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ORIGINAL ARTICLE

Refugia in Patagonian fjords and the eastern Andes during the Last Glacial Maximum revealed by huemul (*Hippocamelus bisulcus*) phylogeographical patterns and genetic diversity

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ABSTRACT

Aim Our aim was to determine the combined impacts of Pleistocene climatic oscillations and glacial periods with recognized biogeographical barriers on the evolutionary history of huemul deer (*Hippocamelus bisulcus*).

Location Southern Chile and Argentina's Andean forest, and Patagonian fjords.

Methods We examined the phylogeography of huemul using 772 bp of the mitochondrial DNA control region sequence from 275 samples (29 locations) collected throughout the distributional range of the species. We grouped samples into clusters based on Bayesian genetic and spatial structure analyses and reconstructed the species' phylogeographical and demographic history.

Results We observed 63 haplotypes that grouped into three clusters (Central Chile, North Patagonia and South Patagonia). All but five haplotypes in North and South Patagonia were distributed locally. Bayesian skyline plots showed that population sizes remained fairly constant until an increase during and after the Last Glacial Maximum. Genetic diversity was generally low, except in three populations in the eastern Andes and on Wellington Island (Patagonian fjords).

Main conclusions Our results suggest that the biogeographical separation of huemul into phylogeographical groups has been heavily influenced by Pleistocene glacial stages, and more recently by habitat fragmentation and isolation. This provides the first evidence that the region west of the Cordilleran ice field was a refugium for at least one species of large mammal during the Pleistocene in southern South America. These results have direct implications for the conservation and management of this endangered deer species.

Keywords
Conservation biogeography, deer, demographic history, *Hippocamelus bisulcus*, mitochondrial DNA, phylogeography, Pleistocene, South America.

INTRODUCTION

From an evolutionary perspective, refugia are regions where organisms persist during periods when their wider geographical range becomes restricted or uninhabitable, generally owing to changes in climatic and environmental conditions (Byrne, 2008). Pleistocene climatic variations profoundly impacted biotic distributions world-wide, restricting many species distributional ranges to such refugia (Byrne, 2008). Although the fossil record often provides evidence of changes in biotic distributions, it generally does not provide information about a species' distributional range during glacial periods. However, demographic changes and the effects of isolation are known to leave genetic signatures in the genomes of extant populations, and these can be studied with molecular markers (Byrne, 2008). Interpreting these genetic patterns in a geographical context (i.e. phylogeography) provides a better understanding of species responses to climate dynamics (Moritz *et al.*, 2000).

Our knowledge of the influence of Pleistocene climatic events on the evolutionary histories of large Neotropical mammals is limited. However, as many widespread populations

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became fragmented during glacial advances and cold periods, it is probable that such events also had an effect on South America's large mammals. Once favourable environmental conditions were re-established, palaeontological evidence indicates that biotic expansion into periglacial areas was probably rapid (Hewitt, 2000). This dynamic process would generally have involved rapid exponential population growth from a relatively small number of founders (Haanes *et al.*, 2010). Such demographic change leaves its signature in the genome, as the equilibrium between genetic drift and mutation is lost (Tajima, 1989; Fu, 1997).

Research into Neotropical invertebrates and small vertebrates has provided data to test biogeographical hypotheses on the origins of current spatial patterns of biodiversity in South America. Several models have been proposed for Ice Age effects on biota (Haffer, 1997), but evidence is accumulating that Pleistocene refugia played a critical role in shaping the genetic and geographical diversity of many extant species, for example freshwater crabs (Xu *et al.*, 2009), sigmodontine rodents (Lessa *et al.*, 2010), galaxiid fish (Zemlak *et al.*, 2011) and *Liolaemus* lizards (Breitman *et al.*, 2012). During Pleistocene glaciations, isolated refugia were created by the contraction and fragmentation of ice-free habitat, rendering otherwise widespread populations allopatrically isolated from each other. During interglacial periods biota expanded from these refugia (Hewitt, 2000), sometimes forming secondary contact zones with conspecific populations (Haffer, 1985).

The chronological records of glaciations in southern South America are among the most complete in the world, with the oldest glaciations occurring in the late Miocene and early Pleistocene (Hulton *et al.*, 2002). During the middle–late Pleistocene, a minimum of eight glaciations occurred, the most extensive of which is known as the Last Glacial Maximum (LGM; 16,000 to 25,000 years ago; Rabassa *et al.*, 2005). During this time, the ice cap covered extensive areas to the west and east of the Andes, with an estimated area of 480,000 km^2, from 38° to 55° S and reaching the Pacific Ocean to the south of parallel 43° S (Hulton *et al.*, 2002). While glaciers advanced to the north throughout the Andes, refugia might have remained in the central valley between the Andes and the Cordillera de la Costa, but their exact location is as yet not well defined. Traditionally, refugia are thought to have occurred exclusively to the north and west of the continental ice field. However, pollen records suggest that the southern coastal range of lowland Chile and Chiloé Island, as well as the southern central depression, were the most likely locations of refugia between 40° and 42° S (Villagran, 1991; Premoli *et al.*, 2000; Rodríguez-Serrano *et al.*, 2008; Victoriano *et al.*, 2008).

The South American deer genus *Hippocamelus* comprises two species, namely the taruca, sometimes called the North Andean huemul (*Hippocamelus antisensis* d'Orbigny, 1834), and the South Andean huemul, or simply huemul (*Hippocamelus bisulcus* Molina, 1782). The huemul is a useful candidate species with which to address questions about the effect of glacial and interglacial dynamics on a large mammal. This species has a wide distribution in Chile and Argentina, occupying mountainous terrain ranging from open habitats in areas of low precipitation to sub-Antarctic rain forests and periglacial coastal habitats typified by the Patagonian fjords (Cabrera & Yepes, 1960; Frid, 2001). Extant huemul populations are fragmented into small, frequently isolated subpopulations (Vila *et al.*, 2006), which are heavily impacted by poaching, habitat loss and fragmentation, and by disturbance and predation by domestic dogs (Corti *et al.*, 2011). Furthermore, the introduction of non-native livestock into the huemul range has reduced its distribution and population size owing to the introduction of exotic diseases (Simonetti, 1995) and to overgrazing by the new competitors (Frid, 2001). For these reasons, the huemul is classified as an endangered species (IUCN, 2012).

When European settlers arrived in South America 500 years ago, the Chilean distribution of huemul was between the Cachapoal River (34.5° S) and the Magellan Strait (54° S; Díaz, 2000), in areas previously ice-covered during the LGM. In Argentina, its historical distribution ranged from 36.5° to 54° S (Díaz, 1990), and current populations exist between 40° and 51° S (Vila *et al.*, 2006). The huemul's current fragmented distribution corresponds to less than 50% of its historical range (Vila *et al.*, 2006) (Fig. 1). For example, a small remnant population in the northernmost distribution (Nevados de Chillán) is *c.* 400 km north of the next closest population located in Nahuel Huapi National Park (Argentina).

In this study, we used mitochondrial DNA (mtDNA) sequence variation to characterize the huemul's molecular diversity based on samples collected across its distribution. We investigated current evidence of phylogeographical structure linked with huemul evolutionary history in order (1) to describe the impacts of the LGM in the southern Andes on the population genetics of this endemic deer, and (2) to describe the evolutionary history and patterns of gene flow among these populations.

