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ORIGINAL ARTICLE

Multiple periglacial refugia in the Patagonian steppe and post-glacial colonization of the Andes: the phylogeography of *Calceolaria polyrhiza*

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ABSTRACT

Aim We perform a phylogeographical study of an endemic Patagonian herbaceous plant to assess whether geographical patterns of genetic variation correspond to *in situ* Pleistocene survival or to glacial retreat and post-glacial expansion. We also seek to determine the locations of potential glacial refugia and post-glacial colonization routes.

Location Southern Andes and Patagonian steppe.

Methods We used *Calceolaria polyrhiza*, a widely distributed Patagonian herbaceous plant that occurs mainly in the understorey of *Nothofagus* rain forests and in the arid Patagonian steppe, as our model system. The chloroplast intergenic spacer *trn*H–*psb*A was sequenced for 590 individuals from 68 populations. Sequence data were analysed using phylogenetic (maximum parsimony, maximum likelihood and Bayesian inference) and population genetic (spatial analyses of molecular variance, mismatch distributions and neutrality tests) methods. Nested clade phylogeographic analyses, and divergence time estimates using a calibrated molecular clock, were also conducted.

Results A total of 27 haplotypes identified in the present study clustered into four primary genealogical lineages, revealing three significant latitudinal phylogeographical breaks. The two high Andean lineages probably split first, during the late Miocene, and the Patagonian lineage split around 4 Ma, coincident with the establishment of the Patagonian steppe. Within each haplogroup, major diversification occurred in the Pleistocene. The Patagonian groups show a pattern consistent with a rapid post-glacial expansion and colonization of the Andean flanks, achieved independently by four lineages. The highest haplotype diversity was found along a longitudinal transect that is remarkably congruent with the limit of the ice-sheet extension during the Greatest Patagonian Glaciation. A north-east expansion is evident, which is probably associated with the 'Arid Diagonal' fluctuations.

Main conclusions Glacial climate fluctuations had a substantial impact on the diversification, distribution and demography of the study species. A scenario of multiple periglacial Pleistocene refugia and subsequent multiple recolonization routes, from eastern Patagonia to the Andean flanks, may explain the phylogeographical patterns observed. However, current genetic structure also preserves the imprints of older events that probably occurred in the Miocene and Pliocene, providing evidence that multiple processes, operating at different spatial and temporal scales, have moulded biodiversity in Patagonia.

Keywords

Arid Diagonal zone, demographic expansion, herbaceous plant, Patagonia, Pleistocene glaciations, secondary contact zone, South America, volcanism.

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INTRODUCTION

Environmental changes during the Quaternary, particularly during the last glacial period, substantially impacted species distribution patterns worldwide (e.g. Hewitt, 2000, 2004). Such impacts are relatively well studied in Europe and North America (e.g. Hewitt, 1996; Taberlet *et al.*, 1998; Alsos *et al.*, 2005; Mraz *et al.*, 2007), but are far less well documented for southern South America (reviewed in Beheregaray, 2008). However, interest in understanding the historical processes impacting on Patagonian organisms is growing (e.g. marsupials, Himes *et al.*, 2008; rodents, Kim *et al.*, 1998; lizards, Morando *et al.*, 2004, 2007; Ávila *et al.*, 2006; fishes, Cussac *et al.*, 2004; Ruzzante *et al.*, 2006; crabs, Xu *et al.*, 2009; gymnosperms, Premoli *et al.*, 2000; Pastorino & Gallo, 2002; Acosta & Premoli, 2010; angiosperms, Muellner *et al.*, 2005; Jakob *et al.*, 2009). With a few exceptions (i.e. Kim *et al.*, 1998; Morando *et al.*, 2004, 2007; Ávila *et al.*, 2006; Jakob *et al.*, 2009), Patagonian phylogeographical studies are restricted to the Andes, and thus address primarily the impact of glaciations in the southern Andes without considering the extensive, arid plains east of the Andean slopes (the Patagonian steppe region). This is particularly true for botanical studies, in which only one publication includes steppe species (Jakob *et al.*, 2009), with others dealing with trees or herbs restricted to the Andes (e.g. Muellner *et al.*, 2005; Marchelli & Gallo, 2006; Acosta & Premoli, 2010).

To further our understanding of the evolutionary history of Patagonian organisms and assess the impact of Pleistocene glaciations on vegetation communities that span the southern Andes and the Patagonian steppe, we selected a widely distributed herbaceous plant that occurs both in the understorey of *Nothofagus* rain forests (i.e. Andean region) and in the arid Patagonian steppe, with enclaves in the high Andean grassland and Monte environments. This distribution includes both glaciated and non-glaciated areas, enabling us to perform phylogeographical and spatial genetic analyses to assess whether genetic patterns correspond to *in situ* Pleistocene survival or to glacial retreat and post-glacial expansion. With respect to the latter scenario, we also assess the locations of potential glacial refugia and recolonization routes. If populations persisted *in situ* during the glacial period, then genetic diversity and unique haplotypes within populations should be high, and comparable to that of populations located beyond the limits of ice sheets. Furthermore, demographic analyses should be consistent with historical population stasis. In contrast, under the second scenario, populations inhabiting previously glaciated areas should share the most common haplotype variants present in source populations following recurrent founder events, and should exhibit low to no haplotype diversity and fewer exclusive haplotypes compared with populations in non-glaciated areas. A strong signal of post-glacial population expansion accompanying the ice-sheet retreat should also be evident.

Geographical context and historical background of Patagonia

Patagonia includes two main ecoregions. The first, the Patagonian steppe, is a large (673,000 km^2), dry, extra-Andean plain covered by grassland and scrubby vegetation that extends from the eastern slopes of the southern Andes to the Atlantic coast. The second is an Andean–Patagonian forest, which is much smaller (248,100 km^2) and covered by woodlands that extend from 35° to 55° S on the eastern and western slopes of the Andes, and contacts the western edge of the Patagonian steppe to the east. To the north, the Andean–Patagonian forest grades into the high Andean grasslands and deserts of western Argentina. Also to the north, the Patagonian steppe is covered by thorn thickets and grades into the Monte phytogeographical province.

The evolutionary history of Patagonia has been shaped by climatic and geological events over the past several million years, such as orogeny, volcanism, cyclical glaciations and concurrent sea-level fluctuations along several basins of the Atlantic coast (Rabassa, 2008; Ramos & Ghiglione, 2008). Pleistocene glaciations (1.8 Ma–10 ka) greatly altered the landscape of this region, both by geographical extension of the ice shield, which may have affected populations located in the Andean region directly, and by concurrent shifts in climate and sea level (Heusser, 1987; McCulloch *et al.*, 2000). The numerous glacial advances and retreats during the Pleistocene are well known (e.g. Rabassa, 2008), for example the Greatest Patagonian Glaciation (GPG; 1–1.2 Ma) and the Last Glacial Maximum (LGM; 20–18 ka). These differed in extent and duration, and thus probably also in their impact on the abundance and distribution of the local biota. It is well known that the ice-sheet extent during the GPG was significantly greater than that during the LGM and the last several glaciations (McCulloch *et al.*, 2000; Rabassa, 2008).

Some have hypothesized that these events forced many species and plant communities to remain in refugia to the north and/or east of the Patagonian steppe during the Pleistocene cold cycles, with dispersal occurring southwards and westwards when the climate warmed and the ice sheet retreated (e.g. Markgraf, 1983; Heusser, 1987). As suggested for some European organisms (e.g. Hewitt, 2004; Pinceel *et al.*, 2005; Huck *et al.*, 2009), species more tolerant of low temperatures may have survived in refugia near the borders of the ice sheet that correspond to the western Patagonian plains.

An outstanding but rarely considered climatic and geographical feature of South America is the 'Arid Diagonal', a narrow area with scarce precipitation (< 250 mm year^{-1}) extending from 42° S at the Atlantic coast to 27° S at the eastern Andes flank and bounding the steppe and the Monte in northern Patagonia. Palaeoenvironmental reconstruction suggests a northward shift of the Patagonian steppe and displacement of the Arid Diagonal in response to cooler climates (Markgraf, 1983; Mancini *et al.*, 2005). Thus, this phytogeographical boundary probably fluctuated during past 2 climatic changes in such a way that, during glacial periods, the Arid Diagonal shifted northwards preceding the extension of the ice sheet, which enabled the north-east expansion of species. During interglacial periods, the Arid Diagonal shifted southwards, causing a concurrent retraction of the north-east boundary of species ranges.

MATERIALS AND METHODS

The study species

Calceolaria polyrhiza Cav. (Calceolariaceae) is a perennial rosulate herb distributed in Argentina, from southern San Juan Province (31° S) to southern Santa Cruz Province (52° S), with a maximal latitudinal extension of 2375 km. It is found from sea level to 3000 m a.s.l. and tolerates diverse climatic conditions. In the north-west, the species occurs in small, isolated populations, but it is more abundant in the south. The northernmost population is isolated from the main distribution range by a distance of 423 km (to the nearest populations in Mendoza Province, 35° S). In Chile, *C. polyrhiza* is less abundant and is found in scattered locations from 35° to 45° S.

