 20 ng of DNA, 0.2 mM of each dNTP (Invitrogen), 4 mM $MgCl_2$ (Invitrogen), 1 × PCR buffer (20 mM Tris–Cl, pH 8.4; 50 mM KCl; Invitrogen), 0.8 μM of each primer, and 5 U of Taq (Invitrogen). The conditions used for the thermal cycling comprised an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 68.5 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension of 10 min at 72 °C. PCR products were purified and sequenced in both directions by Macrogen Inc. (South Korea).

We amplified nine microsatellite loci using the primers described by Esquer-Garrigos *et al.* (2011) (see Supporting information, Table S1). The amplification reactions were performed in a total volume of 10 μL containing 50 ng of DNA, 0.25 mM of each dNTP (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 1 × PCR buffer (20 mM Tris–Cl, pH 8.4; 50 mM KCl; Invitrogen), 0.2 μM of each primer, and 0.5 U of Taq (Invitrogen). The conditions for the thermal cycling were the same as those used by Esquer-Garrigos *et al.* (2011). The size of the amplified alleles was determined using microcapillary electrophoresis in an automatic DNA sequencer (ABI Prism 377; Applied Biosystems) by Macrogen Inc. The analysis of fragments was performed using the GENEMARKER, version 1.85 (SoftGenetics).

Genetic diversity and structure analysis

mtDNA control region

The mtDNA control region sequences obtained were checked, edited, and assembled with PROSEQ (Filatov, 2009). The number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity, and pairwise differences were calculated using ARLEQUIN, version 3.5 (Excoffier & Lischer, 2010). The pairwise F_{ST} (Wright, 1949) values were calculated using the differences between pairs of sequences to examine the differentiation between pairs of springs. The significance of each pairwise value was obtained using 10 000 permutations of the haplotypes between sites. These F_{ST} comparisons were corrected using a Bonferroni-type procedure for identifying truly significant results (Benjamini & Hochberg, 1995) with spreadsheet-based software provided by Pike (2011). A median-joining haplotype network was constructed using NETWORK, version 4.1 (Bandelt, Forster & Röhl, 1999). The global genetic structure was analyzed using SAMOVA (Dupanloup, Schneider & Excoffier, 2002) based on the available genetic and geographical data. SAMOVA defines groups of populations that are geographically homogeneous and maximally differentiated from each other. The data set was partitioned into K groups ($K = 2, 3, \ldots, 12$), maximizing the proportion of total genetic variance as a result of differences between groups of populations (F_{CT}) and obtaining the configuration of K groups exhibiting the largest F_{CT} value. The significance ($\alpha = 0.01$) of this index was evaluated with a permutation test (10 000 permutations).

Microsatellites

Before the analysis, the excess of homozygotes or heterozygotes was reviewed to correct alleles with large peaks and the presence of stutters that generate reading errors, as well as to detect the presence of null alleles for each locus using MICRO-CHECKER, version 2.2.3 (Van Oosterhout *et al.*, 2004). The number of individuals with missing data is indicated in the Supporting information (Table S1).

The genetic diversity is expressed as the mean number of alleles and allele diversity in the population, measured as the number of alleles across loci. The observed heterozygosity (H_O) was obtained from genotype counts, and deviations from Hardy–Weinberg equilibrium (F_{IS}) were evaluated using the expected heterozygosity (H_E).

The degree of genetic structure between pairs of populations was analyzed by calculating F_{ST} (Weir & Cockerham, 1984) using GENETIX, version 4.05 (Belkhir *et al.* 2004). The significance of each comparison was obtained using 10 000 permutations of the allele frequencies between sites. These multiple comparisons were corrected using a Bonferroni-type procedure for identifying truly significant results (Benjamini & Hochberg, 1995) with spreadsheet-based software provided by Pike (2011).