MATERIALS AND METHODS

Sample collection and DNA extraction

Samples were collected throughout the current distributional range of the huemul (Table 1, Fig. 1) following guidelines of the American Society of Mammalogists (Sikes *et al.*, 2011). DNA samples were obtained using one of four methods: (1) skin samples from adults obtained with biopsy darts (Dan-Inject Biopsy Needle, Børkop, Denmark; $n = 75$); (2) muscle or skin tissue from dead animals ($n = 29$); (3) samples from shed antlers of adult males collected in the field and from collections held in museums and national parks – connective tissue was scraped from the base of the antlers (Varas, 2009) and stored in 70% ethanol ($n = 14$); (4) fresh faeces individually collected and preserved in 100% ethanol ($n = 157$; faeces were considered fresh based on their glossiness and dark colour). Sixty per cent of faecal samples were collected from

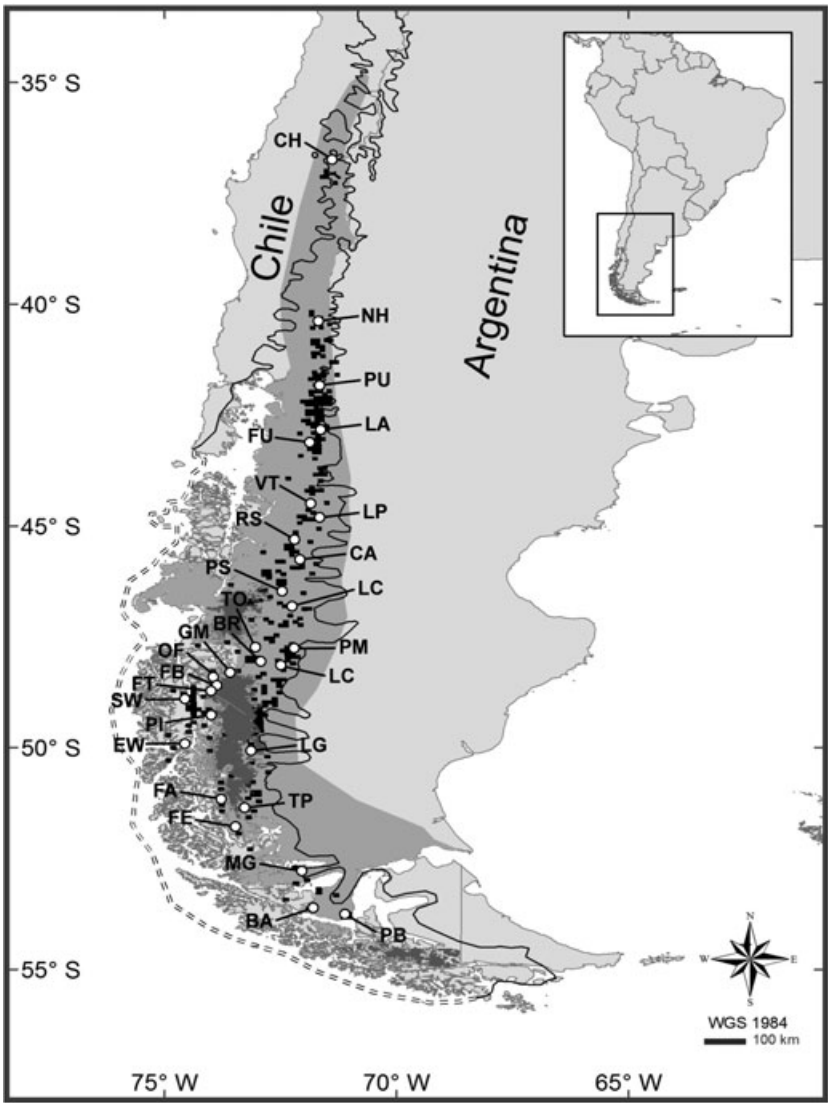

Figure 1 Map with current (black squares) and historical (light grey) distributions of *Hippocamelus bisulcus* populations in Chile and Argentina. Open circles represent sampled localities (see Table 1 for a definition of locality abbreviations). The solid black and grey dashed lines show the limits of the ice coverage during the Last Glacial Maximum.

individuals that were followed in the field until they defecated. Although it cannot be ruled out that some of the remaining 40% of faeces were duplicate samples, collection efforts attempted to reduce this probability. First, based on the collectors' field experience, faeces were discriminated based on their morphology. Second, because home ranges of huemul are *c.* 350 to 444 ha (Gill *et al.*, 2008; Corti *et al.*, 2011), collection of samples less than 1 km apart was avoided as much as possible. Furthermore, all localities where faeces were not collected from known individuals were sampled once, with the exception of Chillán and Tortel, where samples were collected on two separate occasions.

In order to test the robustness of our sampling, the software Genesamp (Sjögren & Wyöni, 1994) was used to calculate the probability of sampling a haplotype with a minimum frequency (usually 1/sample size) given a population's sample and census estimate. This analysis indicated that rare haplotypes (e.g. observed in only one individual among 27 samples in Chillán) could be retrieved with a probability of 0.675 (see Appendix S1 in Supporting Information). While ideally a larger sample size would better reflect the haplotypic composition of a population, the sample size used for this study appears sufficient to retrieve even infrequent variants (the average probability of recovering the haplotypes with minimum frequency is larger than 0.8; Appendix S1).

All samples were stored at –70 °C upon arrival at the Laboratory of Genomics and Biodiversity, University of Bio-Bío, Chillán, Chile. Total genomic DNA was extracted from tissues using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and from faeces using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) in a separate non-genetic-oriented laboratory.

Mitochondrial DNA sequences

The highly divergent peripheral domains of the mitochondrial control region (CR; *c.* 800 bp) were amplified using artiodactyl and huemul-specific primers LPro-Artio (5′-CAG CAC CCA AAG CTG AAA TTC T-3′), L20-Hippo LPro-Artio (5′-GCT CCG TAA AAT TTA AGA GCC-3′),

Table 1 Summary of the *Hippocamelus bisulcus* samples from Chile and Argentina. Localities are ordered from north to south. The type of sample is given (B, biopsy; A, antler; D, dead animals; F, faecal), plus the number of samples per locality. Locality groups were determined by geographical proximity and environmental similarity within each cluster.