Calceolaria polyrhiza is a self-incompatible species pollinated by highly specialized oil-collecting bees – a very unusual plant–pollinator mutualism (Sérsic, 2004; A. Cosacov, A. Sérsic & A.A. Cocucci, unpublished data). Plants have a high seed set and seeds are dispersed mainly by gravity, with no apparent adaptation for long-distance dispersal (Molau, 1988; Fernández *et al.*, 2002).

Sampling

A total of 590 individuals of *C. polyrhiza* from 68 localities covering most of its distribution area were collected (Fig. 1 and Table 1). Sampled individuals were separated by at least 5 m to avoid the collection of clones or close relatives. *Jovellana punctata* Ruiz & Pav., the sister genus to *Calceolaria*, was used as the outgroup in the analyses.

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from silica-gel-dried leaf tissue using a modified cetyl trimethyl ammonium bromide (CTAB) protocol (Cullings, 1992). The chloroplast intergenic spacer *trn*H–*psb*A (primers *trn*H$^{\mathrm{GUG}}$ and *psb*A; Shaw *et al.*, 2005) was sequenced because it showed the highest level of variation among a number of surveyed loci. It was amplified and sequenced with a protocol consisting of 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. Amplification products were purified with Sephadex (GE Healthcare, Piscataway, NJ, USA), and both forward and reverse sequences were obtained using an AB 3730xl automated sequencer (Applied Biosystems, Carlsbad, CA, USA) at Brigham Young University's DNA Sequencing Centre. Electropherograms were edited and assembled using SEQUENCHER 4.6 (Gene Codes, Ann Arbor, MI, USA). Sequences were aligned using the software MAFFT (Katoh *et al.*, 2002) and adjusted by eye. Indels were coded as single binary characters using the simple indel coding method (Simmons & Ochoterena, 2000). GenBank accessions representing all haplotypes are GQ911693–GQ911720.

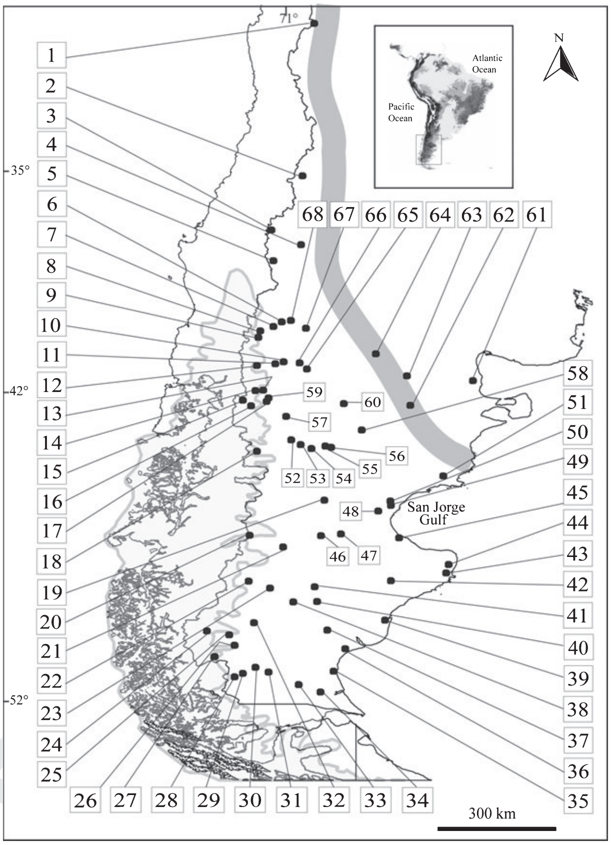

Figure 1 The inset depicts a shaded relief map of South America, with the study area indicated by a box. The map of the study area, expanded, shows the locations of 68 sampled populations of *Calceolaria polyrhiza*. The dark grey slanted strip indicates the Arid Diagonal zone. The extent of the ice cap during the Last Glacial Maximum is indicated by the light grey shaded area. Locality numbers correspond to those in Table 1 (N_{loc}).

Haplotype network and nested clade analysis

Nested clade phylogeographic analysis (NCPA) was performed to separate population structure from population history (Templeton *et al.*, 1995; Templeton, 1998) using ANeCA (Panchal, 2007). A haplotype statistical parsimony network was constructed using TCS (Clement *et al.*, 2000). Two ambiguous connections (loops) in the network were resolved using predictions from coalescent theory (Crandall & Templeton, 1993), which can be summarized by three criteria: (1) frequency criterion – haplotypes are more likely to be connected to haplotypes with higher frequency than to singletons; (2) topological criterion – haplotypes are more likely to be connected to interior than to tip haplotypes; and

Table 1 Collection localities, ecoregions, coordinates, sample size (N_{ind}) and molecular diversity indexes of the sampled populations of *Calceolaria polyrhiza* in southern South America: haplotype diversity (h), nucleotide diversity (π) and mean number of pairwise differences (p). Localities (N_{loc}) are numbered consecutively, as shown on the map in Fig. 1.