The number of genetic groups (K) was evaluated based on the genotypes of individuals using a Bayesian analysis implemented in STRUCTURE, version 2.3.4 (Pritchard, Stephens & Donnelly, 2000). We analyzed from one to 12 groups, with 20 Markov chain Monte Carlo (MCMC) runs of 1 000 000 iterations (with the first 100 000 discarded as burn-in); these parameters were calculated for each value of K under the no admixture model, with correlated allele frequencies among groups (clusters) and the locprior setting, which has been suggested for species with discrete population distributions (Pritchard *et al.*, 2000). The most probable number of populations K was determined using the mean logarithm of the likelihood of the observed data, ln$P$ (Pritchard *et al.*, 2000) (see Supporting information, Fig. S1, Table S2).

Migration rates among springs

BAYESASS, version 1.3 (Wilson & Rannala, 2003) was used to estimate the magnitude and direction of contemporary gene flow among (1) spring populations grouped following the results of the genetic structure analysis and (2) populations in each spring. These analyses used the microsatellite data. For each population, MCMC chains were run once for 3 000 000 generations (900 000 burn in) with a sampling frequency of 2000.

Isolation-by-distance

XLSTAT, version 4.05 (Addinsoft, 2009) was used to perform Mantel tests aiming to evaluate the existence of isolation-by-distance in populations of *O. ascotanensis*. For this, we determined the significance of correlations between matrices of pairwise distances between populations, with 10 000 randomizations. We used the following distance matrices in these analyses: as genetic distances among populations (1) F_{ST} (Weir & Cockerham, 1984) and $F_{ST}/(1 - F_{ST})$ (Rousset, 1997) were calculated based on microsatellite analysis; (2) F_{ST} (Wright, 1949) and $F_{ST}/(1 - F_{ST})$ were calculated using mtDNA control region sequence analysis; and as physical distances, we used (3) the geographical distances between springs and (4) Euclidean distances of the altitude difference in metres between springs. With these matrices, we estimated the correlations shown in Results (Table 4).

Digital elevation model of the salt pan

We determined the altitude patterns in the salt pan using a digital elevation model. A digital satellite image (ASTER GDEM satellite, Global Digital Elevation Model; http://gdex.cr.usgs.gov/gdex/) was analyzed, which provides data on surface altitudes. Using geographic information system (GIS) tools, the information was re-classified in altitude ranges using IDRISI KILIMANJARO (Eastman, 2003). The altitude ranges were classified by zones in accordance with information obtained in the field, identifying low zones as those with altitudes from 3711 to 3720 m a.s.l., medium zones as altitudes from 3721 to 3730 m a.s.l., and high zones as altitudes from 3731 to 3740 m a.s.l. (Fig. 1B). An altitudinal profile of the salt pan was obtained by performing a longitudinal cut at 68°16′W (Figs 1B, 2B). We selected this longitude because it is close to all springs, and also allowed the characterization of the total extension of the salt pan. The altitude was graphed every kilometer from the northern limit. We added a contour line (3740 m a.s.l.) to this profile that defines the border or limit of the salt pan.

RESULTS

mtDNA control region

The mtDNA haplotype sequences of samples from springs V9 and V12 were added to the data set reported by Morales *et al.* (2011), for a total of 291 partial sequences of 919 bp of the mtDNA control region for further analysis. The indices of genetic diversity are shown in Table 1. The pattern observed by Morales *et al.* (2011) was confirmed, with high genetic diversity in *O. ascotanensis*; considering the complete dataset, 94 haplotypes were found, with a haplotype diversity of 0.97. Each of the springs also showed high genetic diversity; the haplotype diversity ranged from 0.78 to 0.97 and the mean number of pairwise differences ranged from 1.33 ± 0.85 to 6.86 ± 3.67. Springs V1 and V11 showed the lowest values (1.33 ± 0.85 and 2.18 ± 1.26, respectively), whereas springs V2 and V12 showed the highest values (6.20 ± 3.03 and 6.86 ± 3.67, respectively). The haplotype network (Fig. 2A) reflected this high diversity in an extended network that showed diversified *O. ascotanensis*

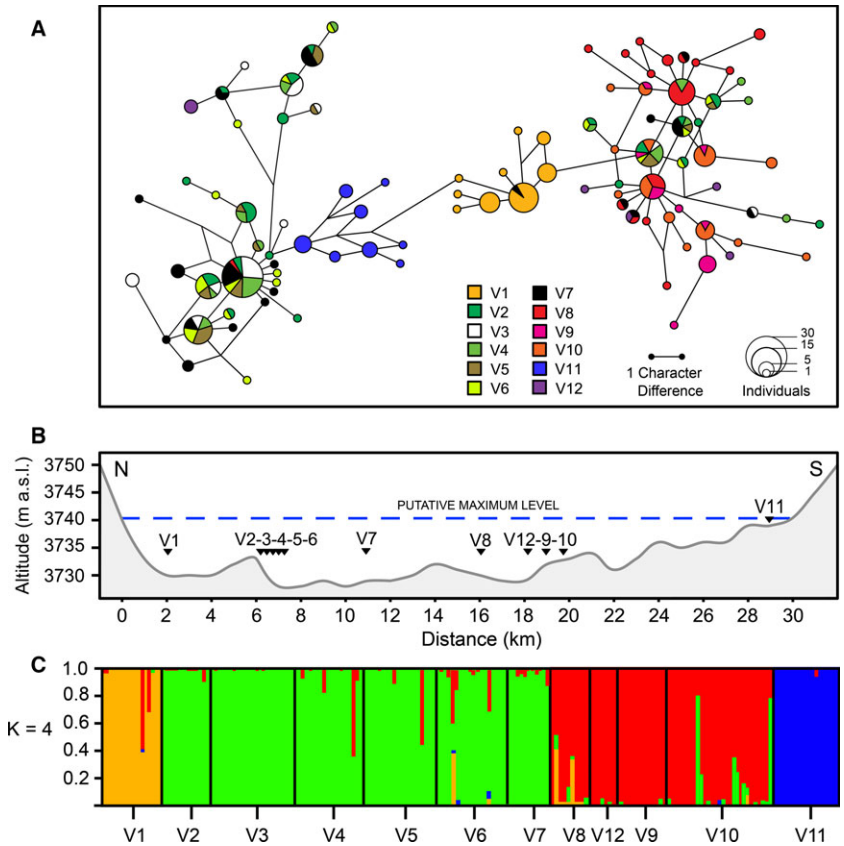

Figure 2. A, median-joining network inferred from the mtDNA control region sequences. B, north–south altitude profile of the Ascotán salt pan. The positions of the springs are indicated. The dashed line indicates the probable maximum water level. C, STRUCTURE plot for $K = 4$, inferred from microsatellites.

populations, although many haplotypes were shared between several springs. The number of pairwise differences ranged between one and 25 (mean 6.53 ± 3.10 mutational steps) (Table 1). However, all the haplotypes found in V1 and V11 were specific to these springs (except for one haplotype that was shared between V1 and V7), forming two clear haplogroups. The haplotypes in these haplogroups were closely related (there are four mutational steps maximum between haplotypes from V1, and six mutational steps maximum between haplotypes from V11). It is worth noting that spring V12 showed a different pattern of genetic diversity than the rest of the springs. The haplotypes from this spring are specific to it (except one that is shared with V7 and V8), although they do not form a haplogroup as V1 and V11 do; most of them are closely related to the more abundant haplotypes of V8, V9, and V10 (Fig. 2A). This spring showed a large number of polymorphic sites (15), which was in the observed range (7–27), even though its sample size was much smaller (Table 1).

The pairwise comparisons between populations (Table 2) showed a similar pattern to that reported by Morales *et al.* (2011); most were significantly different, although there was a group of springs (V2–V7) that were not differentiated (except for the comparison between V3 and V4, which showed a significant but low pairwise F_{ST} = 0.085).