Clusters (abbreviation) *Locality groups (abbreviation)*	Locality, country (abbreviation)	Geographical coordinates	Sample type (no. of samples)
Central Chile Cluster (CCC)			
Chillán (CH1)	Nevados of Chillán, Chile (CH)	36°50′52″ S, 71°15′28″ W	F(27)
North Patagonia Cluster (NPC)			
Puelo Lake (NP1)	Nahuel Huapi National Park, Argentina (NH)	40°47′58″ S, 71°34′35″ W	D(2)
	Lago Puelo National Park, Argentina (PU)	42°08′18″ S, 71°39′37″ W	D(4), F(1)
	Los Alerces National Park, Argentina (LA)	42°48′59″ S, 71°42′54″ W	D(6), F(10)
	Futaleufú National Reserve, Chile (FU)	43°10′32″ S, 71°52′29″ W	F(2)
La Plata Lake (NP2)	La Tapera Village, Chile (VT)	44°41′35″ S, 71°52′18″ W	F(8)
	La Plata Lake, Argentina (LP)	44°52′39″ S, 71°41′32″ W	D(6)
Cerro Castillo (NP3)	Río Simpson National Reserve, Chile (RS)	45°36′07″ S, 72°12′52″ W	D(2), A(12), B(5)
	Cerro Castillo, National Reserve, Chile (CA)	45°58′55″ S, 71°55′43″ W	B(6), F(6)
	Puerto Sánchez, Chile (PS)	46°31′32″ S, 72°37′45″ W	F(5)
South Patagonia Cluster (SPC)			
Cochrane Lake (SP1)	Lago Cochrane National Reserve, Chile (LC)	47°13′06″ S, 72°29′46″ W	B(21), D(1)
Bravo River (SP2)	Perito Moreno National Park, Argentina (PM)	47°48′19″ S, 72°14′17″ W	A(2)
	Tortel Cove, Chile (TO)	47°49′51″ S, 73°18′23″ W	D(1),F(11), B(6)
	Christie Lake, Chile (CL)	48°08′24″ S, 72°26′36″ W	F(5)
	Bravo River, Chile (RB)	48°02′37″ S, 73°01′04″ W	B(8)
Patagonian fjords (SP3)	Jorge Montt glacier, B. O′Higgins National Park, Chile (GM)	48°15′56″ S, 73°27′08″ W	F(12)
	Ofihidro Island, B. O′Higgins National Park, Chile (OF)	48°28′83″ S, 74°00′69″ W	F(6)
	Bernardo fjord, B. O′Higgins National Park, Chile (FB)	48°35′33″ S, 73°54′28″ W	F(4), B(19)
	Témpano fjord, B. O′Higgins National Park, Chile (FT)	48°41′97″ S, 73°59′08″ W	F(4), D(1), B(2)
	Wald Sound, Wellington Island, B. O′Higgins National Park, Chile (SW)	48°49′28″ S, 74°35′37″ W	F(8)
	Pio XI glacier, B. O′Higgins National Park, Chile (PI)	49°14′31″ S, 74°03′18″ W	F(2)
	White Lagoon, Wellington Island, B. O′Higgins National Park, Chile (EW)	49°54′32″ S, 74°34′16″ W	F(4)
Los Glaciares (SP4)	Los Glaciares National Park, Argentina (LG)	49°37′15″ S, 72°55′95″ W	D(4), F(12)
Torres del Paine (SP5)	Amalia fjord, Chile (FA)	50°55′92″ S, 73°49′49″ W	F(2)
	Torres del Paine National Park, Chile (TP)	51°07′43″ S, 73°07′07″ W	B(8), D(1), F(10)
	Encuentro fjord, Chile (FE)	51°31′36″ S, 73°35′24″ W	F(2)
Austral zone (SP6)	Muñoz-Gamero peninsula, Chile (MG)	52°29′14″ S, 72°33′15″ W	F(2)
	Batchelor peninsula, Chile (BA)	53°31′93″ S, 72°14′92″ W	F(11)
	Brunswick peninsula, Chile (PB)	53°45′06″ S, 71°02′06″ W	F(3), D(1)

H540-Hippo (5′-TTC ACG CGG CAT GGT AAT TAA G-3′), L650-Hippo (5′-ATG AAC TTT ATC AGA CAT CTG G-3′), L950Hippo (5′-ACT TAA CTG CAT CTT GAG CAT CC-3′) and HPhe-00020 (5′-ACT CAT CTA GGC ATT TTC AGT GCC TTG C-3′). Amplification from tissue samples was performed using primers complementary to the tRNA flanking the CR (LPro-Artio–HPhe-00020); however, the amplification from faecal samples was performed using pairs of primers that amplify two small regions of *c.* 450 bp each [LPro-Artio or L20-Hippo (forward) and H540-Hippo (reverse), and L650-Hippo or L950Hippo (forward) and HPhe-00020 (reverse)]. These sample sequences were confirmed with two independent rounds of amplification and sequencing. Amplification was performed in 30-μL reactions with *c.* 20 ng genomic DNA, 1× reaction buffer [8 mM Tris-HCl (pH 8.4); 20 mM KCl (InvitrogenGibco, Life Technologies, Rockville, MD, USA)], 2 mM $MgCl_2$, 25 μM each of dGTP, dATP, dTTP and dCTP, 0.5 μM each primer and 0.1U/μ *Taq* polymerase (InvitrogenGibco, Life Technologies, Rockville, MD, USA). Polymerase chain reaction (PCR) amplifications were performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following procedures: initial denaturation at 95 °C for 10 min, followed by 30–35 cycles of 94 °C for 45 s, 60–62° C for 45 s and 72 °C for 60 s, and a final extension at 72 °C for 5 min. PCR products were purified using the GeneClean Turbo for PCR Kit (Q·BIOgene, Carlsbad, CA, USA) following the manufacturer's instructions. Products were sequenced up to

three times in forward and reverse directions using BigDye chemistry (Perkin Elmer, Foster City, CA, USA) in an ABI Prism 3100 semi-automated DNA analyser (Applied Biosystems, Foster City, CA, USA). Sequences were aligned using Geneious pro 5.3.4 (Biomatters Ltd., Auckland, New Zealand) and checked by eye. The number of segregating sites (S), haplotypes (nh), haplotype diversity (h), nucleotide diversity (π) and the average number of nucleotide differences between pairs of sequences (k) were estimated using Arlequin 3.5.1.2 (Excoffier & Lischer, 2010).

Genetic units and intraspecific genealogies

We first evaluated whether there was a phylogeographical signal in the huemul haplotype distribution by comparing G_{ST} (the divergence between populations) with N_{ST} (the divergence between populations accounting for the genetic distance between haplotypes) using Permut 2.0 (Pons & Petit, 1996) and 1,000,000 permutations to assess significance. A Bayesian analysis of population structure accounting for the geographical distribution of huemul was performed with the R package Geneland 1.0.7 (Guillot *et al.*, 2005). The parameters for this analysis were: 5,000,000 Markov chain Monte Carlo (MCMC) iterations, a maximum rate of the Poisson process fixed to 100, uncertainty of the spatial coordinates fixed to 5 km, and the maximum number of nuclei in the Poisson–Voronoi tessellation fixed to 300. These parameters were used for five repetitions of K-values (the number of clusters in the data) in the range 1 to 12. Using the same parameters and the K-values inferred above as a fixed variable, the MCMC algorithm was run 30 times. The mean logarithm of the posterior probability was calculated for each of the 30 runs, and the posterior probability of population membership for each pixel of the spatial domain was computed for the three runs with the highest values. This analysis was complemented with (1) a Bayesian analysis of population structure that compared alternative phylogeographical hypotheses using the software baps (Corander & Martinen, 2006), and (2) an analysis of molecular variance (AMOVA) on alternative population groupings using Arlequin 3.5.1.2 (Excoffier & Lischer, 2010). We also performed a test of isolation by distance (IBD) by comparing an individual pairwise matrix of genetic distances against the corresponding matrix of geographical distances using a Mantel test in the software Alleles In Space (Miller, 2005) and 1000 permutations to assess the significance of the correlation coefficient.

The genealogical relationship between huemul mtDNA haplotypes was described with a haplotype network using the uncorrected median-joining values and the program Network 4.600 (Bandelt *et al.*, 1999). The amount of divergence between pairs of populations (Φ_{ST}) was calculated with Arlequin 3.5.1.2 (Excoffier & Lischer, 2010) using 10,000 permutations to assess significance.

Historical demography

Huemul historical demography was studied by comparing the observed mismatch distributions with those expected under a sudden expansion model (Rogers & Harpending, 1992) using the sum of the squared deviations (SSD) implemented in Arlequin and 1000 bootstrap replicates to assess significance. This software was also used to calculate Tajima's D and Fu's F_S (Tajima, 1989; Fu, 1997; Table 2), which are sensitive to demographic changes. In addition, we reconstructed Bayesian skyline plots using beast 1.4.8 and Tracer 1.0.1 (Drummond & Rambaut, 2007) under the HKY+I substitution model estimated using Modeltest 3.06

Table 2 Genetic diversity indices of 275 *Hippocamelus bisulcus* samples from Chile and Argentina. *n*, number of samples; *na*, number of haplotypes observed; *np*, number of private haplotypes; *h*, haplotype diversity; π, nucleotide diversity; *p*, number of polymorphic sites; Tajima's *D*; Fu's F_S. Standard error values are in parentheses, and statistically significant values are marked with asterisks.