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N_{loc}	Sample locations	Province	Ecoregion	Latitude (°S)	Longitude (°W)	N_{ind}	h (± SD)	π (± SD)	p (± SD)
1	Piedras Negras	San Juan	High Andean grassland	−30.2257	−69.8060	2	0.0000	0.0000	0.0000
2	Valle Hermoso	Mendoza	High Andean grassland	−35.1008	−70.1369	9	0.0000	0.0000	0.0000
3	Epulaufquen	Neuquén	Andean Forest	−36.8382	−71.0127	8	0.0000	0.0000	0.0000
4	Chos Malal	Neuquén	High Andean grassland	−37.3130	−70.1765	8	0.2500 ± 0.1802	0.0071 ± 0.0101	0.2500 ± 0.3113
5	Trolope	Neuquén	High Andean grassland	−37.8245	−70.9644	8	0.0000	0.0000	0.0000
6	La Bomba	Neuquén	Patagonian steppe	−39.7306	−70.4663	8	0.0000	0.0000	0.0000
7	Huiliches	Neuquén	Patagonian steppe	−39.9331	−70.9617	8	0.5357 ± 0.1232	0.0153 ± 0.0160	0.5357 ± 0.4927
8	Lolog	Neuquén	Andean Forest	−40.0704	−71.3365	8	0.0000	0.0000	0.0000
9	Aº Culebra	Neuquén	Andean Forest	−40.2687	−71.3882	8	0.2500 ± 0.1802	0.0071 ± 0.0101	0.2500 ± 0.3113
10	Pilcaniyeu	Río Negro	Patagonian steppe	−41.1012	−70.8110	8	0.0000	0.0000	0.0000
11	La Fragua	Río Negro	Patagonian steppe	−41.1207	−70.9017	8	0.0000	0.0000	0.0000
12	Colonia Suiza	Río Negro	Andean Forest	−41.1787	−71.4310	11	0.0000	0.0000	0.0000
13	El bolsón	Chubut	Andean Forest	−41.9690	−71.2588	8	0.0000	0.0000	0.0000
14	Piltriquitrón	Chubut	Andean Forest	−41.9724	−71.4794	15	0.0000	0.0000	0.0000
15	Pricho	Chubut	Andean Forest	−42.4620	−71.6000	12	0.0000	0.0000	0.0000
16	Cholila	Chubut	Andean Forest	−42.4626	−71.6064	12	0.1667 ± 0.1343	0.0143 ± 0.0147	0.5000 ± 0.4563
17	Cushamen	Chubut	Patagonian steppe	−42.3379	−71.0429	8	0.4643 ± 0.2000	0.0143 ± 0.0154	0.5000 ± 0.4717
18	Lago Vintter	Chubut	Andean Forest	−43.9221	−71.4319	8	0.0000	0.0000	0.0000
19	Los Manantiales	Chubut	Patagonian steppe	−45.4739	−69.5100	8	0.2500 ± 0.1802	0.0143 ± 0.0154	0.5000 ± 0.4717
20	Los Antigüos	Santa Cruz	Patagonian steppe	−46.6093	−71.6435	11	0.0000	0.0000	0.0000
21	Sumich	Santa Cruz	Patagonian steppe	−46.9781	−70.6888	11	0.0000	0.0000	0.0000
22	PN Pto. Moreno	Santa Cruz	Patagonian steppe	−48.0774	−71.6742	8	0.0000	0.0000	0.0000
23	Tamel Aike	Santa Cruz	Patagonian steppe	−48.2856	−71.0549	8	0.6786 ± 0.1220	0.0694 ± 0.0478	2.4286 ± 1.4679
24	Helsinfors	Santa Cruz	Patagonian steppe	−49.6617	−72.8635	8	0.0000	0.0000	0.0000
25	Lago Viedma	Santa Cruz	Patagonian steppe	−49.7879	−72.2225	8	0.0000	0.0000	0.0000
26	Lago Argentino	Santa Cruz	Patagonian steppe	−50.1249	−72.0717	8	0.0000	0.0000	0.0000
27	PN Los Glaciares	Santa Cruz	Andean Forest	−50.4845	−72.6454	13	0.1538 ± 0.1261	0.0044 ± 0.0073	0.1539 ± 0.2287
28	Rupai Pacha	Santa Cruz	Patagonian steppe	−51.1310	−72.0699	2	0.0000	0.0000	0.0000
29	Tapi Aike	Santa Cruz	Patagonian steppe	−51.0203	−71.8368	11	0.6364 ± 0.0895	0.0468 ± 0.0336	1.6364 ± 1.0431
30	El Tero	Santa Cruz	Patagonian steppe	−50.8231	−71.4787	4	0.6667 ± 0.2041	0.0381 ± 0.0350	1.3333 ± 1.0250
31	Ea. La Vanguardia	Santa Cruz	Patagonian steppe	−50.9843	−71.1032	10	0.7111 ± 0.1175	0.0305 ± 0.0248	1.0667 ± 0.7668
32	Tres Lagos	Santa Cruz	Patagonian steppe	−49.4007	−71.5148	8	0.0000	0.0000	0.0000
33	Las Horquetas	Santa Cruz	Patagonian steppe	−51.3814	−70.2412	11	0.3273 ± 0.1533	0.0094 ± 0.0114	0.3273 ± 0.3547
34	Guer-Aike	Santa Cruz	Patagonian steppe	−51.6162	−69.6263	13	0.5091 ± 0.1008	0.0146 ± 0.0150	0.5091 ± 0.4643
35	Río Coig	Santa Cruz	Patagonian steppe	−50.9455	−69.2489	8	0.2500 ± 0.1802	0.0071 ± 0.0101	0.2500 ± 0.3113
36	Monte Leon	Santa Cruz	Patagonian steppe	−50.2333	−68.9167	10	0.4667 ± 0.1318	0.0267 ± 0.0226	0.9333 ± 0.6981
37	Ea. La Julia	Santa Cruz	Patagonian steppe	−49.6354	−69.4266	11	0.3273 ± 0.1533	0.0187 ± 0.0176	0.6546 ± 0.5455
38	Gob. Gregores	Santa Cruz	Patagonian steppe	−48.7354	−70.4024	11	0.4364 ± 0.1333	0.0499 ± 0.0353	1.7455 ± 1.0961
39	San Julián	Santa Cruz	Patagonian steppe	−49.3157	−67.7713	9	0.2222 ± 0.1662	0.0254 ± 0.0221	0.8880 ± 0.6812
40	Ea. 1º Abril	Santa Cruz	Patagonian steppe	−48.7201	−69.7150	8	0.0000	0.0000	0.0000
41	El Puma	Santa Cruz	Patagonian steppe	−48.2521	−69.7832	8	0.4286 ± 0.1687	0.0367 ± 0.0292	1.2857 ± 0.8965
42	Tres Cerros	Santa Cruz	Patagonian steppe	−48.0616	−67.6137	8	0.2500 ± 0.1802	0.0071 ± 0.0101	0.2500 ± 0.3113
43	Pto. Deseado	Santa Cruz	Patagonian steppe	−47.7504	−65.9169	11	0.0000	0.0000	0.0000
44	12 kmPto. Deseado	Santa Cruz	Patagonian steppe	−47.6818	−65.9651	10	0.3556 ± 0.1591	0.0102 ± 0.0121	0.3556 ± 0.3753
45	Caleta Olivia	Santa Cruz	Patagonian steppe	−46.6903	−67.3813	11	0.4727 ± 0.1617	0.0197 ± 0.0182	0.6909 ± 0.5652
46	Pampa Verdún	Santa Cruz	Patagonian steppe	−46.6137	−69.6049	14	0.4835 ± 0.1425	0.0151 ± 0.0150	0.5275 ± 0.4674
47	Las Heras	Santa Cruz	Patagonian steppe	−46.5599	−69.0355	5	0.4000 ± 0.2373	0.0229 ± 0.0228	0.8000 ± 0.6815
48	Ruta 26	Chubut	Patagonian steppe	−45.8235	−67.9718	14	0.7473 ± 0.0659	0.0286 ± 0.0229	1.0000 ± 0.7153
49	La Begonia	Chubut	Patagonian steppe	−45.6523	−67.6157	15	0.6762 ± 0.1049	0.0348 ± 0.0263	1.2191 ± 0.8206
50	Río Chico	Chubut	Patagonian steppe	−45.5079	−67.6237	11	0.0000	0.0000	0.0000
51	Ea. Lochiel	Chubut	Patagonian steppe	−44.7052	−66.1191	11	0.0000	0.0000	0.0000
52	Tecka	Chubut	Patagonian steppe	−43.5683	−70.5679	8	0.5357 ± 0.1232	0.0153 ± 0.0160	0.5357 ± 0.4927
53	Co. Cacique	Chubut	Patagonian steppe	−43.6697	−70.3853	3	0.6667 ± 0.3143	0.0191 ± 0.0238	0.6667 ± 0.6667
54	Paso de Indios	Chubut	Patagonian steppe	−43.7834	−70.2005	4	0.5000 ± 0.2652	0.0143 ± 0.0177	0.5000 ± 0.5191

Table 1 Continued

N_{loc}	Sample locations	Province	Ecoregion	Latitude (°S)	Longitude (°W)	N_{ind}	h (± SD)	π (± SD)	p (± SD)
55	Cajon de Ginebra	Chubut	Patagonian steppe	−43.7559	−69.4767	8	0.0000	0.0000	0.0000
56	El Pajarito	Chubut	Patagonian steppe	−43.7971	−69.3113	4	0.5000 ± 0.2652	0.0143 ± 0.0177	0.5000 ± 0.5191
57	Leleque	Chubut	Patagonian steppe	−42.3159	−71.1523	8	0.0000	0.0000	0.0000
58	El Escorial	Chubut	Patagonian steppe	−43.2321	−68.4456	8	0.0000	0.0000	0.0000
59	El Maitén	Chubut	Patagonian steppe	−42.2069	−71.1062	8	0.0000	0.0000	0.0000
60	Gastre	Chubut	Patagonian steppe	−42.3929	−68.9501	5	0.0000	0.0000	0.0000
61	Sa. Grande	Río Negro	Monte	−41.6629	−65.2664	12	0.3030 ± 0.1475	0.0087 ± 0.0108	0.3030 ± 0.3370
62	Telsen	Chubut	Patagonian steppe	−42.4494	−67.0577	13	0.0000	0.0000	0.0000
63	Samuncurá	Río Negro	Patagonian steppe	−41.5000	−67.1500	2	0.0000	0.0000	0.0000
64	Los Menucos	Río Negro	Patagonian steppe	−40.7998	−68.0443	8	0.0000	0.0000	0.0000
65	Jacobacci	Río Negro	Patagonian steppe	−41.2828	−70.0121	5	0.0000	0.0000	0.0000
66	Comallo	Río Negro	Patagonian steppe	−41.0905	−70.2161	4	0.0000	0.0000	0.0000
67	Piedra del Aguila	Neuquén	Patagonian steppe	−39.9843	−70.0419	11	0.1818 ± 0.1436	0.0052 ± 0.0082	0.1818 ± 0.2534
68	Catan Lil	Neuquén	Patagonian steppe	−39.7296	−70.4703	8	0.7143 ± 0.1227	0.0286 ± 0.0243	1.0000 ± 0.7481

(3) geographical criterion – haplotypes are more likely to be connected to haplotypes from the same population or region than to haplotypes in distant populations (Pfenninger & Posada, 2002). The resulting haplotype network was converted into a hierarchical nested design following Templeton *et al.* (1987) and Templeton & Sing (1993). Clade (D_c) and nested clades (D_n) distances were estimated to assess the association between the nested cladogram and geographical distances among sampled localities (Templeton *et al.*, 1995) using GEODIS (Posada *et al.*, 2000). Null distributions (i.e. under a hypothesis of no geographical association of clades and nested clades) for permutational contingency-table test comparisons were generated from 10,000 Monte Carlo replications, with a 95% confidence level. For significant associations, the latest version of the inference key of Templeton (2004) was used to recognize probable demographic processes and/or historical events of the clades.

Phylogenetic analysis

Phylogenetic relationships among haplotypes and the outgroup specimen were reconstructed using maximum likelihood (ML), under the general time-reversible substitution model (GTR + I + Γ) allowing four Γ categories, using the program PhyML 3.0 (Guindon & Gascuel, 2003; Guindon *et al.*, 2005). Robustness of phylogenetic relationships was evaluated through 1000 bootstrap replications. Bayesian inference was conducted to estimate additional clade support based on the same substitution model. Four Monte Carlo Markov chains starting with a random tree were run simultaneously in two independent runs for 6×10^6 generations using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Trees were sampled every 1000 generations, and convergence diagnostics for log likelihood values and standard deviations of split frequencies were assessed visually using StatView 4.5 (Abacus Concepts, Berkeley, CA, USA). Trees prior to stationarity (burn-in fraction of 0.25) were excluded, and subsequent trees were used to estimate Bayesian posterior probabilities (PP).

