

# Evidence for cryptic glacial refugia from North American mountain sheep mitochondrial DNA

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## Abstract

The separation of populations by ice sheets into large refugia can account for much of the genetic diversity found in present day populations. The evolutionary implications of small glacial refugia have not been as thoroughly explored. To examine refugial origins of North American mountain sheep *Ovis* spp., we analyzed a 604 bp portion of the mitochondrial DNA (mtDNA) control region from 223 *O. dalli* and *O. canadensis*. Major refugia were identified in eastern Beringia and southern North America, and we found evidence for two smaller refugia situated between the Laurentide and Cordilleran glaciers. Our results are the first to demonstrate support for survival of any organism in the latter two refugia. These refugia also appear to have conserved a genetic signal that confirms past hybridization of *O. dalli* and *O. canadensis*.

## Introduction

Ice sheets can serve to fragment and isolate populations in differing refugia (Hewitt, 1996,2000), and this process of vicariance has been attributed as one of the major catalysts for the development of biodiversity in present temperate and arctic environments (Pielou, 1991; Willis & Whittaker, 2000). While isolation in major glacial refugia has been found to account for much genetic and morphological diversity (e.g. Ehrich *et al.*, 2000; Holder *et al.*, 2000; Brunhoff *et al.*, 2003; Flagsted & Røed, 2003; Dobeš *et al.*, 2004; Galbreath & Cook, 2004), quite understandably very little evidence is available to assess the evolutionary importance of smaller refugia. Smaller refugia are often difficult to identify due to a lack of fossil evidence or sufficient detail from studies of glacial limits.

Although examples are rare, genetic evidence has helped verify these 'cryptic' refugial sites and reconstruct their paleoecology. Plants of the *Packera* genus probably survived in southwestern Alberta, Canada in small ice-free areas between the Laurentide and Cordilleran ice

sheets or in nunataks (small ice-free 'islands' extending above the ice sheets) in the same area (Golden & Bain, 2000). Mitochondrial DNA (mtDNA) evidence supports the survival of the endemic Norwegian lemming *Lemmus lemmus* in Scandinavian refugia (Fedorov & Stenseth, 2001). There is strong evidence for the existence of ice-free areas in the northwest of the Canadian arctic archipelago; there is congruence among molecular evidence for refugial populations of rock ptarmigan *Lagopus mutus* (Holder *et al.*, 1999) and the arctic plants *Dryas integrifolia* (Tremblay & Schoen, 1999) and *Saxifraga oppositifolia* (Abbott *et al.*, 2000). The collared lemming *Dicrostonyx groenlandicus* also survived in this region (Fedorov & Stenseth, 2002). This finding has been independently verified by a lemming parasite *Paranoplocephala arctica* (Wickström *et al.*, 2003).

Small populations may have their genetic signal of refugial origin erased if post-glacial migration from other refugial populations is great enough. This may explain the lack of signal from *Rangifer tarandus*, which was hypothesized to have survived glaciation in the Canadian arctic archipelago (Flagsted & Røed, 2003), and in *S. oppositifolia*, which may have survived in northern Norway (Gabrielsen *et al.*, 1997). Thus the signal of past refugial separation may be stronger in

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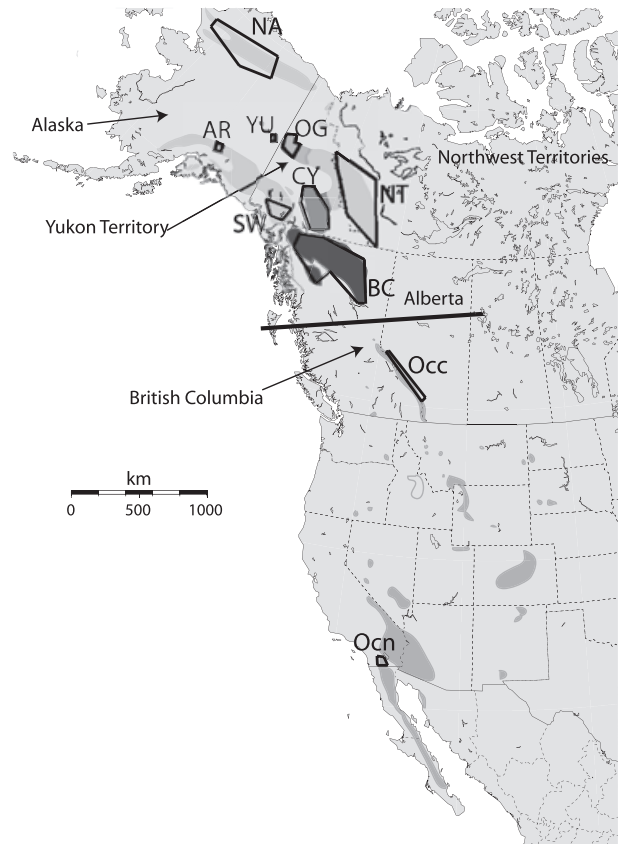
species with limited dispersal abilities (e.g. Fedorov & Stenseth, 2002).

Here we investigate the refugial origins of two species of mountain sheep (*Ovis* spp.) in North America. Mountain sheep are highly philopatric and are ideal study subjects to probe for the existence of cryptic montane refugia because they are superbly adapted to glacial conditions and currently inhabit areas within sight of large ice sheets (Geist, 1971). The treeless landscape of montane glacial environments affords sheep ample foraging opportunity on grasses and sedges and an abundance of suitable escape terrain for predator avoidance.

The two mountain sheep species are recognized based on morphological evidence (Cowan, 1940; Ramey, 1993) and their divergence is thought to be a result of long-term separation by glacial activity. *O. dalli* (thinhorn sheep) occupy mountain ranges in northwestern North America, and *O. canadensis* (bighorn sheep) inhabit areas in southwestern Canada and the United States (Fig. 1). Cowan (1940), Pielou (1991) and Geist (1999) hypothesized that the two North American mountain sheep species were isolated from one another by the ice sheets that separated Beringia and southern North America (Fig. 2). Two subspecies of thinhorn sheep *O. d. dalli* and *O. d. stonei* are also of particular interest in this study. These subspecies are based on regional coat colour differences, however there is complete intergradation between light and dark morphs.