Clusters (abbreviation) *Locality groups (abbreviation)*	*n*	*na*	*np*	*h* ± (SD)	π	*p*	*D*	F_S
Central Chile Cluster (CCC)	27	6	6	0.63 ± (0.68)	0.980 ± (0.76)	8	−1.6441	−2.9857*
Chillán (CH1)	27	6	6	0.63 ± (0.68)	0.980 ± (0.76)	8	−1.6441	−2.9857*
North Patagonia Cluster (NPC)	75	24	22	0.93 ± (0.01)	4.12 ± (3.30)	27	−0.7908	−2.4533
Puelo Lake (NP1)	25	13	10	0.93 ± (0.02)	2.813 ± (1.71)	17	−1.3329	−1.1050
La Plata Lake (NP2)	14	5	4	0.67 ± (0.12)	2.626 ± (1.67)	7	0.7128	0.7697
Cerro Castillo (NP3)	36	8	7	0.80 ± (0.03)	3.965 ± (2.25)	14	0.5614	−1.1134
South Patagonia Cluster (SPC)	173	35	33	0.83 ± (0.02)	2.066 ± (1.28)	33	−1.8965	−3.4834**
Cochrane Lake (SP1)	22	8	7	0.77 ± (0.08)	1.762 ± (1.18)	5	0.2183	−0.1982
Bravo River (SP2)	33	5	4	0.33 ± (0.10)	0.871 ± (0.69)	8	−1.6587	−0.6279
Patagonian fjords (SP3)	62	14	13	0.80 ± (0.03)	2.252 ± (1.39)	14	−0.7169	−2.0283
Los Glaciares (SP4)	16	6	3	0.80 ± (0.06)	2.375 ± (1.52)	8	−0.0537	−0.2258
Torres del Paine (SP5)	23	4	2	0.23 ± (0.11)	0.250 ± (0.32)	3	−1.7325	−2.5257**
Austral zone (SP6)	17	3	2	0.25 ± (0.14)	0.629 ± (0.55)	3	−0.9480	−0.0125
TOTAL	275	63		0.92 ± (0.01)	3.723 ± (2.08)	58	−1.8075	−5.1982

*$P < 0.05$.
**$P < 0.02$.

(Posada & Crandall, 1998). BEAST was run for 10,000,000 iterations sampling every 1000 steps, and assuming a substitution rate per million years for the sequenced fragment of 1.8% (corresponding to a generation time of 3 years; Corti *et al.*, 2011).

RESULTS

Among the 275 individuals analysed, we identified 58 variable positions segregated into 63 haplotypes, and total haplotype (h) and nucleotide (π) diversities of 0.92 and 3.72, respectively (Table 2). The hypervariable domain II was the most variable region in our data, presenting on average about two-thirds of the polymorphisms. The distribution of haplotypes and h and π per locality and region are detailed in Table 2; see also Appendix S2. All sequences were deposited in GenBank under the accession numbers JN870923–JN871197.

The N_{ST} value of 0.555 (SE = 0.433) was significantly higher than the associated G_{ST} value of 0.358 (SE = 0.613) ($P < 0.0001$), indicating the existence of a phylogeographical pattern. GENELAND analysis divided the huemul samples into three geographical regions (Table 3, Fig. 2). Consistent with this result, the variance components of the AMOVA were maximized when the populations were clustered into the same three groups ($\Phi_{CT} = 0.393$, $P < 0.0001$; Table 3) according to the GENELAND results. The Central Chile Cluster (CCC), grouped all Chillán sequences (36°54′ S). The North Patagonia Cluster (NPC), grouped sequences representing localities in the Valdivian temperate forest and Patagonia (40°45′ S and 46°30′ S; NH, PU, LA, FU, VT, LP, RS, CA and PS in Fig. 1). The South Patagonia Cluster (SPC), grouped the remaining sequences representing the Magellanic subpolar forest (47°10′ S and 53°45′ S; LC, PM, TO, CL, RB, GM, OF, FB, FT, SW, PI, EW, LG, FA, TP, FE, MG, BA and PB in Fig. 1). The differentiation estimate between (1) the localities and (2) the GENELAND clusters consistently showed significant Φ_{ST} values between all but one of the pairwise comparisons (Table 4). Chillán (CCC) and Torres del Paine (SPC) were both highly differentiated from the other populations ($\Phi_{ST} = 0.829$). The IBD test found a significant positive correlation between genetic and geographical distances of 0.377 ($P = 0.001$) (see Appendix S3). We conducted a Bayesian analysis of population structure that explicitly tests alternative phylogeographical hypotheses in order to determine which model is best supported by the data. This analysis, conducted in BAPS (Corander & Martinen, 2006), simultaneously compared the following patterns of structure: (1) the

Table 3 Results of analyses of molecular variance (AMOVAs) of mtDNA data from 275 samples of *Hippocamelus bisulcus* from 29 localities in Chile and Argentina. For locality abbreviations, see Table 1. n.s., not significant; asterisks denote statistically significant (***$P < 0.001$). Significance levels are based on 10,000 permutations.

Grouping	Source of variation	Fixation indices	Percentage variance
(1) CH	Among groups	$\Phi_{CT} = 0.32774$ n.s.	32.77
(2) NH, PU, LA, FU, VT, LP, RS, CA, PS, LC, PM, TO, CL, RB, GM, OF, FB, FT, SW, PI, EW, LG, FA, TP, FE, MG, BA, PB	Among populations within groups	$\Phi_{SC} = 0.69133$ ***	46.48
	Among individuals within populations	$\Phi_{ST} = 0.79250$ ***	20.75
(1) CH, NH, PU, LA, FU, VT, LP, RS, CA, PS	Among groups	$\Phi_{CT} = 0.23668$ ***	23.67
(2) LC, PM, TO, CL, RB, GM, OF, FB, FT, SW, PI, EW, LG, FA, TP, FE, MG, BA, PB	Among populations within groups	$\Phi_{SC} = 0.67387$ ***	51.44
	Among individuals within populations	$\Phi_{ST} = 0.75106$ ***	24.89
(1) CH	Among groups	$\Phi_{CT} = 0.39333$ ***	39.33
(2) NH, PU, LA, FU, VT, LP, RS, CA, PS	Among populations within groups	$\Phi_{SC} = 0.61623$ ***	37.39
(3) LC, PM, TO, CL, RB, GM, OF, FB, FT, SW, PI, EW, LG, FA, TP, FE, MG, BA, PB	Among individuals within populations	$\Phi_{ST} = 0.76718$ ***	23.28
(1) CH	Among groups	$\Phi_{CT} = 0.34261$ ***	34.26
(2) NH, PU, LA, FU, VT, LP, RS, CA, PS	Among populations within groups	$\Phi_{SC} = 0.60764$ ***	39.95
(3) GM, OF, FB, FT, SW, PI, EW	Among individuals within populations	$\Phi_{ST} = 0.74207$ ***	25.79
(4) LC, PM, TO, CL, RB, LG, FA, TP, FE, MG, BA, PB			
(1) CH	Among groups	$\Phi_{CT} = 0.30825$ ***	30.82
(2) NH, PU, LA, FU	Among populations within groups	$\Phi_{SC} = 0.62028$ ***	42.91
(3) VT, LP, RS, CA, PS	Among individuals within populations	$\Phi_{ST} = 0.73733$ ***	26.27
(4) LC, PM, TO, CL, RB, GM, OF, FB, FT, SW, PI, EW			
(5) LG, FA, TP, FE, MG, BA, PB			
(1) CH	Among groups	$\Phi_{CT} = 0.29686$ ***	29.69
(2) NH, PU, LA, FU	Among populations within groups	$\Phi_{SC} = 0.61397$ ***	43.17
(3) VT, LP, RS, CA, PS	Among individuals within populations	$\Phi_{ST} = 0.72857$ ***	27.14
(4) LC, PM, TO, CL, RB			
(5) GM, OF, FB, FT, SW, PI, EW			
(6) LG, FA, TP, FE, MG, BA, PB			

Fixation indices: Φ_{CT}, among groups; Φ_{SC}, among localities within groups; Φ_{ST}, within localities.