Molecular diversity and population genetic structure

Haplotype diversity (*h*; Nei, 1987), nucleotide diversity (π; Nei, 1987) and mean number of pairwise differences (*p*; Tajima, 1983) were calculated for the species, for each location, and for every significant haploclade derived from the NCPA, using Arlequin 3.1 (Excoffier *et al.*, 2005). Locations with high levels of genetic variation and unique haplotypes were examined as possible sites of refugia, whereas locations with low levels of genetic variation were examined as possible sites of recent colonization (Taberlet & Cheddadi, 2002).

To investigate hierarchical levels of population structure, two analyses of molecular variance (AMOVA) were performed that consider genetic distances between haplotypes and their frequencies using Arlequin. First, sampling localities were grouped according to the four phylogeographical groups defined by the major clades retrieved by NCPA to determine the amount of variation among and within groups. Then, to test if there were significant differences in the amount of genetic variation among non-glaciated (i.e. possible refugia) and glaciated (i.e. recent colonizations) sites during the GPG, two independent AMOVAs were performed for the Northern and Southern Patagonian clades. Significance levels of variance components were computed using a nonparametric permutation approach with 10,000 replicates.

Demographic history analyses

Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) tests of neutrality were calculated to detect range expansions. Significant negative values of Tajima's *D* and Fu's F_S indicate an excess of low-frequency mutations relative to expectations under the standard neutral model (i.e. strict selective neutrality of variants and constant population size). The significance of both values was calculated from 1000 simulated samples using a coalescent algorithm. In addition, a 'mismatch distribution' analysis to distinguish between models invoking past exponential growth versus historical population stasis was performed (Rogers & Harpending, 1992; Excoffier, 2004). A multimodal distribution of differences between haplotypes is usually found in samples drawn from populations at demographic equilibrium, whereas the distribution is usually [5] unimodal in populations having passed through a recent demographic expansion (Excoffier, 2004). The goodness-of-fit of the observed mismatch distribution to that expected under a sudden expansion model was evaluated using parametric bootstrapping with the sum of squared deviations (SSD). A significant sum of squared differences (SSD; $P \leq 0.05$) indi- [6] cates a departure from the null model of population expansion. Neutrality tests and mismatch distribution analyses were performed for all the phylogroups identified by NCPA using Arlequin.

Divergence time estimates

Based on substitution rates suggested for chloroplast DNA (cpDNA) spacers (Alsos *et al.*, 2005), and one obtained in a phylogenetic analysis of Lamiales (including *Calceolaria* and *Jovellana*) using fossil records (Datson *et al.*, 2008), we assumed substitution rates of 0.8–1% Myr^{-1} to estimate divergence times among haplotypes of the studied populations. This analysis was performed using the program mega 3.0 (Kumar *et al.*, 2004) on a neighbour-joining (NJ) tree with a topology similar to the retrieved ML tree. We used published nucleotide substitution rates because there are neither fossil records nor specific substitution rates available by which to calibrate a molecular clock. Although these estimates are provisional and should be interpreted with caution, they provide approximations that allow us to hypothesize possible scenarios under which lineages would have diverged.

Lineage expansion time does not necessarily have to be coincident with a lineage's divergence time; thus, to estimate the time since the beginning of an expansion, we used $\tau = 2ut$, where t is the time elapsed (in generations) between the initial and current population sizes, and $u = 2\mu k$, where μ is the mutation rate and k is the length of the sequence (Rogers & Harpending, 1992). We assumed a generation time of 2.5 years because the time to first flower occurs 2–3 years after seed germination.

RESULTS

Haplotype network and nested clade analysis

A total of 27 haplotypes were identified (Fig. 2). Statistical parsimony retrieved a well-resolved network, in which four main haplogroups could be distinguished (Fig. 2). The most frequent and widespread haplotype (H1) was found in 44.2% of the individuals and in 52.98% of the sampled populations. It is distributed from the Andean area to the coast (65°–72° W) in the 38°–46° S zone, and is restricted to the central steppe and the coast (65°–70° W) in the 46°–48° S zone. This haplotype forms the core of the 'star-like' network topology (Fig. 2). Haplotype 1 and the immediate descendant haplotype tips ('Northern Patagonian' group; NP) range between 38° and 49° S, and between 65° and 71° W. Out of the 12 haplotypes connected by one step to H1, eight were found in a single population, and four of them (H2, H3, H7, H11) were shared by three or more populations restricted to specific geographical areas following a predominantly latitudinal pattern (Fig. 2).

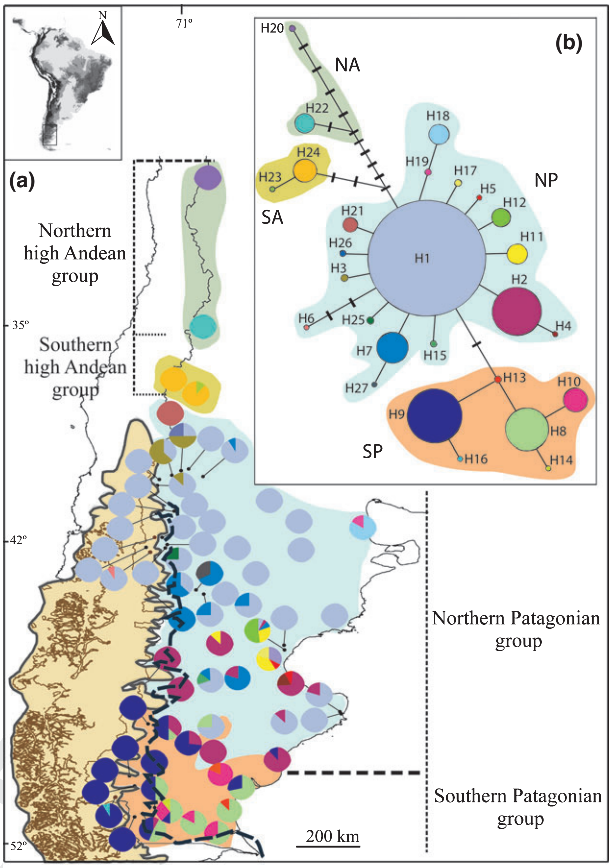

Figure 2 Geographical distribution and genealogical relationships of the 27 cpDNA haplotypes recovered from *Calceolaria polyrhiza* populations from southern South America. (a) Pie charts reflect the frequency of occurrence of each haplotype in each population. Haplotype colours correspond to those shown in panel (b). (b) Statistical parsimony network linking the 27 haplotypes. Haplotypes are designated by numbers, and circle sizes are proportional to haplotype frequencies. Cross hatches represent missing intermediate haplotypes not found in the analysed individuals.

The network also shows three groups of haplotypes connected to H1 by more than one step. Two consist of haplotypes located exclusively in high Andean populations north of 37° S: the 'Northern high Andean' group (NA), consisting of two highly divergent haplotypes (H20 and H22) fixed in the two northernmost populations, respectively, and the 'Southern high Andean' group (SA), consisting of related haplotypes (H23 and H24) found in the two southernmost high Andean populations (Figs 1 & 2).

The third group of haplotypes connected to H1 ('Southern Patagonian' group; SP) consists of haplotypes localized south of 48° S (Fig. 2). Haplotype 13 appears as an internal node connected through one step to H8 or H9, the two most frequent haplotypes in this area (66 and 80 individuals, respectively). The phylogenetic structure of this clade follows a longitudinal pattern in the distribution of its haplotypes, with H16, present in a single western population, and H9, although abundant in the west, decreasing to the east. In contrast, H8 and H10 predominate in the east and decrease to the west.

Nested clade phylogeographic analysis (Fig. 3) shows a significant relationship between genetic and geographical distributions in *C. polyrhiza* (Table 2). Past gradual range expansion followed by fragmentation, or a past larger range followed by extinction in intermediate areas, was inferred for clade 1-3, which included almost all haplotypes of the NP group. Past gene flow and past larger range, followed by extinction of intermediate populations, was inferred for clades 2-2 and 3-1, respectively. Clade 2-2 included all haplotypes of the NP group, whereas clade 3-1 included NP and SP groups. Allopatric fragmentation was inferred for clade 2-1 (all haplotypes of the SP group), for clade 3-3 (the two divergent haplotypes of the NA group), and at the entire cladogram level (Table 2).