Sheep currently inhabit four regions known to have been ice-free during the Wisconsin glaciations (approximately 70 000–10 000 years ago). The two major refugia of Beringia and southern North America are well documented. However, portions of the Mackenzie Mountains of Canada's Northwest Territories (also known as 'Easternmost Beringia'; Dyke & Prest, 1987; Duk-Rodkin & Hughes, 1991), and a region further south in northeastern British Columbia (Catto *et al.*, 1996) also remained ice-free. The glacial history of these regions is complex. Easternmost Beringia was influenced by Laurentide and montane glaciation, and the ice-free area appears to have been effectively cut off from other parts of Beringia during much of the last ice age (Catto, 1996). Northeastern British Columbia was a crossroads for Laurentide and Cordilleran ice sheets and montane glaciers. From investigations near Fort St. John, British Columbia (Fig. 2) Catto *et al.* (1996) found that coalescence of ice sheets did not occur during the last ice age. Since the ice sheets did not advance at the same time in this region a temporally and geographically shifting ice-free zone existed. Numerous isolated foothills of the Rocky Mountains also remained ice-free during glacial maximum.

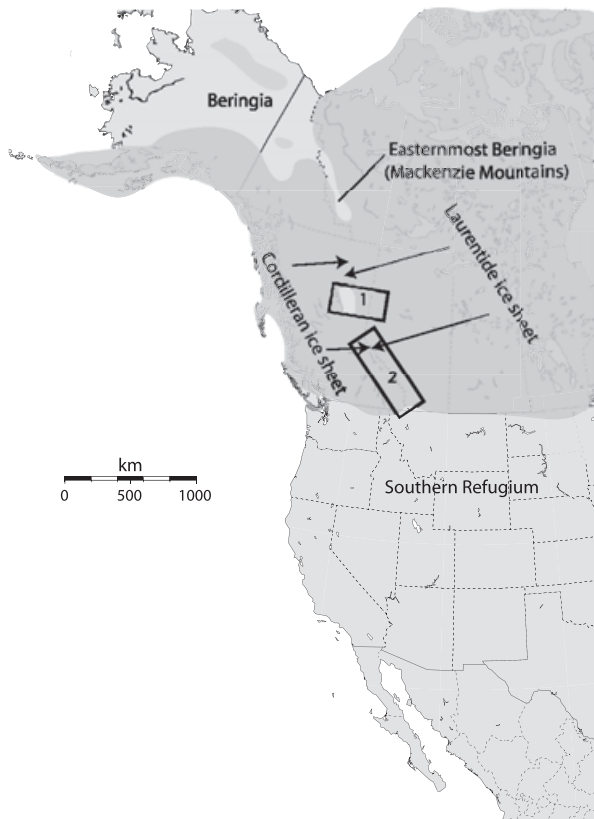
Both of these areas are within the disputed Ice-Free Corridor, a proposed migration route between Beringia and southern North America (e.g. see Pielou, 1991; Mandryk *et al.*, 2001 for a discussion of this region). Most



**Fig. 1** Distribution of mountain sheep (*Ovis* spp.) in North America and regions sampled for mtDNA and relevant territory, province and state names. Shaded areas represent approximate boundaries of sheep habitation. The solid line divides putative species: *O. dalli* to the north and *O. canadensis* to the south. For *O. dalli* darkness of shaded areas represent relative darkness of coat pelage. All *O. dalli* populations except OG, CY and BC are almost exclusively comprised of white coloured sheep (see Materials and methods for site names and further description of colour variation and subspecies of *O. dalli*).

now agree that the Ice-Free Corridor was not open throughout the last ice age since coalescence of ice sheets has been verified at its southern end in central Alberta (Levson & Rutter, 1996) but little is known about the importance of areas in this region as glacial refugia. Other than geologic evidence to support ice-free conditions, we are not aware of any studies that confirm habitation by any organism of the two postulated refugia throughout glaciation. Palynological data, however, confirms a herb and shrub dominated vegetation (containing plants suitable for consumption by sheep) in the Mackenzie Mountains (Szeicz & MacDonald, 2001) during the Lateglacial (ca. 12 000–10 000 C year BP) and Catto *et al.* (1996) postulated that the region in northeastern British Columbia could have supported plant and animal life.

Based on evidence from glacial limits it appears that sheep could have survived glaciation in smaller glacial



**Fig. 2** Glacial coverage of North America during glacial maximum (approximately 21 000 years ago). Glacial limit approximations are based on Duk-Rodkin (1999), Mandryk *et al.* (2001) and Catto *et al.* (1996). North America was glaciated by both the Cordilleran and Laurentide ice sheets. Arrows show general direction of ice sheet advance. Two major refugia existed in Beringia and areas of southern North America. A small ice-free area in the Mackenzie Mountains has also been verified. Box 1 represents the study area of Catto *et al.* (1996), who found that the independently advancing and retreating ice sheets did not coalesce in this region. This region should be viewed as a temporally and geographically fluctuating ice free zone. For example between 14 000 and 10 000 years ago the Cordilleran glacier advanced further east, but by this time the Laurentide glacier had retreated. Little or no evidence is available from the region between Easternmost Beringia and box 1. We have represented this area as covered in ice; however, the exact history of this region is uncertain. The glacial history of the area in box 2 differs from box 1 since coalescence did occur between ice sheets at glacial maximum (e.g. Levson & Rutter, 1996), presumably making survival of sheep in this region impossible.

refugia in the Northwest Territories and British Columbia (Fig. 2). In the context of this paper, we wish to test the null hypothesis that sheep populations survived only in the major refugia as proposed by Cowan (1940), Pielou (1991) and Geist (1999). Recent analysis of nuclear DNA found that populations of *O. dalli* in British Columbia have lower genetic diversity than populations that inhabited major glacial refugia (Worley *et al.*, 2004). This

may indicate survival in a small glacial refugium; however, it is also possible that the result was caused by small number of individuals migrating into this area after glacial retreat. The major refugium hypothesis also applies to the glacial separation of North American *Ovis* species. Interestingly, in his unpublished work Ramey (1993) has some evidence that glacial separation of the species in the major refugia did not occur. Ramey (1993) found that an *O. dalli* haplotype from northern British Columbia was more similar to *O. canadensis* than to *O. dalli* haplotypes. However, this result was based on a single sample from British Columbia and remains difficult to interpret.

To assess refugial origins of populations and reconstruct mountain sheep phylogeography and historical population demography we use a 604 base pair portion of the rapidly evolving mtDNA control region. We use sequences from 223 *O. dalli* and *O. canadensis* from across their range in North America (Fig. 1). If the null hypothesis is accepted and *Ovis* species indeed survived only in the major refugia, then populations currently inhabiting areas outside of the refugia should be of recent origin. This will be reflected in their haplotypes with divergence times showing only recent (post-glacial) separation of populations. When haplotypes are viewed in a haplotype network, we should find no major divisions between the lineages of refugial populations and those of post-glacial origin. Similarly, if the two *Ovis* species have been separated for long periods then we should find a deep division between species haplotypes. Here we also wish to test the validity of *O. dalli* subspecies. Since these subspecies intergrade across their range monophyly is not expected. However, if subspecies groups are supported we should find that analysis of mtDNA molecular variance shows a significant difference between subspecies. Finally, we wish to test to what degree glaciation has affected mountain sheep populations. Since much of current sheep range was covered by glaciers during the ice age, we expect to find evidence for post-glacial rapid expansion in areas covered by ice.