SAMOVA analysis indicated five groups as the best grouping (40.17% of the variation among groups). Three of these groups were composed of individuals from only one spring: V1, V11, and V12. The fourth group was composed of individuals from V2 to V7 and springs V8, V9, and V10 formed the last group. The second best grouping was $K = 4$ groups (38.65% variance among groups), in which V12 was included in the V8–V10 group.

Summarizing, the results of the pairwise F_{ST} analysis, median-joining network, and SAMOVA showed that springs V1 and V11 each formed a distinct group. The pairwise F_{ST} analysis could not differentiate between springs V2 to V7; the network showed that they shared haplotypes and SAMOVA indicated that these six springs formed a genetic group. The discordances between the analyses are a result of the south-centre springs, V8, V9, V10, and V12: the pairwise F_{ST} analysis differentiated all of them, although the SAMOVA analysis grouped springs V8, V9, and V10 together and V12 was still differentiated, even though four out of five haplotypes are closely related to the south-centre group (springs V8, V9, and V10).

Microsatellite analysis

Genotypes were obtained for 202 individuals of the 12 localities (Table 1) using eight loci of the nine analyzed microsatellites (locus B104 showed null alleles in seven of the 12 analyzed localities and was excluded from the analyses). All individuals included in the analyses amplified at least four loci, and 127 out of 202 individuals (62.9%) amplified all analyzed loci (see Supporting information, Table S1).

Genetic diversity varied among localities, showing the same pattern as the mtDNA control region; those of the centre of the salt pan (V2–V10) showed high diversity (H_O ranged between 0.51 and 0.70; H_E ranged between 0.55 and 0.68), higher than those springs from the northern and southern extremes, V1 (H_O: 0.47 and H_E: 0.51) and V11 (H_O: 0.45 and H_E: 0.44). F_{IS} values showed no significant deviation from zero in tests with 10 000 randomizations

Table 2. Estimates of pairwise population genetic differentiation

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
V1	–	**0.12**	**0.147**	**0.145**	**0.139**	**0.109**	**0.127**	**0.035**	**0.13**	**0.091**	**0.199**	**0.134**
V2	**0.457**	–	0.004	0.004	−0.010	−0.003	**0.031**	**0.053**	**0.11**	**0.074**	**0.128**	**0.103**
V3	**0.567**	0.047	–	0.005	−0.01	0	**0.039**	**0.06**	**0.123**	**0.066**	**0.129**	**0.117**
V4	**0.486**	−0.001	**0.085**	–	0.004	−0.001	**0.036**	**0.05**	**0.121**	**0.067**	**0.147**	**0.106**
V5	**0.51**	−0.005	0.005	−0.004	–	−0.001	**0.038**	**0.049**	**0.118**	**0.063**	**0.113**	**0.11**
V6	**0.528**	0.005	−0.006	0.005	−0.041	–	**0.023**	0.02	**0.111**	**0.031**	**0.089**	**0.097**
V7	**0.44**	0.007	0.033	0.001	−0.017	−0.018	–	**0.069**	**0.127**	**0.095**	**0.147**	**0.120**
V8	**0.672**	**0.355**	**0.525**	**0.31**	**0.414**	**0.428**	**0.33**	–	**0.066**	0.007	**0.107**	**0.078**
V9	**0.739**	**0.345**	**0.506**	**0.358**	**0.423**	**0.423**	**0.333**	**0.336**	–	**0.069**	**0.236**	0.007
V10	**0.665**	**0.347**	**0.513**	**0.335**	**0.419**	**0.426**	**0.334**	**0.168**	**0.102**	–	**0.11**	**0.081**
V11	**0.764**	**0.396**	**0.384**	**0.441**	**0.426**	**0.4**	**0.394**	**0.741**	**0.772**	**0.739**	–	**0.226**
V12	**0.64**	**0.127**	**0.27**	**0.192**	**0.209**	**0.222**	**0.158**	**0.398**	**0.323**	**0.319**	**0.632**	–

Pairwise F_{ST} values from mtDNA control region analysis are below the diagonal and pairwise F_{ST} values from microsatellite analysis are above the diagonal. Significant corrected *P*-values are shown in bold.

($P < 0.01$), except in two loci from springs V4 and V6 (Table 1).

The pairwise F_{ST} values among springs were not significant for any of the comparisons between springs V2, V3, V4, V5, and V6 or between pairs V6–V8, V8–V10, and V9–V12 (Table 2). Three springs, V1, V7, and V11, were significantly different from all of the others.

STRUCTURE analysis found high log-likelihood values [ln$P$ (D)] between $K = 4$ and 7 (see Supporting information, Fig. S1, Table S3). With $K = 4$, two independent groups were obtained formed by springs V1 and V11; a third group was formed by springs V2, V3, V4, V5, V6, and V7 and the fourth group was formed by springs V8, V9, V10, and V12 (Fig. 2C). The grouping obtained with STRUCTURE ($K = 4$) was identical to the grouping obtained with SAMOVA with $K = 4$ (mtDNA control region). For this reason, we refer to the group formed by V1 as G1; the group formed by V2, V3, V4, V5, V6, and V7 as G2; the group formed by V8, V9, V10, and V12 as G3; and the group formed by V11 as G4.

Current migration rates

Table 3 and the Supporting information (Table S4) show the migration rates between the genetic groups estimated in the previous analyses and between springs, respectively. In both, most of the migration rates were < 0.05. This finding suggests low gene flow among the genetic groups and among springs and also that most individuals had a local origin (rates > 0.9).

Isolation-by-distance

The Mantel tests (Table 4) showed a high correlation between the genetic distances estimated with microsatellites and those estimated with the mtDNA control region (F_{ST} $r = 0.712$, $P < 0.01$), indicating a similar tendency in the two markers. This result is congruent with the similar pattern of differentiation found in the pairwise F_{ST} analysis for the mtDNA control region and microsatellites (Table 2); of the 66 total pairwise comparisons, 48 were significant for the mtDNA control region, whereas 51 were significant for microsatellites.

The correlations of the genetic and geographical distances using the altitude differences of the springs as covariates were high and significant (F_{ST} mtDNA control region, $r = 0.584$, $P < 0.01$; F_{ST} microsatellites, $r = 0.578$, $P < 0.01$). By contrast, there was no significant relationship using geographical distance as covariate. The correlations performed with $F_{ST}/(1 - F_{ST})$ provided systematically lower correlations and partial correlations between genetic distance and the other distances (Table 4).

Table 3. Migration rates estimated for each genetic group based on the microsatellite dataset

From/ to	G1	G2	G3	G4
G1	**0.9095**	0.00451482	0.0054046	0.00654569
G2	0.0165486	**0.962471**	0.022322	0.0061734
G3	0.0634574	0.0291139	**0.964708**	0.00725005
G4	0.0104944	0.00390003	0.00756548	**0.980031**

The columns correspond to the locality of origin of the individuals and the rows are the locality of destination. The auto-recruitment rate (local origin) is shown in bold.

Digital elevation model

The elevation model of the Ascotán salt pan (Fig. 1B) clearly indicates the portion of the salt pan with greater altitude (i.e. above 3730 m a.s.l.). Spring V11 was located in this zone. Altitude decreased toward the north of the salt pan; however, the northern extreme where spring V1 is located was above 3730 m a.s.l. The lowest zone of the salt pan is the northwest and currently contains several bodies of water but is without *O. ascotanensis* populations (Fig. 1A). Finally, we observed that (Fig. 2B) the central springs are located at similar and lower altitudes but are separated by areas of higher elevation, which appear to be geographical barriers to dispersion. Springs V2 and V3 in the northern centre and V10 in the southern centre are at higher altitudes than their neighbours (V4–V7 and V8–V9, respectively), althogh with a difference of no more than 2 m.

DISCUSSION

Genetic diversity and structure in *O. ascotanensis*

The analyses of genetic structure coincided in determining four genetic groups in the *O. ascotanensis* species, both with microsatellites and with the mtDNA control region, confirming the pattern obtained by Morales *et al.* (2011). Although there were a few variations in the best grouping, they were exclusively a result of the mitochondrial genetic diversity of the south-centre springs; these four springs share haplotypes, although to a lesser extent than V2–V7. For this same reason, V12 is a differentiated group in some analyses. Additionally, the mitochondrial marker did not indicate differences between the individuals of springs V2–V7, whereas the F_{ST} pairwise analysis with microsatellites

Table 4. Mantel tests to compare genetic distances and physical distances (geographical distance and altitude difference)

Matrix 1	Matrix 2	Covariable	*R*	*P*-value
Molecular marker comparison				
F_{ST} microsatellites	F_{ST} mtDNA CR		0.712	< 0.01
$F_{ST}/(1 - F_{ST})$ microsatellites	$F_{ST}/(1 - F_{ST})$ mtDNA CR		0.638	< 0.01
Partial Mantel tests				
F_{ST} mtDNA CR	Geographical distance	Altitude difference	0.584	< 0.01
$F_{ST}/(1 - F_{ST})$ mtDNA CR	Geographical distance	Altitude difference	0.522	< 0.01
F_{ST} microsatellites	Geographical distance	Altitude difference	0.578	< 0.01
$F_{ST}/(1 - F_{ST})$ microsatellites	Geographical distance	Altitude difference	0.553	< 0.01
F_{ST} mtDNA CR	Altitude difference	Geographical distance	0.078	NS
$F_{ST}/(1 - F_{ST})$ mtDNA CR	Altitude difference	Geographical distance	0.122	NS
F_{ST} microsatellites	Altitude difference	Geographical distance	0.091	NS
$F_{ST}/(1 - F_{ST})$ microsatellites	Altitude difference	Geographical distance	0.071	NS

mtDNA CR, mitochondrial DNA control region; *r*, correlation coefficient; NS, not significant.

showed that the individuals of spring V7 are different in terms of everything else (Table 2). This result suggests that the mtDNA control region reflects an ancient connection between spring V7 and the more northern springs, which would have allowed gene flow, whereas the microsatellite variation suggests that this spring has been isolated recently.

The contemporary migration rates suggested low gene flow among groups and that most of the individuals were of local origin (Table 3). The individuals of G4 (spring V11) showed very low migration rates, and almost all individuals had a local source (> 98%). This pattern could be a result of the geographical location of this spring, separated by 9.9 km from the nearest spring (V10) and located at the highest altitude in the salt pan (3740 m a.s.l.). The altitude and geographical distance could also affect the migration rate among springs within genetic groups: in group G2, spring V3 provided a significant number of migrant individuals to the rest of the springs of that group, and, in group G3, a similar trend was observed, with spring V10 acting as a source of migrants (see Supporting information, Table S4). This pattern could have been facilitated by the closeness between springs of the same genetic group and the higher altitude at which these source springs are located (Table 1 and Fig. 2B). The geographical distance could be the most significant factor explaining this migration pattern because the large extensions of evaporative surfaces between springs represent geographical barriers that would accentuate the isolation-by-distance pattern.

The environmental changes that occurred in this area during the Pleistocene may have influenced the genetic structure of *O. ascotanensis*. In this period, the basin of the Ascotán salt pan probably contained a lake, possibly during the humid periods between 17 and 11 kyr BP (Bills *et al.*, 1994; Keller & Soto, 1998; Placzek *et al.*, 2006; Blard *et al.*, 2011). During this time, one large population would have existed, allowing the generation of the high genetic diversity that is currently observed. In the Holocene (8.5 kyr BP), a hyper-arid period began, which produced the desiccation of the large Altiplanic lakes (Latorre *et al.*, 2002). The springs slowly became isolated. Based on the microsatellite results and the altitude of the springs, we may infer the way in which the isolation of the different populations was generated. The first spring to become disconnected may have been V11 because it is at a greater altitude. Then, V1 would have become isolated and, finally, those of the north-centre (V2–V7) and south-centre (V8–V10) would have separated. The springs within these groups would have remained connected sporadically, most likely as a consequence of the South American summer monsoon (Zhou & Lau, 1998). The temporal connection of these springs would facilitate dispersion of individuals between them, allowing gene flow. Thus, both groups would behave as metapopulations (Morales *et al.*, 2011) composed of different local populations in the different springs and connected by corridors that are formed temporarily (Levins, 1969; Hanski & Gilpin, 1991; Hanski, 1998). This temporal connection between springs would have been repeated at least over the last 700 years, a time period that has shown fluctuations in the precipitation in this region with dry and wet periods of different durations (Morales *et al.*, 2012). Such dynamics would allow high diversity to be maintained in these central groups of the salt pan and decrease the effect of genetic drift. Therefore, altitude could have been a key factor that

facilitated spring isolation in the past when the water level dropped. However, currently, the geographical distance would be a key factor involved in the temporal connections or disconnections of the springs because the extensive evaporitic areas between springs and areas of higher elevation are the barriers that cannot be surpassed.

Similar genetic diversity and structure results have been reported in other species of the same family Cyprinodontidae; for example, currently, populations of the genus *Cyprinodon* inhabit isolated springs in an elevation gradient in the Ash Meadows Wildlife Refuge (Mojave Desert), although they were part of a single, larger population in the past. In this system, fragmentation began with decreasing water level over the last 20 000 years; hence, elevation played an important role in the ancient fragmentation process (Duvernell & Turner, 1999; Martin & Wilcox, 2004), just as in *O. ascotanensis*. Another study that analyzed the effect of geographical isolation on the genetic diversity and structure of populations considered the species *Alcolapia grahami* (Kavembe, Machado-Schiaffino & Meyer, 2014). The populations of this species are patchy, fragmented and small, and are present in several isolated alkaline lagoons in Lake Magadi in Kenya. As in the present study, the populations of *A. grahami* contain high genetic diversity, representing remnants of an ancient, much larger population that existed during the persistence of the Pleistocenic paleolake Orolonga (which split between 13 000 and 7000 years BP). Although these cases represent good examples in which hydrological mechanisms can explain genetic differentiation, *O. ascotanensis* is an excellent example of a species that underwent similar processes but at a very small geographical scale because it is possible to find differentiated and even divergent genetic groups that are only 4–9 km apart, in a total extension of < 30 km (Figs 1, 2B).

CONCLUSIONS

In the present study, we provide evidence of the existence of high genetic structure in the species *O. ascotanensis*, with four well-differentiated populations. These patterns would be highly associated with a fragmentation process of this species and the salt pan aquatic system that began after the Last Maximum Glacial and that continues in the present, which has been modulated by geography (altitude and distance between springs) and hydrographical history. This microscale model may reflect the process that occurred during the late Pleistocene in the Altiplano and could be useful for understanding the patterns of differentiation in the genus *Orestias* and other aquatic taxa

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Plot of the mean logarithm of the likelihood of the observed data estimated *sensu* Pritchard *et al.* (2000) and calculated in STRUCTURE HARVESTER (Earl & vonHoldt, 2012).
Table S1. Number of individuals with missing loci included in the analysis.
Table S2. Microsatellite loci used in the present study.
Table S3. Indices to estimate the true *K*, *sensu* Evanno, Regnaut & Goudet (2005) and calculated in STRUCTURE HARVESTER (Earl & vonHoldt, 2012).
Table S4. Migration rates estimated for each spring, based on the microsatellite dataset.