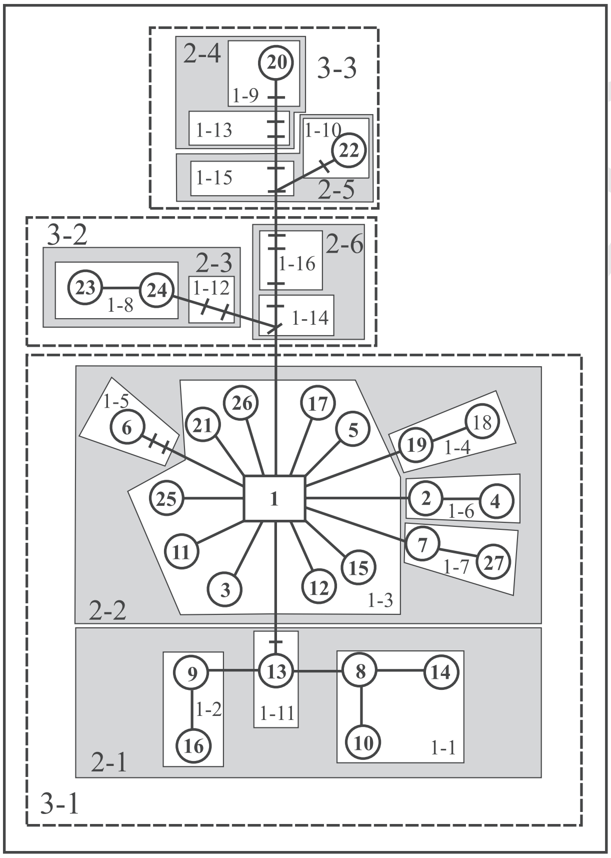

Figure 3 Statistical parsimony network and resulting set of nested clades of the 27 cpDNA haplotypes found in *Calceolaria polyrhiza*. Haplotypes are designated by numbers; cross hatches represent missing intermediate haplotypes not found in the analysed individuals.

Phylogenetic analysis

Topologies retrieved by maximum likelihood reconstruction and Bayesian inference are congruent with the topology resulting from NCPA (Figs 3 & 4). These analyses also provide further support for a deep split between clade 3-1 (all haplotypes south of 38° S; NP and SP groups), and clades 3-2 and 3-3 (all haplotypes north of 37° S; SA and NA groups). These two high Andean lineages formed a paraphyletic grade of two highly supported clades at the base of the tree.

Within clade 3-1, haplotypes of the NP group (clade 2-2) did not form a monophyletic group and showed little phylogenetic structure, whereas the SP group (clade 2-1) was recovered as monophyletic, with distinctive eastern and western subclades (Fig. 4).

Molecular diversity and population genetic structure

Haplotype diversity (h) ranged from 0.0 to 0.75, with an average of 0.21. Nucleotide diversity (π) ranged from 0.0 to 0.07, with an average of 0.01. The mean number of pairwise differences among haplotypes within populations (p) ranged from 0.0 to 2.43, with an average of 0.36 (Table 1). The highest haplotype diversity was found within coastal populations of the San Jorge Gulf (populations 48, 49), and within populations located along a longitudinal transect at *c.* 71° W across the Patagonian steppe (populations 23, 29, 30, 52, 53, 68; Fig. 5). With the exception of population 23, these populations all contained private haplotypes (Fig. 2).

Populations with the lowest haplotype diversity were found along the western margin and along a northern strip of the distribution range, where the majority of populations were monomorphic. Western monomorphic populations, which are located within or very close to areas glaciated during the GPG, possess one of four of the more frequent haplotypes (H1, H2, H7, H9), whereas monomorphic populations located in the north-western corner (i.e. north of the northern limit of the ice sheet) possess exclusive haplotypes connected to H1 by one to nine steps. Monomorphic populations located at the north-eastern edge possess the common haplotype H1 (Table 1 and Fig. 2).

The highest mean number of pairwise differences among haplotypes was found in three populations located between 48° and 49° S (populations 23, 38, 41; Table 1), along a nearly diagonal line. These populations possess haplotypes from NP and SP groups (Fig. 2).

Standard diversity indices estimated for clades derived from the NCPA are shown in Table 3. The Patagonian clade (clade 3-1) presented higher levels of haplotype and nucleotide diversity than the two high Andean clades (clades 3-2 and 3-3). Within the Patagonian group, the highest level was found in

Table 2 Inferences of historical processes affecting genetic structure in *Calceolaria polyrhiza* populations from southern South America, based on nested clade phylogeographic analysis. Hierarchically nested clades, geographical groups (NP, Northern Patagonia; SP, Southern Patagonia; NA, Northern high Andes; SA, Southern high Andes), results of permutation contingency tests, NCPA inference chain, and inferred events are shown.

Clade	Group	χ^2 statistic	*P*-value	Inference chain	Inferred event
1-3	NP	1183.29	< 0.01	1-2-3-5-15-21 NO	Past gradual range expansion followed by fragmentation or a past larger range followed by extinction in intermediate areas
2-1	SP	133.91	< 0.01	1-2-3-4-9 YES	Allopatric fragmentation
2-2	NP	1109.85	< 0.01	1-2-3-5-6-7-8 YES	Past gene flow followed by extinction of intermediate populations
3-1	NP + SP	525.66	< 0.01	1-2-3-5-15-21 NO	Past larger range followed by extinction in intermediate populations
3-3	NA	10.00	< 0.05	1-19 NO	Allopatric fragmentation
Total	NP + SP + NA + SA	1181.00	< 0.01	1-19 NO	Allopatric fragmentation

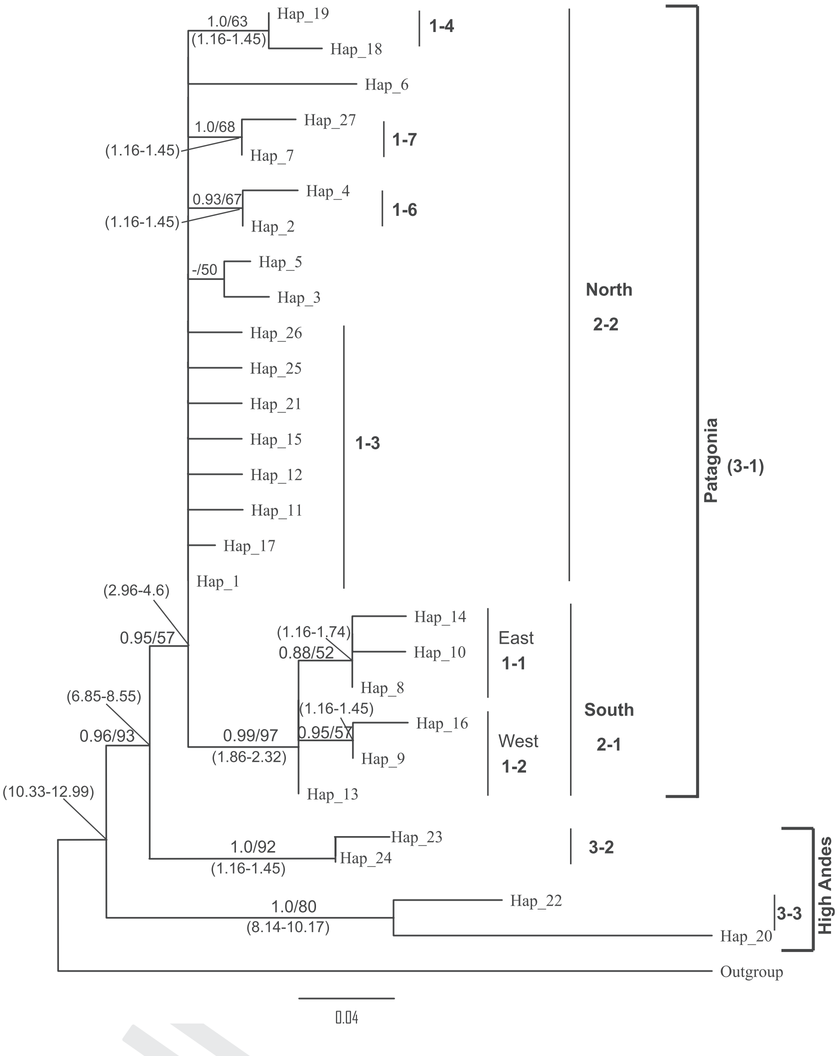

Figure 4 Maximum likelihood tree ($\ln L = -669.1609$) for the 27 cpDNA haplotypes of *Calceolaria polyrhiza*. Numbers above branches are support values from Bayesian inference/bootstrap resampling. Numbers below branches or given for each node are the estimated divergence times in million years ago. The black bars on the right indicate the corresponding clade number in the nested clade phylogeographic analysis.

the southern clade (clade 2-1; SP group), particularly in the south-east (clade 1-1). The lowest haplotype and nucleotide diversity values were found in the Southern high Andean clade (clade 3-2), whereas the highest mean number of pairwise nucleotide differences per population was found in the Andean region, particularly in the Northern high Andean clade (3-3; Table 3).