## Materials and methods

We sampled sheep from eight areas across the range of *O. dalli* (Fig. 1). Three locations were sampled in Alaska (in brackets: abbreviation for population and number of samples from location): Alaska Range (AR, 3), Yukon Charley Rivers National Preserve (YU, 4) and Northern Alaska (NA, 4). Five geographic regions were sampled in Canada: Ogilvie Mountains (OG, 9); Mackenzie Mountains, Northwest Territories (NT, 16); Pelly Mountains, Central Yukon (CY, 18); Southwest Yukon (SW, 12); and British Columbia (BC, 22).

We sampled Rocky Mountain bighorn *O. c. canadensis* from the Canadian Rocky Mountains (Occ, 11) and incorporated one sequence from Genbank (accession number AY091486, Hiendleder *et al.*, 2002) also from

that region. We also used Genbank samples (accession numbers AF076911–AF076917, Boyce *et al.*, 1999) of seven haplotypes from southern California *O. c. nelsoni* (Ocn, 124) desert bighorn in our analysis.

Two subspecies of *O. dalli* are currently recognized based on differences in colour morphology. Nearly all individuals in AR, YU, NA, NT and SW have a white coat and these populations are classified as *O. d. dalli*. *O. d. stonei* individuals have dark coat coloration, however the degree of darkness is variable and is never consistent within a population. Sheep representing this subspecies are found in BC and CY. The OG population has individuals of either all white or darker coloration and so it is not easily classified into either subspecies.

We based population divisions on geographic factors, i.e. mountain range, and for *O. dalli* sheep major differences in ram colour morphology. All *O. dalli* sheep from Alaskan populations are predominantly white, while very few sheep in SW and NT have some grey colour on their flanks. Approximately 35–40% of OG sheep have dark colouration on their flanks, and the rest of the population is completely white or white with dark tails. All CY sheep are dark in coloration, but are on average lighter than sheep in BC (Sheldon, 1911; Loehr, unpublished data). In all of these populations a great deal of individual variation is also prevalent, such that the darkest sheep in OG look similar to the lighter sheep in CY; a similar relationship exists between CY and BC (also see Fig. 1).

Most samples of *O. dalli* were taken from horn core samples of hunter-killed rams in Yukon, British Columbia and Northwest Territories from 1994 to 2000. Alaskan samples comprised whole blood samples of both rams and ewes collected between 1999 and 2002. Rocky mountain *O. canadensis* samples also comprised horn core samples. Horn corings were stored dry in sealed paper envelopes, while blood samples were stored at  $-20^{\circ}\text{C}$  in EDTA. Genomic DNA was extracted from approximately 0.5 mL of horn material per sample using a tissue extraction kit (Qiagen, Crawley, West Sussex, UK). Blood samples were extracted using a phenol chloroform technique (Sambrook *et al.*, 1989) from 200  $\mu\text{L}$  of blood.

The human primers L15999 (5'-ACC ATC AAC ACC CAA AGC TGA-3') and H16498 (5'-CCT GAA GTA GGA ACC AGA TG-3') were used to amplify the control region of mtDNA in the 5' to 3' direction. Polymerase chain reactions (PCR) were carried out in 50  $\mu\text{L}$  reactions containing 5  $\mu\text{L}$  DNA template, 0.4  $\mu\text{M}$  of each primer, 100  $\mu\text{M}$  dNTP's, 1.5 mM  $\text{MgCl}_2$  and 1 unit *Taq* polymerase (Bioline, London). PCR profiles comprised 32 cycles of 94  $^{\circ}\text{C}$  denaturing for 30 s, 50  $^{\circ}\text{C}$  annealing for 30 s, and extension at 72  $^{\circ}\text{C}$  for 40 s. Cycles were preceded by 3 min at 94  $^{\circ}\text{C}$  and terminated with 5 min at 72  $^{\circ}\text{C}$ . PCR products were gel purified (Qiagen) and quantified before sequencing. Purified PCR products were sequenced using primers as above and run on an ABI 3730. Editing and alignment was carried out using the

software SeqScape (Applied Biosystems, Foster City, USA).

A Siberian snow sheep *O. nivicola* sample was used as an outgroup for analysis. It was chosen because *O. nivicola* are the closest interspecific relative of *O. dalli* and *O. canadensis*. A minimum spanning network was constructed using the pair-wise number of differences among haplotypes as implemented in Arlequin ver. 2.001 (Schneider *et al.*, 2000).

To determine population structure based on species/subspecies groups we used Analysis of Molecular Variance (AMOVA) as implemented in Arlequin (Excoffier *et al.*, 1992). *O. canadensis* subspecies comparisons were not made since sampling from *O. canadensis* sheep was less extensive. A detailed mtDNA analysis of *O. canadensis* subspecies has been done by Ramey (1993).

Haplotype divergence times were calculated with Mdiv (Nielsen & Wakely, 2001). This program employs a Bayesian framework using the Hasegawa–Kishino–Yano nucleotide substitution model (HKY 1985) to correct for multiple hits, and produces a joint estimation of multiple demographic parameters ( $\theta$ , nonequilibrium-based migration rate, time to most recent common ancestor (TMRCA) and time of divergence for any pair of populations). These two estimates differ because haplotypes can start to diverge before a single population diverges into two or more populations. Using both estimates it is possible to estimate the time that haplotypes began to diverge and when populations separated or experienced secondary contact.

To calculate the time since the divergence of haplotypes we used a molecular clock of 24% per locus per million years, which has been estimated for domestic and wild sheep from the complete length of the mtDNA control region (Hiendleder *et al.*, 2002). Since the control region evolves too rapidly for comparison based on fossil evidence of separate species, an indirect method was used. Hiendleder *et al.* (2002) first estimated the protein-coding sequence distance between the most divergent lineages of sheep (*O. canadensis* and *O. aries*). This was then referenced against molecular divergence of the complete mtDNA genome for cow (*Bos* spp.) and sheep, and then further referenced using fossil evidence of a divergence of the *Ruminantia* and *Cetacea* 60-million-year ago. To be conservative we also consider a much faster mutation rate estimate of 38% per locus per million years, which has been estimated using cattle mtDNA from a 240-bp portion of the same portion of the control region that we sequenced (Troy *et al.*, 2001).