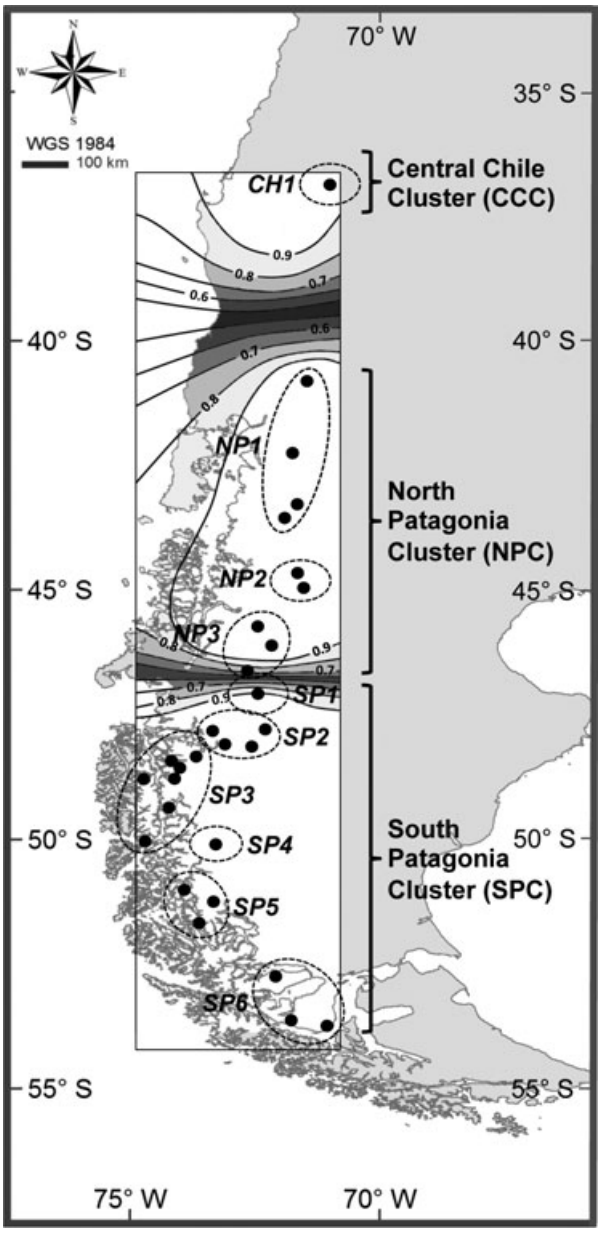

Figure 2 Results of the GENELAND analysis based on mitochondrial DNA data with K fixed at 3. The inset map shows the posterior probability of individuals of *Hippocamelus bisulcus* in Chile and Argentina. Regions with the greatest probability of inclusion are indicated by white, whereas diminishing probabilities of inclusion are proportional to the degree of dark grey. Black dots represent sampling sites and the dashed ovals show the locality groups. See Table 1 for a definition of locality group abbreviations.

Table 4 Population pairwise Φ_{ST} values (below the diagonal) and P-values (above the diagonal) for the population comparisons (top) and the Bayesian cluster comparisons (bottom) for 275 samples of *Hippocamelus bisulcus* from Chile and Argentina.

		Central Chile Cluster (CCC)	North Patagonia Cluster (NPC)			South Patagonia Cluster (SPC)					
Locality groups (abbreviation)			*Puelo*	*La Plata*	*Cerro*	*Cochrane*	*Bravo*	*Patagonian*	*Los*	*Torres del*	*Austral*
Region (abbreviation)	*n*	*Chillán*	*Lake*	*Lake*	*Castillo*	*Lake*	*River*	*fjords*	*Glaciares*	*Paine*	*zone*
Chillán (CH1)	27	–	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Puelo Lake (NP1)	25	0.808	–	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
La Plata Lake (NP2)	14	0.738	0.184	–	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Cerro Castillo (NP3)	36	0.544	0.327	0.253	–	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Cochrane Lake (SP1)	22	0.758	0.691	0.636	0.403	–	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Bravo River (SP2)	33	0.770	0.720	0.627	0.331	0.429	–	0.00 ± 0.0	0.00 ± 0.0	0.144 ± 0.03	0.00 ± 0.0
Patagonian fjords (SP3)	62	0.631	0.612	0.525	0.340	0.411	0.165	–	0.00 ± 0.0	0.009 ± 0.01	0.00 ± 0.0
Los Glaciares (SP4)	16	0.682	0.549	0.461	0.214	0.323	0.121	0.164	–	0.00 ± 0.0	0.00 ± 0.0
Torres del Paine (SP5)	24	0.829	0.767	0.657	0.346	0.525	0.016	0.188	0.200	–	0.00 ± 0.0
Austral zone (SP6)	16	0.821	0.740	0.655	0.404	0.589	0.463	0.374	0.406	0.644	0.00 ± 0.0
Central Chile Cluster (CCC)	27	–	0.00 ± 0.0	0.00 ± 0.0							
North Patagonia Cluster (NPC)	75	0.551	–	0.00 ± 0.0							
South Patagonia Cluster (SPC)	173	0.614	0.395	–							

division found by GENELAND; (2) all samples north of Lake General Carrera (LGC; also known as Lake Buenos Aires in Argentina) against all samples south of the lake; (3) the samples of Chillán against all the rest; and (4) no detectable population structure [this scenario corresponds to the boundaries set by Sérsic *et al.* (2011) applied to our data]. This analysis showed that the GENELAND solution best fitted

the data with a posterior probability of 0.999, indicating that while all hypotheses had the same prior probability to be supported by the data (i.e. 0.25), only a division into three groups as described here is supported by our data.

The haplotype network analysis generated a network with a maximum of 18 steps between the most distant sequences (Fig. 3). Overlaying the GENELAND partition on the haplotype network reveals a close correspondence between the haplotypic genealogy and the geographical divisions found in the data. On the basis of their geographical origin and their genetic similarity, haplotypes in NPC and SPC can be further subdivided into three and six subgroups, respectively (Table 1). SPC presents a dominant haplotype (H35, see Appendix S2) occurring south of LGC. H35 was shared by samples of all southern localities except by those in the Austral zone. Interestingly, the samples originating from the Patagonian fjords contained a very high number of private haplotypes (n_p = 13, Table 2). Finally, a minor discrepancy was found in the presence of four shared haplotypes between NPC and SPC. Such a discrepancy may reflect either translocated lineages or lineages that previously dispersed into their current sampling locations.

All demographic analyses found a consistent signature of population expansion in the recent past. The analyses, based on summary statistics, showed negative values of Fu's F_S and Tajima's D for all populations (Table 2), while the mismatch distributions were all unimodal with a modal peak close to the recent past (Fig. 4a–c). The Bayesian skyline plot found a pattern of a long history of constant population size followed by demographic expansions that occurred in the recent past (Fig. 4d–f). Interestingly, NPC shows evidence of a population increase earlier than in both SPC and CCC (Fig. 4b & 4e, Table 2), and while the latter two were passing through their most recent expansions, NPC shows signs of a demographic reduction.

DISCUSSION

Patagonia was shaped by a complex geological history, including Andean uplift, volcanism, marine introgressions, and extreme climatic oscillations (reviewed in Breitman *et al.*, 2012). This area is not a biogeographical barrier for several species, but its environmental conditions have isolated and led to the differentiation of some smaller-sized species (Lessa *et al.*, 2010; Zemlak *et al.*, 2011; Breitman *et al.*, 2012). Although these small vertebrates have limited dispersal and shorter generational times than huemul, they are indicative of the selection strength and isolation pressures that have impacted some species in this region's biota.

Past environmental changes in Patagonia have modified ecosystems (Rabassa *et al.*, 2005) and species distributions (Sérsic *et al.*, 2011). Current regional phylogeographical patterns have been shaped by population contraction and recovery, range contractions and expansions, and the formation of secondary contact areas (Sérsic *et al.*, 2011). Our results show phylogeographical patterns comparable to those previously described for invertebrates and small vertebrates. This is the first evidence for the survival of a large mammal such as the huemul in these southern refugia during the Pleistocene.

Genetic diversity and phylogeography

The assumption that huemul are genetically depauperate (Corti *et al.*, 2011), while partially true at a local scale, is not supported across the range of the species. Our study shows that there is high genetic diversity among huemul populations, and that such a pattern probably arose as a result of the philopatric behaviour of females (Corti, 2008). However, peripheral populations in the huemul distribution may experience low levels of genetic diversity as a consequence of

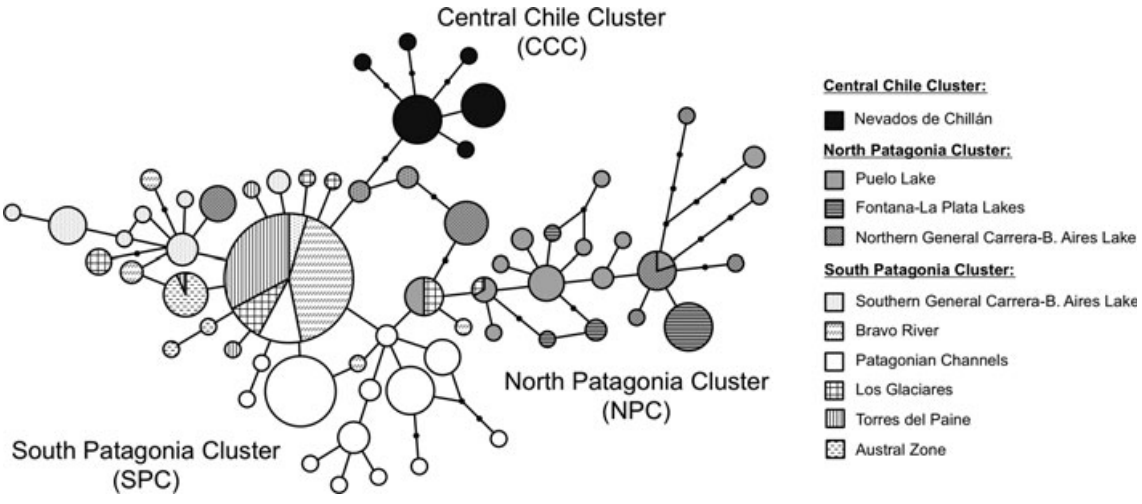

Figure 3 Minimum spanning network of *Hippocamelus bisulcus* haplotypes. Circle sizes correspond to haplotype frequencies. The sphere colours correspond to different regions, where black represents Central Chile Cluster (CCC), light grey North Patagonia Cluster (NPC), and white South Patagonia Cluster (SPC). Loops in the network correspond to recurrent mutations.

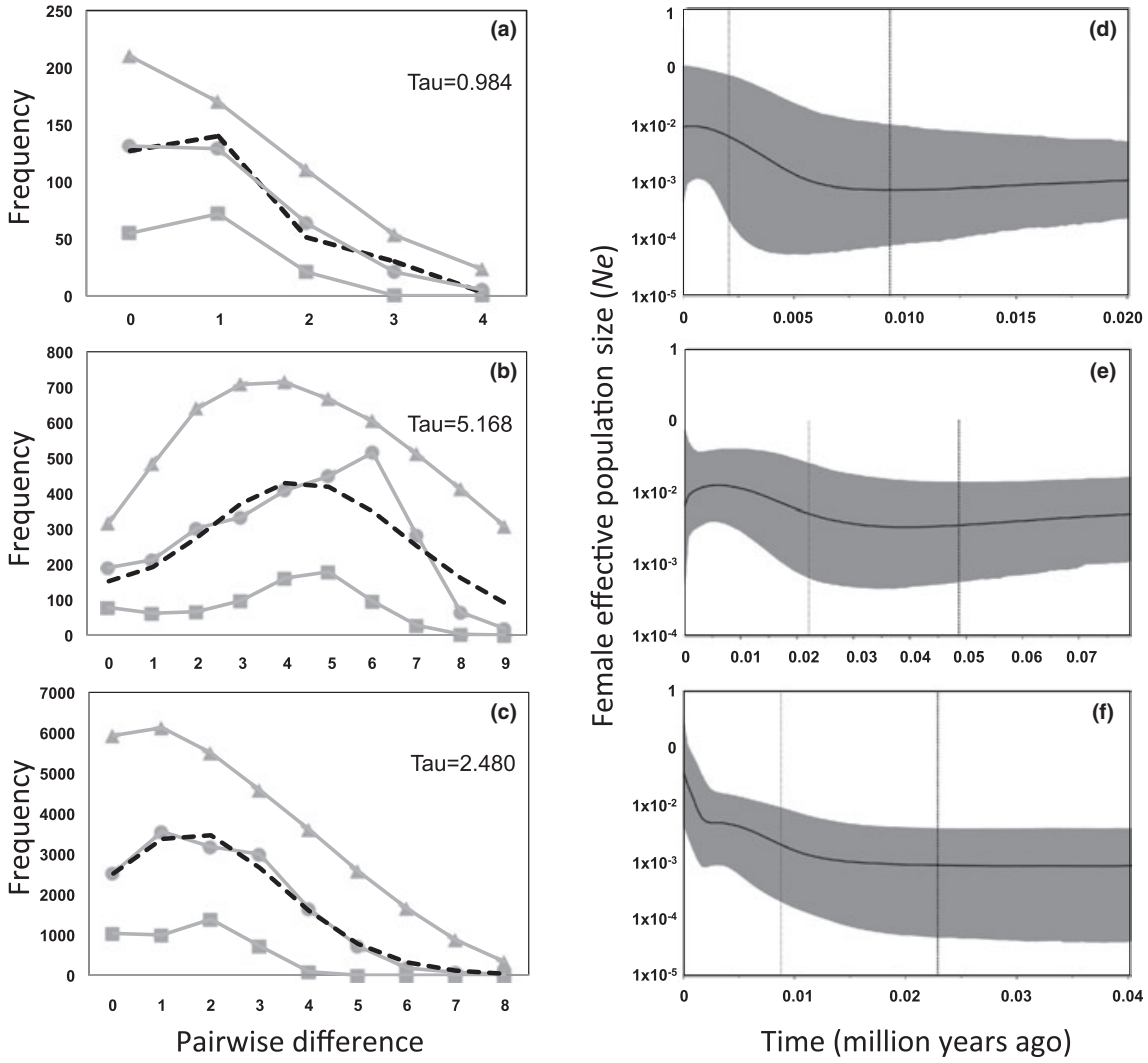

Figure 4 Pairwise nucleotide mismatch distributions and Bayesian skyline plots of *Hippocamelus bisulcus*. Mismatch distributions are shown for (a) the Central Chile Cluster (CCC), (b) the North Patagonia Cluster (NPC) and (c) the South Patagonia Cluster (SPC). Light grey lines reflect the modelled mismatch distributions under a scenario of exponential population growth. Symbols are as follows: triangle, upper 95% confidence interval (95% CI); circle, modelled curve; square, lower 95% CI. Bayesian skyline plots are shown for (d) CCC, (e) NPC and (f) SPC. The solid line is the median estimate of the female effective population size (*Ne*), and the grey area shows the 95% highest probability density (HPD).

their small population sizes and isolation. Peripheral populations are thought to occupy ecologically marginal environments, and may suffer founder effects and inbreeding (Hoffmann & Blows, 1994). Such populations are, however, of evolutionary importance, because they may be locally adapted (e.g. regarding climate tolerance) and have high levels of genetic differentiation, which are important for the maintenance of the biodiversity of the species (Lessica & Allendorf, 1995; Shafer *et al.*, 2011).

We have shown that the huemul, as a whole, has a high degree of mitochondrial haplotype ($n = 63$, $h = 0.92 \pm 0.01$) and nucleotide ($\pi = 3.723 \pm 2.08$) diversity, the latter being similar to that reported in North American mountain goat (*Oreamnos americanus*, 3.7%; Shafer *et al.*, 2011), which also inhabits mountainous areas with significant glacial influence. However, it is higher than the values reported for more mobile species such as moose (*Alces alces*, 1.8%; Hundertmark *et al.*, 2002). While these differences may reflect a comparatively larger historical female effective population size (*Ne*) in huemul, they may equally reflect differences in the mating and/or dispersal behaviour of these different species.

Although the huemul exhibits substantial genetic diversity, our data suggest that population structure may explain the disparity between species-wide and regional nucleotide diversity. Such a pattern is also evident for other Neotropical biogeographical regions (i.e. Maule, Valdivian temperate forest and Magellanic subpolar forest). However, further examination of these results reveals that (1) NPC populations (north of LGC) are more diverse than populations in SPC and CCC

(Table 2; Fig. 3), and (2) that the distribution of genetic diversity in the clusters is likely to be explained by recent population dynamics. Interestingly, the timing of population expansions observed in each of the clusters (Fig. 4a–c) is not the same (i.e. CCC: *c.* 6000 years ago; SPC: *c.* 16,000 years ago; NPC: *c.* 33,000 years ago). These differences lead us to infer an older age for the NPC populations, a hypothesis supported by the existence of a large haplotype diversity occurring at intermediate frequencies (Table 2).

The distribution of haplotypes in huemul populations does not follow the expected north–south distributional gradient. Northern populations (e.g. CCC) are expected to harbour a higher genetic diversity than southern populations owing to habitat persistence at lower latitudes during the LGM. However, our data show that CCC is the least diverse of the three clusters found (Table 2) and shows various haplotypes at low frequency (Fig. 3). This observation, complemented by the results of the demographic inference, suggests that CCC might have recently been derived from its geographically closest population, NPC. Such a hypothesis suggests that NPC is the ancestral population from which SPC and CCC derived. This hypothesis is supported by the higher genetic diversity of NPC, the intermediate frequency of many of its extant haplotypes, and the older dates of its demographic expansion relative to SPC and CCC. By contrast, the founders of SPC dispersed southwards towards the Patagonian fjords, from where they moved inland, eventually covering the current range of SPC. A colonization route from northern Patagonia through the fjords into the rest of southern Patagonia could explain the high genetic diversity of the otherwise small huemul populations in the fjords and the large dominant haplotype in SPC that differs from the fjord's haplotypes by one mutation.

Population structure of huemul

The use of mtDNA markers in combination with palaeoclimatic reconstruction provides detailed insights into the evolution of species during Pleistocene climatic oscillations. MtDNA is a powerful tool for phylogeographical studies because of its sensitivity to genetic drift owing to its small effective population size ($Ne_{\mathrm{mtDNA}} \approx Ne_{\mathrm{nuclear\ DNA}}/4$) and its high evolutionary rate in regions such as the CR. Female huemul are philopatric, while males are the main source of gene flow (Corti *et al.*, 2011), further allowing the mtDNA to detect subtle levels of population structure that may not be evident with biparentally transmitted nuclear markers of larger *Ne* (Avise, 2000). Our data suggest that there is a moderate amount of genetic structure among huemul populations corresponding to CCC, NPC and SPC.

The huemul's CR sequence variation revealed three distinct genetic groups (the average Φ_{ST} between them was 0.52; Table 4) geographically separated by the LGC, which divides the NPC and SPC populations. This pattern was detected with all the statistical methods implemented in this study, namely GENELAND, AMOVA and the G_{ST} versus N_{ST} comparison. Moreover, a population comparison also results in significant differentiation (Table 4), suggesting that individuals within a cluster are more likely to reproduce with each other than with individuals in other clusters, but the chance of mating between all individuals within a cluster is not the same (the average Φ_{ST} between populations in NPC and between populations in SPC is 0.415 and 0.335, respectively). These population differences within a cluster probably arise as a result of the geographical distances amongst them. Consistent with this hypothesis, we found a significant positive correlation between the genetic and geographical distance between all individuals ($r = 0.377$, $P = 0.001$) such that geographical distance explains *c.* 14% of the variance in genetic distance. This IBD pattern most likely reflects differences between samples of the northernmost and southernmost clusters, but it also captures some of the differences between populations within clusters (i.e. those that cause the large Φ_{ST} between populations within clusters; Table 4). This result is consistent with a species with low dispersal rates, such as is recorded for huemul (Corti, 2008). The northernmost huemul population in the CCC inhabits the Mediterranean-climate type area of Chile between 32° and 37° S. This area is characterized by mild and rainy winters and warm and dry summers. Furthermore, populations in North Patagonia from Nahuel Huapi National Park on the northern shores of LGC inhabit Valdivian temperate forest ecosystems. Parts of the southern Andes in this region have mountainous woodland and shrubland ecosystems, and also areas of temperate broadleaf and mixed forest represented by sub-Antarctic *Nothofagus* forests (Dinerstein *et al.*, 1995; González *et al.*, 2006).

LGC (46°32′ S, 72°33′ W; 130 km long and 3–21 km wide) appears to be the most important geographical barrier for the huemul, reducing gene flow between populations on either side of the lake (Fig. 5). During intermittent cold and warm periods, glaciers probably retreated and expanded rapidly, as observed for the period 13,000–14,000 years ago, when glaciers retreated 90–125 km to within about 20 km of their present margins (Turner *et al.*, 2005). Consistent with these observations, we found that among the various phylogeographical hypotheses tested with our data, the partition of the genetic variation according to GENELAND had the highest statistical support.

Refugia and post-glacial colonizations

The biotic consequences of climate change have attracted considerable attention (Hewitt, 2000; Weider & Hobæk, 2000). In particular, the 'refugia debate' centres on the possible retraction of habitats to limited areas that served as refugia for many species, especially at lower latitudes (Lessa *et al.*, 2003). Comparative phylogeographical studies have identified refugia in North America (Soltis *et al.*, 2006), the Arctic (Weider & Hobæk, 2000) and the European Alps (Tribsch *et al.*, 2005), among other regions. However, there are few studies in South America south of 45° S. Recent

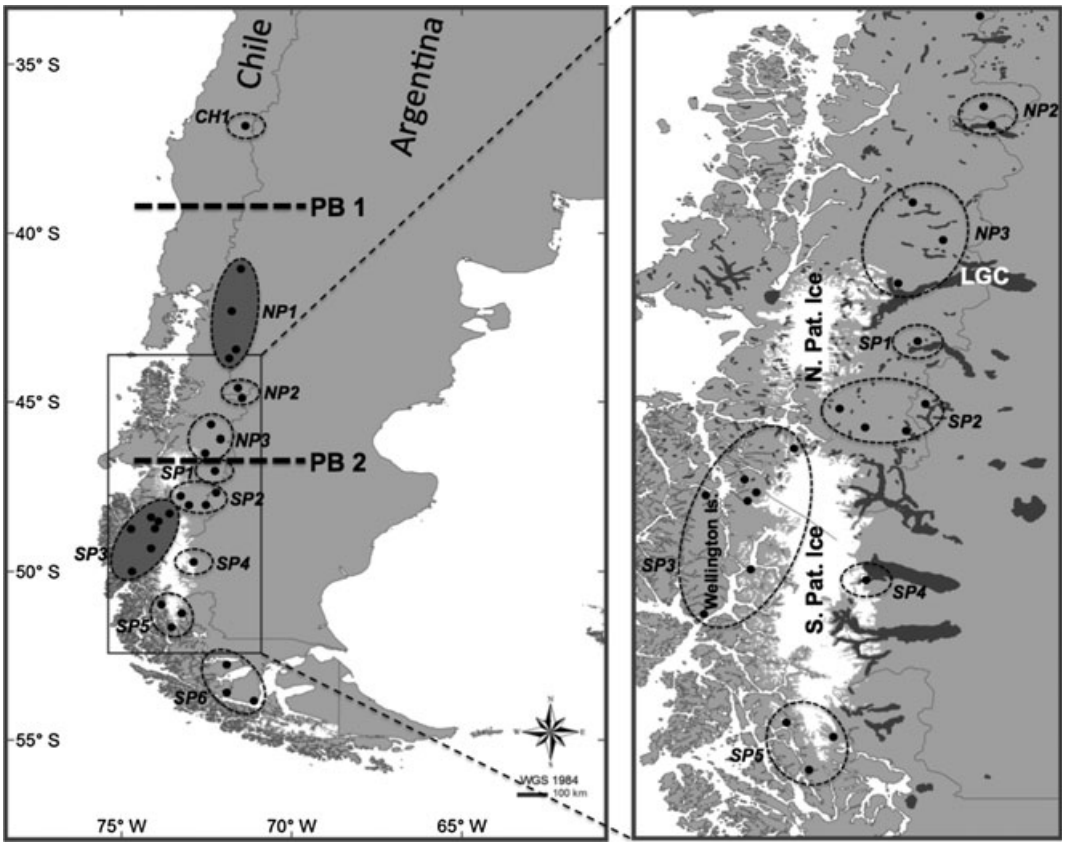

Figure 5 Phylogeographical breaks and refugia of *Hippocamelus bisulcus* in Chile and Argentina. The map shows the locations, clusters, phylogeographical breaks (PB 1 and PB 2) and, in the dark grey ovals, the Eastern Andes refugium (*NP1*) and the Patagonian fjords refugium (*SP3*) discussed in the text. The enlarged image shows the location of Lake General Carrera (LGC), North Patagonian icefields (N. Pat. Ice), South Patagonian icefields (S. Pat. Ice) and Wellington Island.

work suggests that terrestrial Patagonian taxa and some aquatic species probably survived glacial periods in southern refugia or alternatively recolonized the area from northern latitudes (Lessa *et al.*, 2010; Sérsic *et al.*, 2011; Zemlak *et al.*, 2011). Furthermore, it has recently been shown that populations of some South American species (e.g. *Liolaemus* lizards; Breitman *et al.*, 2012) survived several glaciation–deglaciation processes *in situ* without showing demographic fluctuations.

Our results support the hypothesis that the post-glacial colonization of huemul probably occurred from multiple refugia in the Patagonian north-east region (NPC) and the Patagonian fjords in the recent past. A variety of processes and directional range shifts suggest a mosaic of phylogeographical patterns, far more complex than the traditionally proposed north–south colonization route (Sérsic *et al.*, 2011). These refugia were located mainly in ice-free areas along the coast and in the Andes. Around 11,000 and 10,000 years ago, the occurrence of *Nothofagus betuloides* pollen increased by between 50% and 70%, suggesting that trees were the dominant vegetation at Tempano fjord (FT, Table 1) and on Wellington Island (SW, EW, Table 1) in the Patagonian fjords. This implies that the climate at the time was similar to that at present, in terms of both temperature (5 to 8 °C) and precipitation (Ashworth *et al.*, 1991). Under such conditions it is probable that huemul populations thrived in these refugia (e.g. the Patagonian fjords, the area that currently corresponds to Wellington Island and the site near Puelo Lake), as suggested by our demographic analyses, and do not support the suggestion that the Patagonian fjords were entirely uninhabitable owing to the presence of ice (Hulton *et al.*, 2002).

During the LGM the sea level was *c.* 120 m lower than at present. This lower sea level exposed the shelf around the Pacific Patagonian fjords, increasing the area of ice-free terrestrial habitats (Ashworth *et al.*, 1991; Rodríguez-Serrano *et al.*, 2008). Such areas probably served as land bridges, allowing the movement of animals from the mainland onto what are currently islands. The final stage of deglaciation occurred *c.* 10,000 years ago, a time when LGC discharged nearly 2000 km^3 of fresh water into the Pacific Ocean marking the final separation of the North and South Patagonian ice fields (McCulloch *et al.*, 2000; Turner *et al.*, 2005).

Implications for conservation

Currently, 1500–2000 huemul are estimated to live in Chile and the far south of Argentina (IUCN, 2012). Their limited geographical range has been strongly affected by recent

anthropogenic activities, resulting in a population decline to less than 10% of the estimated former population size of the species (Cabrera & Yepes, 1960). In recent years, the population of Chillán has been the focus of significant conservation efforts (Povilitis, 1998), although it has yet to show signs of significant recovery.

Our research supports the existence of three relatively distinct ecotypes of huemul. Examination of past climatic events suggests a long period of geographical separation of these three forms, resulting in their contrasting demographic histories. Given the dynamic history of huemul during the last 50,000 years, we suggest that the clusters should be considered to be three emerging ecotypes, with a broad contact area between NPC and SPC around LGC. Consequently, we suggest that these groups should be managed separately to preserve potential allelic combinations involved in local adaptation. Additional analyses at nuclear microsatellite loci will provide a higher resolution of the genetic differentiation among these populations and potentially lead to more appropriate conservation actions.

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SUPPORTING INFORMATION

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

Appendix S1 *Hippocamelus bisulcus* populations analysed for mtDNA control region variation, including population identifiers and sample sizes.

Appendix S2 Distribution of the 63 control region haplotypes observed in 275 *Hippocamelus bisulcus* samples.

Appendix S3 Correlation plot between the matrix of genetic distances and the matrix of geographical distances for all pairs of individuals of *Hippocamelus bisulcus*.

BIOSKETCH

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Author contributions: J.C.M. developed the ideas and obtained funding for the project; R.L., P.C., A.V. and J.C.M. collected the samples; V.V. conducted the DNA analyses; P.O., V.V. and J.C.M. analysed the data; and P.O., P.C. and J.C.M. wrote the paper. All authors read, commented on and approved the final version.

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