The AMOVA revealed that 65.88% of the variation was explained by differences among the geographical groups (i.e. NA, SA, NP and SP), whereas inter- and intrapopulation differences explained 23.14% and 10.98% of the variation, respectively (Table 4). Within Southern Patagonia, 40.95% of the variation was explained by differences between glaciated and non-glaciated areas, whereas in Northern Patagonia no

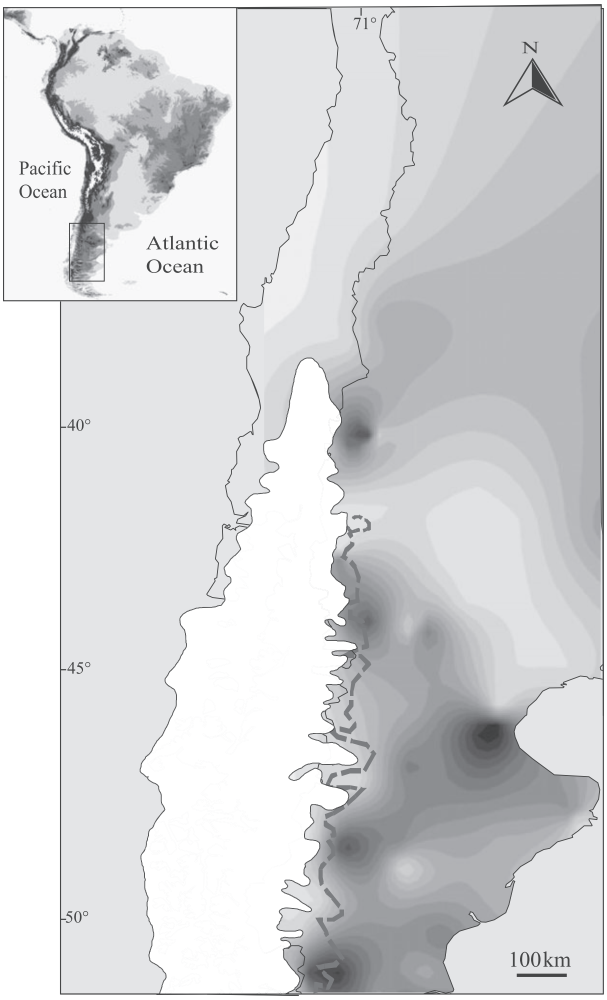

Figure 5 Surface plot of haplotype diversity along *Calceolaria polyrhiza* populations from southern South America. Areas with the highest levels of genetic diversity are in black. The shaded area and the dashed line indicate the limits of the Last Glacial Maximum and the Greatest Patagonian Glaciation, respectively.

significant effect of glaciation in the partition of the molecular variation was found (Table 4).

Demographic history analyses

Demographic analysis showed evidence of range expansion in the Patagonian clade (clade 3-1), particularly in the north (clades 2-2 and 1-3) and in the south-west (clade 1-2) and in the species-level analysis (Table 3). Although mismatch analysis was consistent with a model of population expansion for the high Andean group, the more conservative estimate of population expansion, F_S, did not indicate a departure from population equilibrium (Table 3).

Divergence time estimates

Divergence times estimated for supported clades are shown in Fig. 4. According to these estimates, NA lineages (H20 and H22) and the remaining haplotypes diverged between 10.33 and 12.99 Ma (late Miocene; Lourens *et al.*, 2004), whereas SA lineages (H23 and H24) diverged from the Patagonian group around 6.85–8.55 Ma. The estimated time of diversification within the NA clade is between 8.14 and 10.17 Ma (late Miocene). In contrast, haplotypes nested within SA diverged more recently, around 1.16–1.45 Ma (Pleistocene). Diversification of haplotypes in Patagonia began around 4.6–2.96 Ma (late Pliocene), and most of the main subclades of NP and SP originated almost simultaneously during the Pleistocene, between 1.16 and 1.45 Ma (Fig. 4).

Based on the location of the crest of the unimodal mismatch distribution (τ), all demographic expansions began in the late Pleistocene, *c.* 16–158 ka (Table 3).

DISCUSSION

Phylogroups

Phylogeographical analyses identified four geographically structured phylogroups of *C. polyrhiza* haplotypes, and two of them are strongly divergent. Distribution of these phylogroups follows a latitudinal, and largely allopatric, pattern. Genetic diversity and historical processes vary within these four groups, suggesting different and independent evolutionary pathways. Overall, NCPA suggests that the evolutionary history of *C. polyrhiza* exhibits a phylogeographical footprint consistent with past fragmentations and allopatric differentiation.

The four phylogroups – two exclusively from the high Andes and two from Patagonia – correspond to three latitudinal phylogeographical breaks. The first break occurs at 35° S and divides the high Andean populations into a Northern (NA) and a Southern (SA) clade. This latitudinal break coincides with breaks reported in previous phylogeographical studies based on different organisms (lizards, Ávila *et al.*, 2006; Morando *et al.*, 2007; grasses, Jakob *et al.*, 2009; trees, Azpilicueta *et al.*, 2009). The northernmost clade, NA, occurs between 30° and 35° S and includes two highly divergent haplotypes found exclusively in the two northernmost populations. Within this clade, the highest mean number of pairwise differences between haplotypes is observed, and a history of allopatric fragmentation is inferred. In addition to this genetic gap, there is also a discontinuity in the species' distribution. The same geographical and genetic gap has also been reported in two groups of Patagonian lizards (Morando *et al.*, 2004; Ávila *et al.*, 2006), suggesting a shared vicariant history among these organisms. The second high Andean phylogroup (SA) is located at *c.* 37° S. It is strongly divergent from the Northern clade (NA) and includes two exclusive and closely related haplotypes; the most frequent of these is shared by two close populations. The second phylogeographical break is located south of 38° S, the latitude at which the northern limit of the distribution of haplotype H1 is found. All populations located south of this geographical break have haplotypes nested within the Patagonian clade. Interestingly, this break

Table 3 Diversity indices and results of demographic analyses used to test range expansion in *Calceolaria polyrhiza* clades. Haplotype (h) and nucleotide (π) diversity, mean number of pairwise differences (p), Tajima's D, Fu's F_S, sum of squared differences (SSD), mismatch distribution parameter τ, and estimated ranges of expansion time are shown.

		Diversity indices			Demographic analyses				
Clades	Geographical area	h ± SD	π ± SD	p ± SD	D	F_S	SSD	T	Expansion time (ka)
3-1	**Patagonia**	0.737 ± 0.017	0.044 ± 0.027	1.833 ± 1.056	−1.050	**−9.200***	**0.020**	3.59	16.438–153.681
2-1	Southern Patagonia	0.608 ± 0.027	0.027 ± 0.022	1.241 ± 0.981	1.349	0.659	**0.049**	2.76	94.520–118.150
1-1	East	0.433 ± 0.051	0.009 ± 0.010	0.442 ± 0.400	0.075	0.256	0.017*	–	–
1-2	West	0.025 ± 0.025	0.001 ± 0.002	0.025 ± 0.084	−1.054	**−2.020***	**0.000**	3.00	102.739–128.424
2-2	Northern Patagonia	0.569 ± 0.026	0.016 ± 0.013	0.739 ± 0.549	**−1.785****	**−14.35****	**0.005**	0.79	27.054–33.818
1-3	All	0.276 ± 0.033	0.006 ± 0.007	0.205 ± 0.311	**−1.712****	**−12.01****	**0.004**	3.00	102.739–128.424
1-4	Northeast	0.303 ± 0.148	0.007 ± 0.008	0.303 ± 0.337	−0.195	0.297	**0.236**	3.00	102.739–128.424
1-6	South	0.058 ± 0.039	0.001 ± 0.003	0.059 ± 0.131	−0.899	−1.138	**0.000**	3.00	102.739–128.424
1-7	Central	0.077 ± 0.070	0.002 ± 0.003	0.077 ± 0.153	−1.156	−1.094	**0.000**	3.00	102.739–128.424
–	**High Andes**	0.595 ± 0.067	0.098 ± 0.056	4.216 ± 2.160	0.845	5.71	0.025*	–	–
3-2	Southern high Andes	0.125 ± 0.106	0.003 ± 0.050	0.125 ± 0.202	−1.162	−0.700	**0.042**	2.08	98.325–122.907
3-3	Northern high Andes	0.327 ± 0.153	0.053 ± 0.035	2.291 ± 1.358	−0.171	4.634	0.150*	–	–
Total		0.76 ± 0.016	0.049 ± 0.029	2.201 ± 1.219	**−1.450***	**−10.53****	**0.016**	3.70	126.712–158.390

Results consistent with demographic expansion are shown in bold.
*$P < 0.05$; **$P < 0.01$.

Table 4 Results of the analysis of molecular variance (AMOVA) for 68 populations of *Calceolaria polyrhiza* grouped into four geographical regions (Northern high Andes, Southern high Andes, Northern Patagonia and Southern Patagonia) based on cpDNA sequence data. For the Northern and Southern Patagonian groups, additional AMOVAs were performed to test genetic subdivision among populations located in areas covered versus uncovered by ice during the Greatest Patagonian Glaciation. Degrees of freedom (d.f.), sum of squares (SSD), variance components (VC), percentage of total variance (% total) and significance value (P) are given for each hierarchical level.