We calculated indicators of within population genetic diversity (haplotype diversity and nucleotide diversity) using Arlequin. To probe for evidence of population expansion we calculated Fu's  $F_S$  test (Fu, 1997), which is considered a sensitive indicator of population expansion. It compares the number of haplotypes observed with the number of haplotypes expected in a random sample. This test assumes that recombination does not

occur and uses an infinite-sites model. Further analysis of population expansion was done using mismatch distribution analysis (MDA). MDA assesses the shape of the distribution of the number of observed differences between pairs of DNA sequences. A population that has experienced rapid growth exhibits a unimodal wave when results from MDA are graphed. MDA produces an age expansion parameter ( $\tau$ ), which is a relative measure of the time (in generations) since population expansion and is useful to date the initiation of rapid population growth (see Rogers & Harpending, 1992; Schneider & Excoffier, 1999). MDA also produces an estimate of effective population size at the start ( $\theta_0$ ) and at the end ( $\theta_1$ ) of expansion.

## Results

There were 64 unique haplotypes defined by 90 polymorphic sites, including 84 transitions and 6 transversions in the 223 *O. dalli* and *O. canadensis* sequences we analyzed. The mean number of pair-wise differences between sequences was 17.31 (SD  $\pm$  7.72) and nucleotide diversity was 0.035 (SD  $\pm$  0.017). Population specific results are presented in Table 1.

We found extensive paraphyly between *O. dalli* and *O. canadensis*. The minimum spanning network (Fig. 3) showed that *O. dalli* haplotypes from BC and NT and *O. canadensis* haplotypes from southern Canada were more closely related to each other than to haplotypes from their putative species. A large percentage of *O. dalli* genetic variation was explained by dividing populations into their respective mountain ranges, showing that molecular variance was geographically structured (Table 2). Divisions based on species or *O. dalli* subspecies resulted in no significant difference (Table 3).

The shortest divergence times were estimated at 7000 years for OG and YU, and 11 000 years for CY and SW (Table 4). We found a divergence time of about 68 000 years for the BC and southern Canada *O. canadensis* haplotypes. The divergence time between these two populations is of particular interest. To be conservative we also calculated the divergence time using the much faster mutation rate calculated for cattle (Troy *et al.*, 2001). The faster mutation rate resulted in a

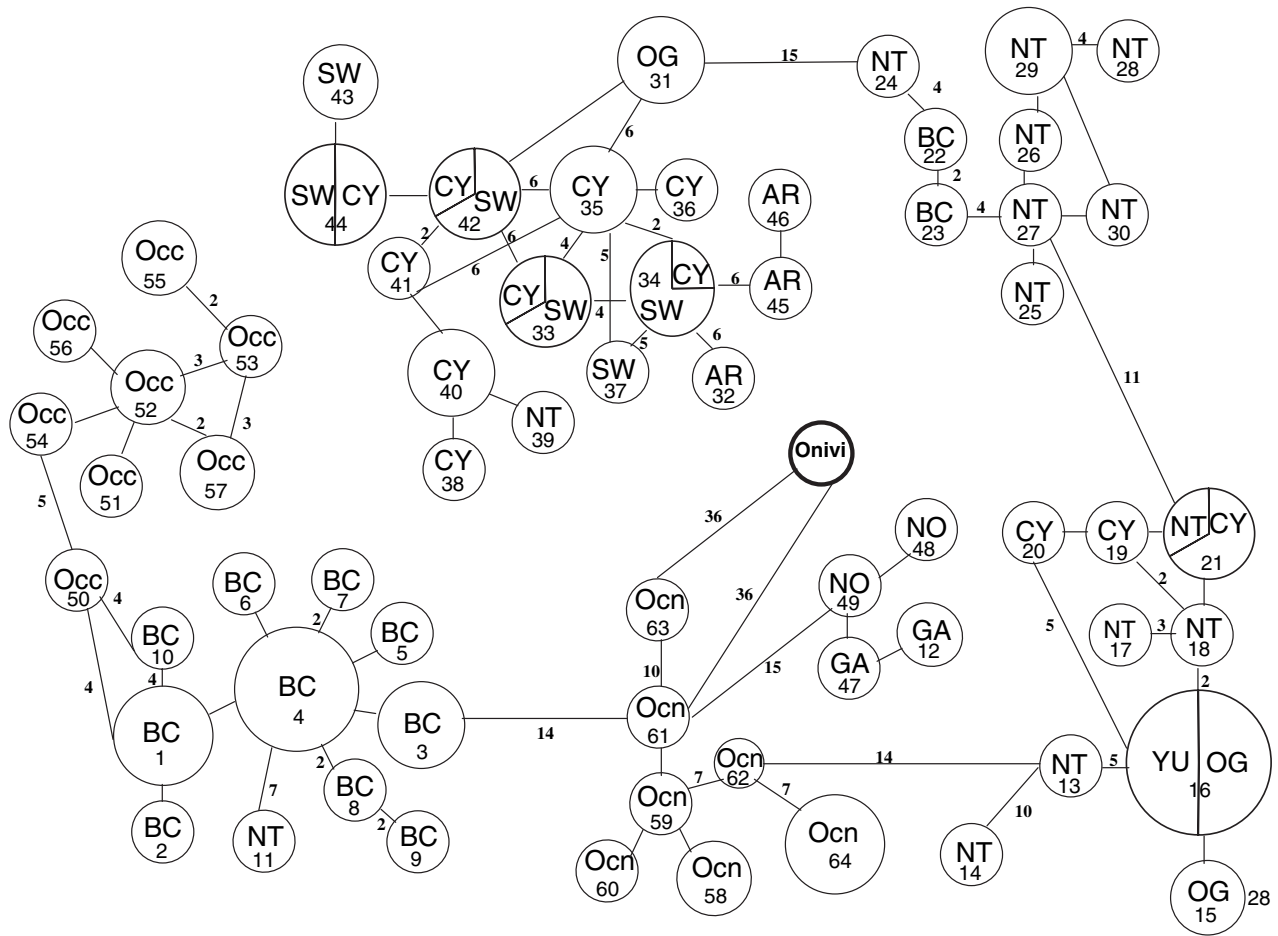
divergence time of 43 305 years before present. Again using the mutation rate of 24% calculated for sheep we estimated the TMRCA. The TMRCA for BC/Occ was about 431 000 (Table 4). A similar TMRCA was found for most populations surveyed. However, we found a relatively short TMRCA for populations at the opposite ends of mountain sheep distribution. *O. dalli* sheep from northern Alaska and *O. c. nelsoni* sheep from the southern US shared a common ancestor around 250 000 years ago. In the haplotype network, *O. c. nelsoni* haplotypes had the least number of steps to Siberian snow sheep *O. nivicola*.

To test for evidence of population growth we used Fu's  $F_S$  test. This test is considered significant at  $\alpha = 0.05$  when  $P = 0.02$  (Fu, 1997). Population growth was found for NA and Occ and bordered on significance for NT (Table 5). For these populations MDA also indicated population growth since the null hypothesis of population expansion was not rejected. Results for Fu's  $F_S$  and  $P$  (SSD) were also consistent in the case of Ocn, since both tests rejected the possibility of growth. The OG population also appears to not have experienced growth since the  $P$  (SSD) result neared significance and  $\theta_0$  and  $\theta_1$  were also nearly identical (Table 5).

There were inconsistent test results for the CY, SW and AR populations. Fu's  $F_S$  test detected no growth for these populations, but rapid population expansion could not be ruled out by the MDA test. This was also the case for BC. However, the star shaped pattern of haplotypes from BC suggests a population bottleneck making the population expansion scenario more likely (Fig. 3). Twenty of the 22 BC samples were found in the star shaped haplogroup (see Fig. 3, haplotypes 1–10). The remaining two haplotypes (22 and 23) were similar to an NT haplogroup and are a probable result of contact between NT and BC about 48 000 years ago. We then tested the BC haplotypes 1–10 again for evidence of population expansion. In this case Fu's test was significant ( $F_S = -4.77$ ,  $P = 0.003$ ) demonstrating strong support for population expansion. Population growth was also supported by MDA analysis ( $P$  (SSD) = 0.31). The value of  $\tau$  was estimated at 1.83 (95% CI = 0.42–2.79) which was similar to that estimated for all BC haplotypes.

**Table 1** Genetic diversity for *O. dalli* and *O. canadensis* populations based on mtDNA control region. Number of samples ( $N$ ), number of unique haplotypes (Hn), haplotype diversity ( $h$ ), nucleotide diversity ( $\pi$ ), number of segregating sites ( $S$ ) (the \* indicates that a transversion was observed in the population) and mean number of pair-wise differences ( $d$ ) between haplotypes.

	OG	NT	CY	SW	AR	YU	NA	BC	Occ	Ocn
$N$	9	16	18	12	3	4	4	22	11	124
Hn	3	14	12	6	3	1	4	12	8	7
$h$ ( $\pm$ 95%CI)	0.722 (0.097)	0.975 (0.035)	0.948 (0.033)	0.894 (0.054)	1.000 (0.272)	0	1.000 (0.177)	0.896 (0.045)	0.946 (0.054)	0.773 (0.025)
$\pi$ ( $\pm$ 95%CI)	0.020 (0.011)	0.024 (0.013)	0.027 (0.014)	0.011 (0.006)	0.017 (0.014)	0	0.004 (0.003)	0.013 (0.007)	0.008 (0.005)	0.013 (0.007)
$S$	22*	47*	35*	14	11*	0	3	39	11*	20*
$d$ (SD)	10.89 (5.47)	11.24 (5.39)	12.69 (6.00)	5.14 (2.68)	7.33 (4.73)	0	1.67 (1.22)	6.97 (3.40)	3.02 (1.70)	6.56 (3.12)



**Fig. 3** Minimum spanning network of haplotypes, rooted with *Ovis nivicola*. Occ and Ocn populations are *O. canadensis*, all others are *O. dalli* (see methods and Fig. 1). *O. d. stonei* populations are CY and BC. Intergradation between subspecies occurs in OG. All remaining *O. dalli* populations represent the *O. d. dalli* subspecies. Circle size is proportional to the number of times the haplotype was observed. An exception is the Ocn haplotype group. Since the number of samples in this population was very high, haplotypes are represented in proportion to number of samples from the same population. Numbers inside circles refer to haplotype identification number. Numbers next to lines refer to number of steps between haplotypes. If no number is present then one step separates haplotypes.

Molecular variance of <i>O. dalli</i> and <i>O. canadensis</i>				
Criteria for division	Components	% of total variation	Fixation indices	P
Mountain range	Among populations (i.e. mountain ranges)	67.5	$\Phi_{st}$ 0.67	<0.001
	Within populations	32.5		
Species	Among species groups	9.4	$\Phi_{ct}$ 0.09	0.18
	Among populations within species groups	58.9	$\Phi_{sc}$ 0.65	<0.001
	Within populations	31.7	$\Phi_{st}$ 0.63	<0.001

**Table 2** Analysis of molecular variance (AMOVA) of *O. dalli* and *O. canadensis* populations based on mountain range and species.

**Discussion**

**Multiple refugia and gene flow between species**

Our results support the possibility of four glacial refugia. As expected, two major refugia were verified: one

northwest of the major ice sheets in Beringia, and a second in southern North America. Support for long-term isolation in these large refugia is found in population divergence times. Divergence times of 167–307 000 years for *O. c. nelsoni* from southern North America and *O. dalli* populations from Beringia suggest

**Table 3** AMOVA of *O. dalli* based on mountain range and subspecies.

Criteria for division	Molecular variance of <i>O. dalli</i> only			P
	Components	% of total variation	Fixation indices	
Mountain range	Among populations (i.e. mountain ranges)	57.8	$\Phi_{st}$ 0.58	<0.001
	Within populations	42.1		
Subspecies	Among subspecies groups	-11.1	$\Phi_{ct}$ -0.11	1.0
	Among populations within subspecies groups	69.8	$\Phi_{sc}$ 0.63	<0.001
	Within populations	41.3	$\Phi_{st}$ 0.53	<0.001

that contact has not occurred since the mid Pleistocene. During the last ice age the mass of ice separating these two refugia were thought to have completely divided *O. dalli* from *O. canadensis* (Pielou, 1991; Geist, 1999). Our evidence does not support this hypothesis, instead the existence of two additional refugia in the Mackenzie Mountains and northeast British Columbia is probable. This region appears to have allowed historic gene flow between the two species, and harboured populations in refugia during the last glacial maximum.

There is evidence for contact between NT, CY, OG and BC that predates glacial maximum, suggesting a refugium in the Mackenzie Mountains existed (Table 4). A divergent haplogroup is also dominated by NT haplotypes (see Fig. 3 haplotypes 22–30). Two BC haplotypes exist in this haplogroup, however based on divergence estimates this is a result of contact between these populations prior to glacial maximum. Since divergence estimates can only be approximate there is still a possibility that gene flow also occurred after glacial maximum. Although this refugium may not have been completely separated from Beringia, it appears evident that within this region there has been considerable differentiation of haplotypes, supporting a hypothesis of continual long-term habitation of the Mackenzie Mountains.

The BC *O. dalli* population also had divergence times with adjacent populations that predated glacial maximum. Interestingly, this population appears to be a product of hybridization between species since haplotypes similar to both *O. dalli* and *O. canadensis* were present. However, most haplotypes in this *O. dalli* population were more similar to *O. canadensis* haplotypes than to other *O. dalli*. This finding contradicts Geist's (1971) hypothesis of *O. dalli* range expansion from a Beringian refugium into British Columbia. A second explanation may be that the BC population was colonized by *O. canadensis* haplotypes after ice sheets retreated. Evidence against this comes from an estimated divergence time between BC *O. dalli* and *O. c. canadensis* that predates glacial maximum by about 50 000 years.

Colour morphology also suggests that a post-glacial colonization of BC by *O. canadensis* is unlikely. *O. dalli* in BC share similar coat pelage with sheep further north in CY and both of these populations differ greatly from the

coat colour of *O. canadensis*. If BC were initially colonized by *O. canadensis* and sheep from CY then migrated south and interbred with *O. canadensis* (resulting in a change in morphology to resemble CY *O. dalli*) then CY haplotypes would have been found in BC. However, post-Wisconsinan glaciation gene flow between these populations is not supported: no CY haplotypes were found in BC. Nuclear DNA microsatellite evidence also showed a high degree of population structure between BC and CY suggestive of limited gene flow (Worley *et al.*, 2004). However, it is still possible that a small number of migrants introduced the present coat colour to the BC population, and a strong selective advantage lead to it spreading throughout the population. Unfortunately, horn morphology and body size do not give a clear indication of refugial origin since BC sheep are intermediate in size between *O. dalli* and Occ sheep (Geist, 1971; Ramey, 1993).

We propose a third explanation, which is most congruent with the evidence available. Although parts of the range occupied by BC *O. dalli* remained ice-free (Catto *et al.*, 1996), no large ice-free areas have been identified in the southern Canadian Rocky Mountains currently inhabited by *O. canadensis* (e.g. Levson & Rutter, 1996). The mtDNA from *O. canadensis* supports a scenario in which the southern Canadian Rockies were recolonized after ice retreated (Luikart & Allendorf, 1996). Thus we propose that contact occurred between *O. canadensis* and *O. dalli* prior to glacial maximum. As ice advanced the BC *O. dalli* population survived glaciation in small ice-free areas described by Catto *et al.* (1996), while *O. canadensis* populations in the Canadian Rockies were overcome by ice and went extinct. The star shaped pattern in the haplotype network for BC (Fig. 3) indicates a possible population bottleneck (Slatkin & Hudson, 1991) consistent with this scenario. Rapid expansion for BC was estimated at 7300 years ago. This relatively recent onset of population growth agrees well with melting of ice in this region, which has been dated to about 10 000 years ago (Catto *et al.*, 1996). Indicators of nuclear genetic diversity were relatively low for BC populations, also lending some support to a bottleneck hypothesis (Worley *et al.*, 2004). Results should be interpreted with some caution since extinction events,

**Table 4** Below diagonal: Mdiv estimates of divergence times for population pairs. Numbers in parentheses are 95% credibility intervals of the estimate. Divergence times before present are presented below credibility intervals. Above diagonal: Mdiv estimate and number of years to most recent common ancestor. The times in years (in bold) were obtained based on a mutation rate for the control region (24% per million years) and an average generation time of 3 years.

	OG	NT	CY	SW	BC	YU	AR	NA	Occ	Ocn
OG	–	2.34 <b>297 694</b>	4.40 <b>326 070</b>	4.93 <b>296 750</b>	3.97 <b>402 040</b>	8.83 <b>253 029</b>	4.54 <b>283 750</b>	5.45 <b>319 336</b>	5.1 <b>348 633</b>	3.25 <b>341 504</b>
NT	0.30 (0.17–1.94)	–	2.74 <b>309 580</b>	2.40 <b>301 687</b>	2.64 <b>399 011</b>	2.23 <b>311 152</b>	1.96 <b>309 823</b>	1.97 <b>348 341</b>	2.54 <b>355 865</b>	1.55 <b>341 283</b>
CY	0.95 (0.45–4.87)	1.39 (0.43–4.73)	–	5.77 <b>355 488</b>	3.75 <b>445 649</b>	4.57 <b>308 237</b>	3.37 <b>333 638</b>	3.81 <b>402 332</b>	3.97 <b>411 991</b>	3.05 <b>427 358</b>
SW	1.72 (0.89–4.88)	1.32 (0.48–4.86)	0.18 (0.00–1.86)	–	4.39 <b>463 687</b>	5.46 <b>277 266</b>	2.09 <b>144 504</b>	6.43 <b>426 155</b>	5.74 <b>484 237</b>	3.90 <b>418 438</b>
BC	1.74 (0.56–4.85)	1.65 <b>998</b>	11 <b>336</b>	2.10 (0.79–4.84)	–	3.87 <b>349 711</b>	3.66 <b>438 914</b>	3.97 <b>407 855</b>	4.99 <b>457 417</b>	2.78 <b>378 268</b>
YU	0.23 (0.06–1.95)	0.28 (0.19–4.86)	3.86 (0.58–11.7)	4.16 (0.84–9.74)	1.06 (0.46–4.86)	–	4.51 <b>237 245</b>	9.50 <b>239 974</b>	5.6 <b>272 708</b>	2.77 <b>282 771</b>
AR	0.97 (0.35–4.88)	39 <b>156</b>	260 <b>349</b>	211 <b>250</b>	95 <b>786</b>	4.24 (0.76–9.74)	–	4.88 <b>376 167</b>	4.90 <b>430 026</b>	2.72 <b>423 583</b>
NA	4.16 (0.76–9.74)	1.16 (0.28–4.87)	0.81 (0.38–4.85)	1.32 (0.33–4.85)	1.87 (0.55–4.84)	2.23 <b>042</b>	4.18 (0.64–9.74)	–	4.86 <b>284 258</b>	2.27 <b>253 306</b>
Occ	4.08 (0.43–7.78)	213 <b>956</b>	219 <b>646</b>	379 <b>099</b>	219 <b>852</b>	5.72 (1.12–9.76)	3.87 (0.90–7.79)	4.30 (0.35–5.87)	–	1.86 <b>256 477</b>
Ocn	2.12 (0.82–5.86)	270 <b>401</b>	316 <b>517</b>	338 <b>917</b>	68 <b>567</b>	4.48 (0.62–7.81)	3.87 (0.90–7.79)	2.60 <b>352</b>	1.03 (0.22–4.86)	–
	222,766	242,201	219,964	306,854	182,331	177,625	302,115	167,383	142,027	

**Table 5** Indicators of historical population demography. Fu's  $F_s$  is an indicator of population growth. Other demographic parameters (95% CI) were calculated using MDA:  $\tau$  (time estimate for onset of expansion),  $\theta_0$  (population size estimate at onset of growth) and  $\theta_1$  (estimate after growth).  $P$  ( $SSD_{obs}$ ), measures the probability of deviation from a hypothesis of rapid population growth according to the mismatch distribution (see Fig. 4). Years since expansion initiation are estimated from  $\tau$ . No statistics were calculated for YU, since all haplotypes were the same in this population.

	OG	NT	CY	SW	BC	AR	NA	Occ	Ocn
Fu's $F_s$ (P value)	7.75 (0.99)	–3.85 (0.03)	0.07 (0.48)	1.08 (0.72)	–0.82 (0.40)	0.81 (0.41)	–2.18 (0.01)	–2.97 (0.02)	11.42 (0.99)
$\tau$	7.00 (2.53–11.25)	11.83 (4.91–26.83)	3.00 (0.98–9.58)	7.55 (3.82–12.38)	2.00 (0.00–5.07)	11.72 (6.49–30.72)	1.84 (0.00–3.80)	3.21 (1.27–4.24)	12.27 (5.02–23.26)
$\theta_0$	2.10 (0.00–2.84)	4.86 (0.00–29.2)	12.32 (0.00–29.2)	0.00 (0.00–2.16)	1.46 (0.00–7.70)	0.00 (0.00–17.00)	0.00 (0.00–1.35)	0.00 (0.00–2.61)	0.00 (0.00–10.48)
$\theta_1$	2.10 (0.22–59.13)	21.60 (12.41–191.19)	14.61 (3.31–310.61)	13.22 (9.04–3301.97)	38.71 (4.80–6773.3)	841.09 (24.06–7013.6)	3776.3 (3761.7–6946.3)	5010.60 (32.70–9225.63)	21.23 (16.49–156.40)
$P$ ( $SSD_{obs}$ )	0.07 (0.22–59.13)	0.59 (12.41–191.19)	0.19 (3.31–310.61)	0.41 (9.04–3301.97)	0.11 (4.80–6773.3)	0.29 (24.06–7013.6)	0.44 (3761.7–6946.3)	0.16 (32.70–9225.63)	<0.001
Years since expansion initiation	25 584	43 238	10 964	27 595	7309	42 825	6724	11 732	44 846

genetic drift, or a selective sweep may have altered the composition of haplotypes in populations since the end of the ice ages and could confound our results.

### Historic population demography

During the last ice age, overall habitat available to sheep was limited by the extent of ice sheets. With the retreat of ice, new territory was made available, allowing some populations to expand. Rapid population growth was most obvious for the Occ, NA and NT populations (Table 5, Fig. 4). Contradictory results were initially found for BC, but strong evidence for population growth was found when the haplogroup distinct to BC was analyzed. All populations with signs of rapid expansion now occupy regions that had extensive glacial coverage (Figs 1 and 2). Initiation of population expansion was roughly dated to the period during and following glacial maximum (22 000–10 000 years ago). The exception is NT, which appears to have experienced expansion prior to glacial maximum (Table 5). This may hint that glacial maximum occurred there earlier than elsewhere. Temporal estimation of expansion events based on a single locus must, however, be interpreted with caution (Hillis *et al.*, 1996). The Ocn and OG populations showed no sign of population growth (Table 5, Fig. 4), which appears consistent with the reduced presence or complete absence of glaciers in the regions these populations now occupy (Fig. 2).

Historical demography for the CY, SW and AR populations remains less clear. Much of the current habitat occupied by these populations was covered by ice (Fig. 2). Therefore, it might be expected that population growth would be easily detected. However, we found contradictory results for population expansion (see Results and Table 5). The discrepancies found between  $F_S$  and  $P$  (SSD) may be a result of a genetic bottleneck occurring in a population after the initial expansion. Such a pattern has potential to erase the signal of earlier expansion events leading to a lower probability of significant  $F_S$  tests (Excoffier & Schneider, 1999). However, it is also possible that these populations have not expanded greatly in size. For example, during glacial maximum there could have been large sheep populations in areas of Beringia near glaciers, which then shifted into newly available territory as the glaciers retreated. At the same time their original territory may have become unsuitable due to growth of forests as the climate moderated.

### Taxonomy

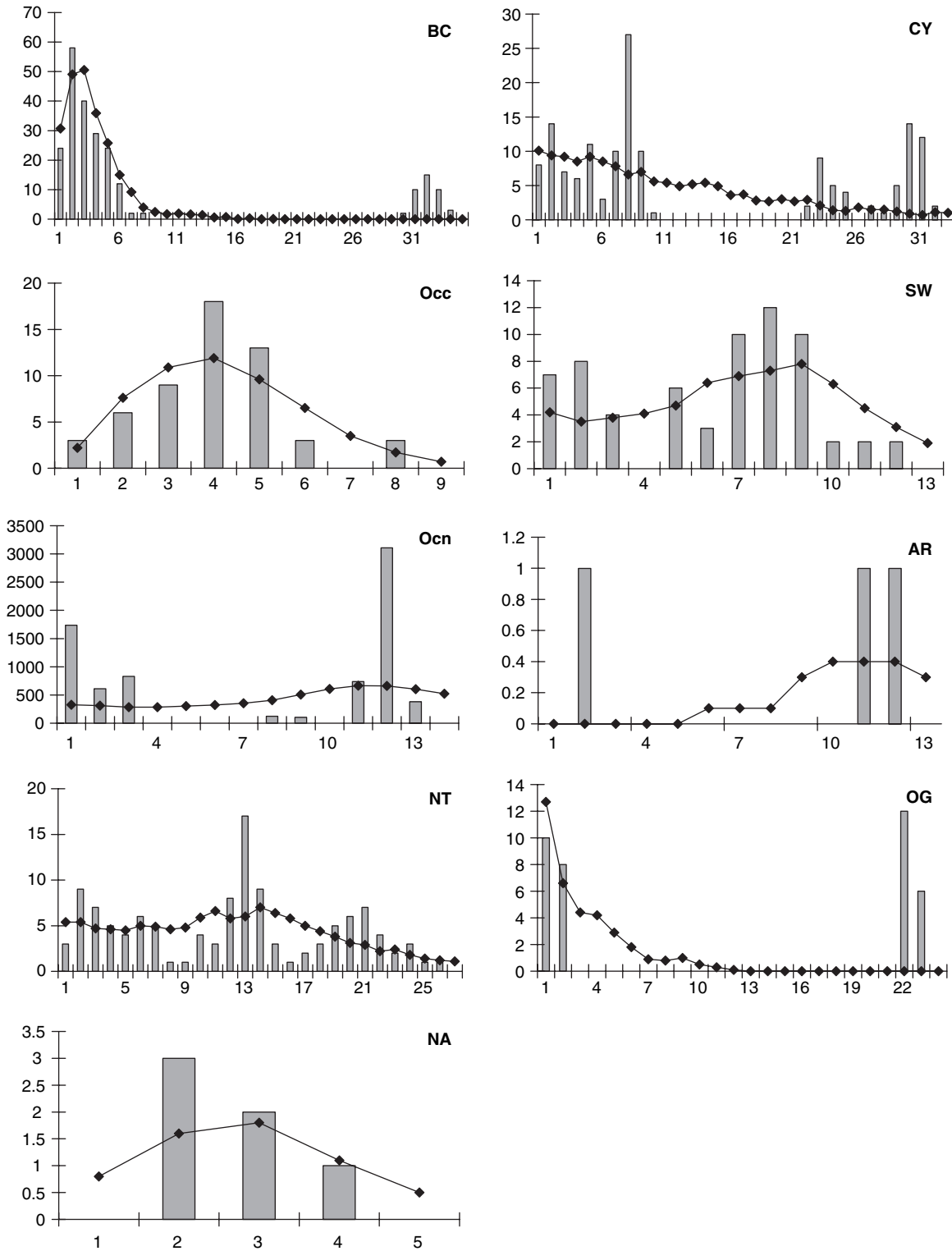
Paraphyly was found between *O. dalli* and *O. canadensis* and was also evident in *O. dalli* subspecies (Fig. 3). AMOVA at the species level produced nonsignificant results (Table 2), and for *O. dalli* the variance based on subspecies was negative, indicating that some individuals

were more closely related to members of the other subspecies than they were to their own (Table 3). This confirms earlier unpublished work by Ramey (1993), also reporting paraphyly. Species classification is currently based on analysis of skull morphology (Cowan, 1940) but fewer significant differences between species were found upon reanalysis of the same data (Ramey, 1993). At the molecular level *O. dalli* and *O. canadensis* do not appear to fulfil species criteria since their mtDNA lineages are not reciprocally monophyletic (e.g. Moritz, 1994). However, basing species on mtDNA monophyly may be overly stringent (Crandall *et al.*, 2000). Instead species status may be better based on criteria of historic and current genetic and ecological exchangeability. Crandall *et al.* (2000) suggested that evidence for inexchangeability can support species division, while evidence for exchangeability supports synonymization. Our results indicate that historic gene flow has occurred, thus the species have demonstrated historic exchangeability. Current gene flow between putative species can be assessed with future research involving nuclear DNA markers. Some evidence for ecological inexchangeability may be found in differences in skull, horn, body size and colour morphology. On the other hand at their closest geographic point of contact the habitat and life history traits of these species are very similar indicating exchangeability (Geist, 1971).

Current taxonomy groups *O. dalli* populations into *O. d. stonei* or *O. d. dalli*. There is some support for this classification in nuclear DNA. Worley *et al.* (2004) found significant subspecies differentiation by grouping BC and CY together as Stone's sheep and all others as Dall's sheep. However, mtDNA and the clinal nature of colour morphology do not support this grouping. In addition OG differs in colour morphology from Dall's sheep populations, yet it is geographically and genetically isolated from other dark sheep populations. In sum our evidence confirms that the OG, CY and BC populations have been launched on independent evolutionary paths: isolation has occurred geographically and genetically for long time periods, and colour morphology has diverged. The current *O. d. stonei* subspecies designation does not appear useful to describe the evolutionary history of these populations, and we recommend that they be managed as separate entities.

### Conclusions

Pleistocene glaciations are a major catalyst of the evolution of diversity (e.g. Pielou, 1991; Avise & Walker, 1998; Holder *et al.*, 2000), and our research demonstrates the importance of looking beyond well-known major glacial refugia to smaller ice-free areas to establish their evolutionary importance. We have shown that for North American mountain sheep such areas may have preserved a record of these species' evolutionary history. It appears that these small refugia have allowed for



**Fig. 4** Mismatch distribution for sequences from *Ovis dalli* and *O. canadensis* populations. Bars represent observed values and lines expected values for a model of sudden population expansion. Y axis: number of pairs, X axis: number of pair-wise differences. When graphed a population that has undergone rapid population expansion will exhibit a unimodal wave in the distribution of pair-wise genetic differences. In our data this trend is most evident in Occ and least evident in Ocn.

hybridization between species as well as stimulation of diversification processes. As might be expected, the signature left by isolation in small glacial refugia is often a genetic bottleneck (e.g. Fedorov & Stenseth, 2001, 2002), and our results from British Columbia show this same trend. The Mackenzie Mountain population on the other hand appeared to have maintained high levels of genetic diversity. This may be due to larger refugial size, or possibly post glacial admixture of divergent haplotypes. High genetic polymorphism was also maintained in *Packera contermina* despite nunatak isolation, which may have been a result of hybridization or life history traits specific to the species (Golden & Bain, 2000). Small refugial populations commonly show morphological differentiation due to random drift or directional selection while in isolation (e.g. Clarke *et al.*, 2001; Fedorov & Stenseth, 2001, 2002). In one proposed refugial population (BC) morphological differentiation was evident, while the other had no obvious indications of differentiation (NT).

Our findings are the first to suggest that an organism could have survived the Wisconsinan glaciation in refugia in northern British Columbia and the Mackenzie Mountains, and demonstrate the importance of mtDNA as a tool to further knowledge about glacial refugia and ice age paleoenvironments and paleoecology. Our finding is of relevance to reconstructing the evolutionary histories of other North American species, which may have been affected by similar processes. Although previous research has resulted in consensus that the environment of ice-free areas in this region was very severe, and probably unsuitable to at least human habitation (e.g. Catto, 1996, Catto *et al.*, 1996, Mandryk *et al.*, 2001), we have provided new insight into the paleoecology of the region, since our evidence suggests that the region could have been inhabited by a large mammal for the duration of the last ice age.

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