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Source of variation	d.f.	SSD	VC	% Total	P-value
Among regional groups	3	335.627	1.144	65.88	< 0.001
Among populations	64	235.566	0.402	23.14	< 0.001
Within populations	523	99.701	0.190	10.98	< 0.001
Total	590	670.895	1.736		
Southern Patagonia					
Glaciated versus non-glaciated	1	28.901	0.407	40.95	< 0.001
Among populations	15	37.084	0.244	24.51	< 0.001
Within populations	133	45.662	0.343	34.53	< 0.001
Total	149	111.647	0.994		
Northern Patagonia					
Glaciated versus non-glaciated	1	0.842	0.011	3	n.s.
Among populations	45	104.023	0.247	64.97	< 0.001
Within populations	367	53.164	0.145	38.03	< 0.001
Total	413	158.029	0.381		

n.s., not significant.

also agrees with results of phylogeographical studies in plants (Muellner *et al.*, 2005; Marchelli & Gallo, 2006; Azpilicueta *et al.*, 2009) and lizards (Morando *et al.*, 2007).

The Patagonian clade is large and includes two primary haplotype groups: the Northern (NP; 39°43′–48° S) and Southern (SP; 48°–51° S) Patagonian clades. The limit between these subclades defines a third phylogeographical break, which is located along a diagonal line that approximates the present course of the Chico River in Santa Cruz province, Argentina. Along this line, haplotypes from the Northern and Southern Patagonian clade were found syntopically at three locations. These populations showed the highest mean number of pairwise differences, as expected for populations that include distantly related haplotypes (Avise, 2000; Petit *et al.*, 2002). This suggests a secondary contact zone between the two Patagonian clades (NP and SP).

Divergence times and a possible past scenario

Our results suggest that the initial divergence among *C. polyrhiza* lineages occurred before the onset of the major Quaternary glaciations. Thus, not only Pleistocene glaciation cycles, but also earlier volcanic and tectonic events that occurred during the late Miocene and Pliocene (e.g. Ramos & Ghiglione, 2008) probably influenced the diversification of this species. This result is highly congruent with the time-scale suggested for the diversification of the genus *Calceolaria*, which would have originated after the major uplifting of the Andes, *c.* 20 Ma (Molau, 1988; Datson *et al.*, 2008). Based on our dating estimates, the Northern high Andean lineages became isolated *c.* 11 Ma (late Miocene). Around this period, strong tectonic activity was taking place at the Pacific coast, promoting the uplifting of the Andes and the formation, in southern Mendoza (*c.* 35° S), of a major landscape discontinuity (Las Loicas trough) west of a volcanic arc (Ramos & Kay, 2006).

The second split is estimated to have taken place *c.* 7.7 Ma, and separated the Southern high Andean haplotypes from Patagonian lineages (*c.* 37° S). This break coincides with the Cortaderas lineament, an area of extensive volcanic and tectonic activity during the late Miocene (Ramos & Kay, 2006; Ramos & Ghiglione, 2008). Because these geological events produced remarkable climatic and landscape changes, they probably contributed to the differentiation of the high Andean lineages.

The origin of the large clade that includes all haplotypes present in the Patagonian steppe is estimated to have occurred *c.* 3.7 Ma (middle Pliocene), after the final uplifting of the southern Andes (*c.* 5 Ma). This major geological event reduced the westerlies precipitation regime, causing the aridization of the eastern plains and the establishment of the Patagonian steppe (Barreda *et al.*, 2008; Ramos & Ghiglione, 2008). A phylogeographical study of a shrub (*Anarthrophyllum desideratum*) characteristic of the steppe vegetation, for which there is a specific calibrated molecular clock, also suggests that differentiation in the steppe started *c.* 4 Ma (A.N. Sérsic *et al.*, [9] in prep.). The main diversification of the Northern and Southern Patagonian clades, as well as the differentiation of the two South high Andean haplotypes, is estimated to have occurred between 1.16 and 1.45 Ma. Thus, these diversifications were probably associated with climatic and landscape changes that occurred during the Pleistocene glacial cycles. Interestingly, these results are highly concordant with the estimated origin (*c.* 1.3 Ma) of three recently diverged grass species of the genus *Hordeum* characteristic of Patagonian steppe communities (Jakob *et al.*, 2009). Although our divergence time estimates mesh with the other studies cited here, limitations in our ability to estimate divergence times more accurately also limit the robustness of our inferences; these inferences should be accepted with caution and, as hypotheses, remain open for testing and refinement by future studies.

Multiple Pleistocene refugia and post-glacial expansion

Our analyses provide several lines of evidence for identifying potential Pleistocene refugia for *C. polyrhiza*. These include: (1) the lack of genetic variability in previously glaciated areas [10] as a result of bottlenecks; (2) the high genetic variability and the presence of private haplotypes along a transect located along the limit of the ice sheet and in central-eastern Patagonia; (3) the pattern of haplotype overlap and range expansion in south-western and northern Patagonia; and (4) the fact that the estimation of the time since exponential [11] growth occurred suggests that demographic expansions began after the GPG ice sheet retreated.

In *C. polyrhiza*, the highest haplotype diversity was found in a restricted area of central-eastern Patagonian steppe (in the zone of the San Jorge Gulf) and in the vicinity of four points along a longitudinal transect located at *c.* 71° S, which is remarkably congruent with the limit of the ice-sheet extension during the GPG. High haplotype diversity in populations suggests either that these populations have persisted for a long time, being sufficiently large to maintain the observed level of genetic variation, or that the area was recolonized by a number of lineages present in source refugia (e.g. Hewitt, 1996; Petit *et al.*, 2002; Mraz *et al.*, 2007). However, in the area near the Gulf of San Jorge and at three of the four points along the longitudinal transect, the presence of private haplotypes is indicative of population persistence rather than of recolonization from refugia, as also concluded by other studies (e.g. Pinceel *et al.*, 2005; Mraz *et al.*, 2007; Ronikier *et al.*, 2008; Huck *et al.*, 2009). Moreover, all but two previously glaciated sites (those with populations 16 and 27) are monomorphic for one of the four most common haplotypes (H1, H2, H7, H9), and these haplotypes are also found in putative source populations located in the Patagonian steppe outside the GPG and LGM limits. These haplotypes define four latitudinal zones, and each haplotype is the most abundant within the populations in the previously glaciated areas in the respective latitudinal zone. This pattern strongly suggests post-glacial colonization of the formerly glaciated western areas by four independent genetic lineages dispersing from the non-glaciated eastern Patagonian steppe. This pattern is also congruent with patterns of genetic diversity observed in most temperate plants and animals, which typically show low levels of diversity in previously glaciated areas owing to repeated bottlenecks during colonization (e.g. Hewitt, 1996, 2004; Taberlet *et al.*, 1998; Ronikier *et al.*, 2008). In southern Patagonia, the pattern of decreasing genetic diversity from east to west is quite evident, and the AMOVA suggested that glaciations influenced the genetic structure of populations located in the southern Patagonian steppe. In the northern steppe, however, the effect was not statistically significant owing to the prevalence of monomorphic populations both in the west (i.e. previously glaciated areas) and at the eastern edge of the range, an area affected by fluctuations in the Arid Diagonal (see below).

Demographic analyses indicated a strong signal of demographic expansion in the Patagonian clade, particularly in its northern (1-3) and south-western (1-2) subclades. In addition, the Northern Patagonian clade showed a star-like topology centred on the most widespread haplotype – a pattern suggesting a rapid expansion of the ancestral haplotype over a large geographical area (Avise, 2000). Moreover, estimation of the time since exponential growth occurred suggests that all detected demographic expansions began 16–158 ka. These populations therefore appear to be recovering from bottlenecks that occurred during the GPG (1–1.2 Ma) and the LGM (18– [12] 20 ka; McCulloch *et al.*, 2000).

Based on current patterns of genetic variation, along with results of demographic analyses, we propose the existence of multiple periglacial refugia (*sensu* Holderegger & Thiel-Egenter, 2009) in the Patagonian steppe during the Pleistocene, as has also been hypothesized in previous studies (e.g. Premoli *et al.*, 2000; Marchelli & Gallo, 2006). We suggest at least three distinct refugia in the immediate vicinity of the ice sheet, located in south-western Neuquén (*c.* 40° S), central-western Chubut (*c.* 43° S) and south-western Santa Cruz (*c.* 51° S) provinces. Because a fourth area with high diversity (located *c.* 48° S) contains haplotypes from the Northern and Southern Patagonia clades and does not have private haplotypes, we suggest that it was recently recolonized from different source populations. This area, located along a diagonal following the course of the Chico River, is proposed as a secondary contact zone between NP and SP. The Chico River, at present, is an intermittent stream with a much-reduced discharge, but during Pleistocene glaciations it was a powerful, glaciofluvial current draining to the east into the Atlantic Ocean (Hernández *et al.*, 2008; Martínez & Coronato, 2008). During glacial melting, the Chico River's upper watershed tributaries, such as the huge palaeolake Caldenius (47°–48° S), shifted their direction of drainage from the Atlantic to the Pacific Ocean, reducing greatly the Chico River discharge (Tatur *et al.*, 2002; del Valle *et al.*, 2007). The changing geology and hydrology of this region may well have influenced the underlying processes of fragmentation, recolonization and secondary contact of NP and SP lineages, giving rise to the observed pattern of genetic variation.

The great molecular diversity found in populations from the eastern range margin of *C. polyrhiza*, along the coastline and primarily near the San Jorge Gulf, is likely to be the remnant of a more extensive easterly distribution. During glacial cycles, the coastline fluctuated around four longitudinal degrees to the east (Rabassa, 2008). As also proposed for *Hordeum* species (Jakob *et al.*, 2009), *C. polyrhiza* probably survived Pleistocene glaciations in a much larger, but now submerged, eastern part of the Patagonian steppe. Coastline fluctuations associated with glacial periods would have caused alternating cycles of fragmentation and expansion in populations of *C. polyrhiza*, probably resulting in repeated vicariance events with concomitant differentiation of populations.

With respect to populations located beyond the northern limit of the ice sheet (north of 38° S), it is possible that the persistence of small and isolated populations was favoured during the Pleistocene glaciations. The long-lasting isolation of these populations is indicated by the high number of private and fixed haplotypes, and by the high genetic divergence among them (cf. Avise, 2000). In fact, the area located at 35° S has previously been postulated as a relictual zone characterized by fragmented, high-elevation forests (Donoso, 1993); our results corroborate the existence of this zone. In addition, it is well documented that the region north of 36° S was characterized by alpine valley-type glaciation, [13] with glaciers confined to valleys and not extending beyond the mountain front (Rabassa, 2008). It is possible that ice-free areas could have favoured the persistence of small, isolated populations. Such a scenario would explain the current pattern of genetic diversity in high Andean populations of *C. polyrhiza* and also agrees with previous studies on plants and animals (Marchelli & Gallo, 2004; Himes *et al.*, 2008; Azpilicueta *et al.*, 2009).

Fluctuation of the Arid Diagonal

Most plant communities in this region migrated northwards during Pleistocene cold cycles and recolonized their habitats at higher latitudes when the climate warmed (Markgraf, 1983). Current genetic patterns observed on the north-east edge of the distribution range of *C. polyrhiza* strongly support this hypothesis, and may be related to fluctuations in the Arid Diagonal and associated displacements of the phytogeographical boundary between Monte and Patagonian steppe (Mancini *et al.*, 2005). The high frequency of monomorphic populations for H1 on the north-east edge of the distribution of the species suggests a recent colonization of this area, probably during the LGM. In addition, the geographical zone where these populations occur shows a diagonal pattern coincident with the Arid Diagonal. The genetic divergence of a single *C. polyrhiza* population from the Monte phytogeographical province (population 61) suggests a long-lasting persistence and isolation in this area. This population could be the remnant of an older north-east expansion, which was followed by extirpation and southward retraction of the geographical range. Supporting this hypothesis, the NCPA suggested past gene flow followed by the extinction of intermediate populations for this area. Although the location of the Arid Diagonal in the Pleistocene is uncertain, we suggest that current genetic patterns of *C. polyrhiza* may reflect past fluctuations of this important climatic barrier in Patagonia.

CONCLUSIONS

This phylogeographical study of *C. polyrhiza* reveals the influence of contrasting and complex geological and climatic events contributing to patterns of diversification and distribution in this Patagonian species. Glacial climate fluctuations substantially impacted the diversification, distribution and demography of *C. polyrhiza*, yet the phylogeographical structure preserves the imprints of older events such as Miocene and Pliocene volcanism and orogeny. The initial divergence among *C. polyrhiza* lineages probably took place during the late Miocene, in the high Andes, from where populations expanded southwards. The divergence of the Patagonian lineages (*c.* 4 Ma) was probably related to a major climatic change in the eastern plains, when the rise of the Andes had progressed enough to decrease westerly precipitation, with consequent aridization and the establishment of the current steppe. Within the Patagonian lineages, major diversifications seem to be related to climatic and landscape changes during the GPG and LGM. However, the effects of the Pleistocene glaciations apparently varied along the geographic range of this species.

Finally, our results suggest that a model of multiple periglacial refugia may be proposed for Patagonian organisms. Recolonization of the Andes flanks by *C. polyrhiza* was presumably achieved independently by four lineages confined to specific latitudinal zones, revealing the existence of latitudinal migration corridors and supporting the hypothesis of multiple recolonization routes from eastern Patagonian steppe to the Andean flanks.

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BIOSKETCH

Andrea Cosacov is a graduate student studying for a PhD in biology at the University of Córdoba and is a member of the Floral Biology group of the Instituto Multidisciplinario de Biología Vegetal (IMBIV). This study is part of her PhD thesis on geographical variation in the phenotypic traits and phylogeography of *Calceolaria polyrhiza*. The authors of this paper constitute an interdisciplinary team of molecular and field researchers interested in the ecology, evolution, systematic and conservation of several groups of plants (http://patagonia.byu.edu/index.aspx).

Author contributions: A.C., A.N.S. and A.A.C. conceived the idea and collected the samples; A.C., V.S. and L.A.J. performed the molecular analyses; A.C. analysed the data statistically; A.C., A.N.S. and A.A.C. led the writing; L.A.J. and V.S. improved the final version of the manuscript.

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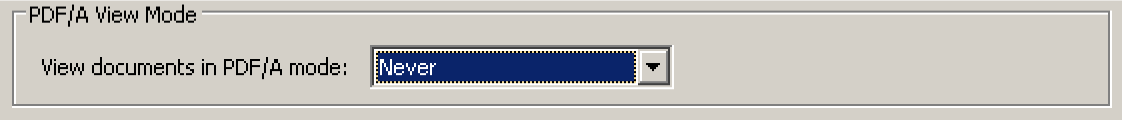

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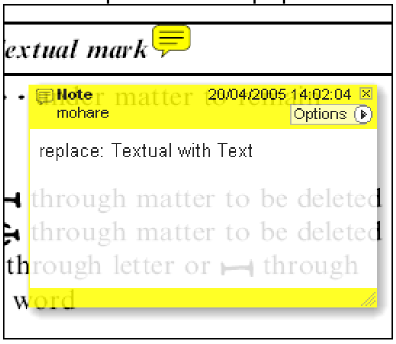
Marks a point on the paper where a note or question needs to be addressed.



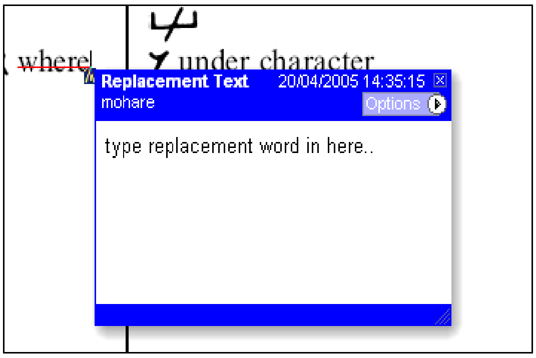
replace: Textual with Text

How to use it:

1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
4. Click the X in the top right hand corner of the note box to close.

Replacement text tool — For deleting one word/section of text and replacing it

Strikes red line through text and opens up a replacement text box.



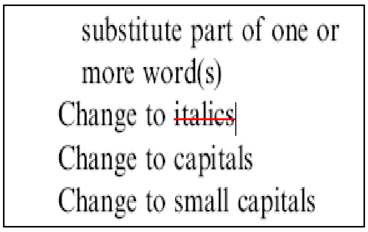
type replacement word in here..

How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Replace Text (Comment) option
5. Type replacement text in blue box
6. Click outside of the blue box to close

Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



substitute part of one or
more word(s)
Change to ~~italics~~
Change to capitals
Change to small capitals

How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

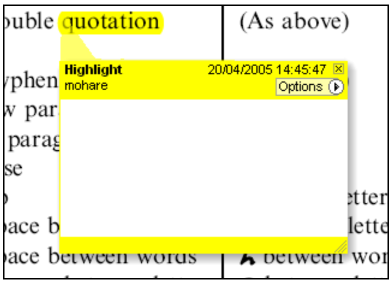
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the ‘standard business’ selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

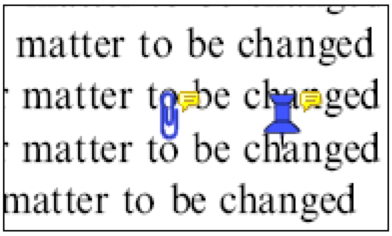


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.

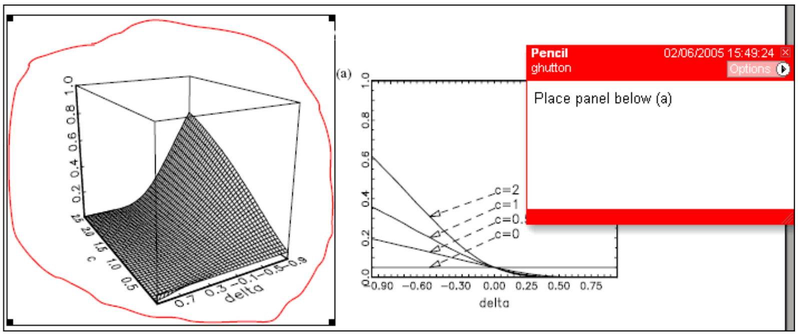


How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
4. Select appearance of icon (paperclip, graph, attachment or tag) and close

Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



Place panel below (a)

How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: