ORIGINAL ARTICLE

Phylogeography and the origins of range disjunctions in a north temperate fish, the pygmy whitefish (*Prosopium coulterii*), inferred from mitochondrial and nuclear DNA sequence analysis

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ABSTRACT

Aim To investigate the degree of phylogeographical divergence within pygmy whitefish (*Prosopium coulterii*) and to test hypotheses concerning the origin of disjunct populations within North America.

Location North America from western Alaska to Lake Superior.

Methods Mitochondrial (ATPase subunit VI) and nuclear (ITS-1, ITS-2) DNA sequence variation was assessed across the species' North American range to test for the existence of distinct phylogeographical groupings of pygmy whitefish associated with known glacial refugia. Coalescent simulations of the mitochondrial DNA (mtDNA) data were used to test hypotheses of population structure.

Results This species is composed of two monophyletic mitochondrial clades across its North American range. The two mtDNA clades differed by an average 3.3% nucleotide sequence divergence. These clades were also distinguished by ITS-2, but the relationships among lineages were not resolved by the ITS-1 analysis. Coalescent analyses rejected the null hypothesis of the current disjunct distributions being a result of fragmentation of a single widespread ancestral lineage across a variety of effective population sizes and divergence times.

Main conclusions The current range disjunctions of pygmy whitefish in North America probably resulted from isolation, genetic divergence, and selective dispersal from at least two major Pleistocene glacial refugia: Beringia and Cascadia. More recent isolation and dispersal from an upper Mississippi refugium is suggested by relationships within one of the clades and by distributional evidence from co-distributed species. The Beringian and Cascadian refugia have played major roles in the zoogeography of Nearctic temperate aquatics, but the roles of smaller refugia appear more variable among other species.

Keywords
ATPase subunit VI, coalescent analysis, conservation biogeography, fish, internal transcribed spacer, North America, Pisces, Pleistocene refugia, post-glacial dispersal, *Prosopium*.

INTRODUCTION

Intraspecific range disjunctions occur when a taxon has geographic occurrences that are separated by large geographic distances, and they are one of the oldest observations in biogeography (Lomolino *et al.*, 2006). Range disjunctions have provoked much thought in biogeography because they suggest that separate geographic areas were once connected, that long-distance dispersal has occurred between such areas, or that a once more broadly distributed taxon has become extinct in intervening areas. In fact, range disjunctions, along with resolution of the processes involved in plate tectonics, orogeny and glaciation, as well as improved methods of phylogenetic reconstruction, provided a major impetus to the origin and the development of the field of vicariance biogeography (Riddle, 1996; Lomolino *et al.*, 2006).

Broadly distributed species present a special challenge to conservation because these taxa ordinarily exhibit non-uniform ecological and genetic characteristics over their ranges, and strategies for conservation of biodiversity developed for one portion of the geographic range may not be appropriate for other portions of the range. This is especially true for species with disjunct distributions, because the very existence of disjunctions is strongly suggestive of historical ecological and/or genetic independence. The importance of incorporating historical independence into conservation has long been recognized, and resulted in several concepts and criteria for identifying major intraspecific units of biodiversity such as evolutionarily significant units (ESUs, e.g. Ryder, 1986; Waples, 1991) and related concepts (Crandall *et al.*, 2000; Green, 2005). Although there is as yet no consensus on the best means to capture intraspecific diversity, a shared attribute of all proposed definitions is a lack of gene flow evidenced by marked genetic distinction among groups of populations.

The pygmy whitefish [Salmonidae: *Prosopium coulterii* (Eigenmann and Eigenmann, 1892)] is a small, inconspicuous inhabitant of lakes and occasionally rivers of northern North America, and of a small portion of eastern Russia (Scott & Crossman, 1973; Chereshnev & Skopets, 1992). In north-western North America, *P. coulterii* possesses a discontinuous native distribution: some populations occur in the extreme portions of south-western Alaska, including the Chignik and Ugashik river drainages, but it is absent from the eastern and northern regions of Alaska. In north-western Canada, it is distributed in the upper Yukon River system within Yukon Territory (but is absent from downstream segments in north-central Alaska). In Yukon Territory, it is also known from a single lake (Elliot) in the Peel River drainage, which is a Mackenzie River tributary. It is found in the Alsek River drainage, which is a smaller watershed that has its headwater reaches in southern Yukon, and drains south through north-west British Columbia into the North Pacific Ocean. Within British Columbia, its distribution also includes lakes in the Peace and Liard river drainages in the Mackenzie River system, as well as lakes in the Pacific drainages within Cascadia: the Columbia, Fraser and Skeena river systems (Scott & Crossman, 1973; McPhail & Lindsey, 1986). The distribution of *P. coulterii* in the Columbia River system includes scattered lakes in south-central British Columbia, Flathead Lake Montana, as well as 15 lakes in Washington State, but it has been extirpated from six of these, and it is currently listed as a sensitive species by the Washington Department of Fish and Wildlife (Hallock & Mongillo, 1998). It is also known from Waterton Lakes in the extreme south-west corner of the Canadian Province of Alberta, which is part of the Hudson Bay drainage (Lindsey & Franzin, 1972). Remarkably, *P. coulterii* is also native to western Lake Superior, but it has not been reported from Manitoba, North Dakota and Saskatchewan, or from inland lakes in Minnesota, Ontario, and Wisconsin (Hubbs & Lagler, 1964; Scott & Crossman, 1973) although there is a recent record from a single small lake in north-western Ontario (P. Blanchfield, D. Watkinson and E.B. Taylor, unpublished data). Hence, the native distribution of the *P. coulterii* consists of three disjunct North American fragments: south-west Alaska (SA), Cascadia/southwest Mackenzie/Alsek/Yukon (CMAY) and Lake Superior (LS). The populations in south-west Alaska are separated from those within CMAY by *c.* 950 km, while populations occurring in CMAY are separated from Lake Superior by *c.* 1900 km. These North American disjunctions within *P. coulterii* have long intrigued zoogeographers (Eschmeyer & Bailey, 1955; McCart, 1970; Lindsey & Franzin, 1972; Lindsey & McPhail, 1986; Underhill, 1986; Mandrak & Crossman, 1992). For instance, McCart (1970) reported at least two morphological types of pygmy whitefish in lakes of the Bristol Bay region of Alaska and suggested that these forms represented fish that had colonized the lakes independently from distinct glacial refugia. These two putative groups of pygmy whitefish from the Alaskan lakes matched morphological groups from fish collected in other portions of North America and were called the 'southeastern' and 'northwestern' groups (McCart, 1970; Lindsey & Franzin, 1972). McCart (1970) hypothesized that the geographic pattern of morphology in pygmy whitefish and the sympatric occurrence of the two types in some western Alaskan lakes indicated that the northwestern group evolved in ice-free areas somewhere in the Yukon River–Bering Sea region and that the southeastern group evolved somewhere in the vicinity of the lower Columbia River south of the Wisconsinan ice sheet. The LS pygmy whitefish are morphologically intermediate to the southeastern and northwestern groups, an observation that has been used as evidence of persistence of the species during the Wisconsinan glaciation in a third, eastern refugium (McCart, 1970). Lindsey & Franzin (1972) and Bird & Roberson (1979), however, reported some exceptions to the geographic pattern reported by McCart (1970) from additional collections and suggested that extant *P. coulterii* may stem from isolation and post-glacial dispersal from up to six distinct refugia.

In this study we investigated *P. coulterii* from across its North American range in a phylogeographic analysis of mitochondrial and nuclear DNA sequences to test the hypothesis that its current range disjunctions are concomitant with population sets that were isolated in, and subsequently dispersed from, separate refugia during the Pleistocene glaciations (cf. Väinöla *et al.*, 1994; Bernatchez & Wilson, 1998; Taylor *et al.*, 1998; Fedorov & Stenseth, 2002; Jaramillo-Correa *et al.*, 2004; Waltari & Cook, 2005; Gagnon & Angers, 2006; Hoarau *et al.*, 2007). This hypothesis leads to the prediction that populations from each of the three disjunct population assemblages (SA, CMAY and LS, Fig. 1) will form at least three well-discerned groups in a phylogenetic analysis (i.e. they will constitute distinct phylogroups) with approximately equal divergence from one another.

MATERIALS AND METHODS

Field collections and DNA extraction

Pygmy whitefish were collected from 30 localities from across the species' North American range, including the Chignik and

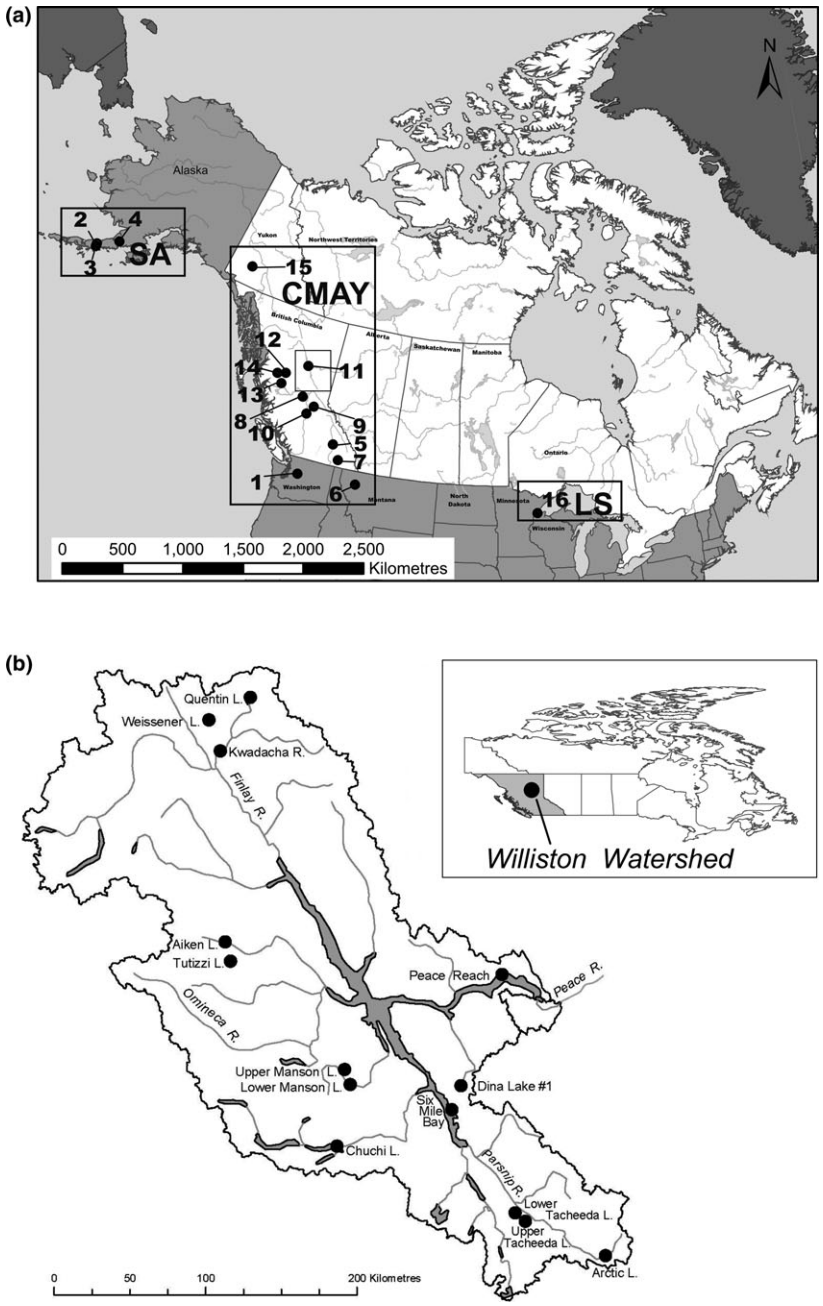

Figure 1 Map showing (a) pygmy whitefish (*Prosopium coulterii*) sampling localities across North America and (b) multiple sampling localities within the Williston Reservoir watershed, British Columbia, Canada. The boxed areas represent the approximate distributional limits of the: (1) south-west Alaska (SA), (2) Cascadia/south-west Mackenzie, Alsek, Yukon (CMAY), and (3) Lake Superior (LS) population assemblages of pygmy whitefish. The inset box within the CMAY area represents the area expanded in panel (b). Numbers of sampling localities follow those in Table 1.

Ugashik (SA), the Cedar, Columbia, Fraser, Peace, Skeena and Alsek river drainages (CMAY), as well as Lake Superior in the Great Lakes/St Lawrence River drainage (LS) (Fig. 1, Table 1). Pygmy whitefish in Chignik Lake occur as high-gill-raker (*c.* 19) and low-gill-raker (*c.* 14) forms (McCart, 1970). Our samples from this locality were collected using beach seines and appear to be of the low-raker form only [average number of total gill rakers on the first arch (± SD) = 13.3 (0.89, 30), P. Kong and E.B. Taylor, unpublished data]. Samples consisting of adipose or caudal fin clips were taken from specimens and preserved in absolute ethanol, and the remainder of each specimen was placed in 10% formalin. Total DNA was extracted from fin clips using the DNeasy tissue kit (Qiagen Inc., Mississauga, Canada) and stored at −20 °C until analysis.

Mitochondrial and nuclear DNA sequence analysis

A 627-bp fragment of the mitochondrial ATPase subunit VI gene was amplified by polymerase chain reaction (PCR) using primers H9208 5′-TAT-GCG-TGT-GCT-TGG-TGT-GCC-A-3′ and L8558 5′-AGC-TTC-TTC-GAC-CAA-TTT-ATG-AG-3′, described by Giuffra *et al.* (1994). The 25-μL PCRs contained 2.5 μL of 10× PCR buffer (New England Biolabs, NEB, Pickering, Canada), 0.2 mM of each dNTP, 0.3 μM of each primer and 0.25 units of *Taq* polymerase (NEB). The PCR

Table 1 Map locations for sampling locations (see Fig. 1), drainages and hypothesized phylogroup inclusion [southern Alaska (SA), Cascadia/Mackenzie/Yukon (CMAY) and Lake Superior (LS)], lakes sampled, sample sizes (*n*), clade affiliation (Fig. 3), and sequence haplotype presence (*H*) for 160 pygmy whitefish (*Prosopium coulterii*) assayed for variation at the ATPase subunit VI mitochondrial gene. Haplotype designations follow those in Fig. 3, and numbers in parentheses beside the haplotypes indicate their abundance in the sample.

Map location	Drainage (hypothesized phylogroup)	Lake	*n*	Clade	*H*
1	Cedar River (CMAY)	Chester Morse	5	2	H6(5)
2	Chignik River (SA)	Black	6	1	H23(1), H24(1), H25(4)
3	Chignik River (SA)	Chignik	22	1	H24(10), H25(12)
4	Ugashik River (SA)	Ugashik	6	1	H22(1), H25(3), H26(1)
5	Columbia River (CMAY)	Arrow	7	2	H6(5), H10(1), H16(1)
6	Columbia River (CMAY)	Flathead	4	2	H5(1), H6(3)
7	Columbia River (CMAY)	Kootenay	4	2	H16(2), H19(2)
8	Fraser River (CMAY)	Cluculz	2	2	H16(1), H17(1)
9	Fraser River (CMAY)	Jack of Clubs	5	2	H16(5)
10	Fraser River (CMAY)	McCleese	4	2	H16(4)
11	Peace River (CMAY)	Arctic	6	2	H16(6)
11	Peace River (CMAY)	Aiken	5	2	H6(4), H16(1)
11	Peace River (CMAY)	Dina	6	2	H16(6)
11	Peace River (CMAY)	Lower Manson	6	2	H6(3), H16(3)
11	Peace River (CMAY)	Peace Reach	8	2	H14(1), H16(4), H17(1)
11	Peace River (CMAY)	Upper Manson	4	2	H6(2), H16(2)
11	Peace River (CMAY)	Chuchi	6	2	H4(1), H6(4), H9(1)
11	Peace River (CMAY)	Quentin	6	2	H15(5), H16(1)
11	Peace River (CMAY)	Kwadacha River	3	2	H15(2), H16(1)
11	Peace River (CMAY)	Lower Tacheeda	4	2	H1(1), H2(1), H3(1), H7(1)
11	Peace River (CMAY)	Upper Tacheeda	7	2	H6(7)
11	Peace River (CMAY)	Monkman	3	2	H13(1), H6(2)
11	Peace River (CMAY)	Six Mile Bay	6	2	H14(1), H16(4), H18(1)
11	Peace River (CMAY)	Tutizzi	5	2	H6(1), H11(1), H12(1), H16(2)
11	Peace River (CMAY)	Weissener	5	2	H6(4), H8(1)
12	Skeena River (CMAY)	Chapman	5	2	H10(1), H6(2), H16(2)
13	Skeena River (CMAY)	Owen	5	2	H16(5)
14	Skeena River (CMAY)	Tyhee	3	2	H10(2), H16(1)
15	Alsek River (CMAY)	Aishihik	2	1	H21(2)
16	Great Lakes Basin (LS)	Superior	12*	2	H20(7)

*Five fish were determined to belong to clade 2 by *Sty* I restriction analysis.

conditions consisted of a preliminary denaturing stage of 1 min at 94 °C; followed by 40 cycles of 1 min at 94 °C, 1 min 15 s at 48 °C, and 1 min at 72 °C; followed by 5 min at 72 °C. Individuals representing 17 of the haplotypes identified in the mtDNA analysis (see below) were amplified for both nuclear ribosomal internal transcribed spacers (ITS-1 and ITS-2). Fragments of 615 and 367 bp of ITS-1 and ITS-2, respectively, were PCR-amplified using the primers described by Phillips *et al.* (1995) and the PCR conditions described by Presa *et al.* (2002). Both for mtDNA and for ITS fragments, the PCR products were purified using the Qia Quick PCR purification kit (Qiagen Inc.) and were sequenced in one direction using primer L8558 (mtDNA) or forward primers in the case of ITS-1 and -2 on an ABI 3730 automated sequencer (Life Technologies Corp., Carlsbad, CA, USA). All sequences have been deposited in GenBank under accession numbers HQ616435–HQ 616461 for ATPase VI, HQ616462–HQ616478 for ITS-1, and HQ616479–HQ616495 for ITS-2.

After resolution of the major mtDNA clades (see below) a few additional samples were subjected to restriction enzyme digestion using *Sty* I following the manufacturer's (NEB) assay conditions. This enzyme has the recognition sequence CCWWGG and cut the ATPase VI fragment of clade 1 fish into two fragments (*c.* 132 and 495 bp in size) and that of clade 2 fish into three fragments (84, 132 and 411 bp) that were distinguished on 2% agarose gels.

Following sequence alignment, a preliminary distance analysis using the unweighted pair group method with arithmetic averages (UPGMA) was used on a matrix of nucleotide difference numbers between all pairwise combinations of sequences to identify mtDNA haplotypes in MEGA 4.0 (Tamura *et al.*, 2007). A phylogenetic analysis was subsequently conducted on 26 haplotypes using the neighbour-joining (NJ) distance method (Tamura–Nei substitution model) in MEGA 4.0. A representative of the round whitefish (*Prosopium cylindraceum*) was used as the outgroup.

Confidence for the NJ analysis was estimated using 1000 bootstrap pseudo-replicates, as well as the interior branch test (1000 replicates). A maximum parsimony (MP) analysis was conducted on the 26 haplotypes and outgroup using PAUP* 4.0b10 (Swofford, 2001). The MP analysis employed 1000 heuristic search replicates, with the starting tree for each replicate obtained randomly, and tree bisection–reconnection (TBR) branch swapping. Confidence in the MP analysis was ascertained with 1000 bootstrap pseudo-replicates, with each pseudo-replicate consisting of four heuristic search replicates; the starting tree for each was obtained randomly, and TBR branch swapping was used.

Tree-based phylogenetic analyses assume a bifurcating pattern of ancestor/descendant relationships and, therefore, they will not resolve evolutionary histories among very closely related haplotypes, especially if ancestral haplotypes are extant. In such cases, the haplotypes will be related by an evolutionary network as opposed to a bifurcating tree (Posada & Crandall, 2001). A statistical parsimony network with 95% connection limits was constructed using 26 mitochondrial ATPase subunit VI haplotypes (H1–H26, see Results) using the program TCS 1.21 (Clement *et al.*, 2000).

Both ITS sequence sets were aligned using the clustal module in MEGA 4.0, using a gap opening penalty of 10 and a gap extension penalty of 3. Separate phylogenies were subsequently estimated using the NJ method, and the Kimura-2 parameter distance model, with confidence assessed using 1000 bootstrap pseudo-replicates in MEGA 4.0.

Combined mitochondrial and nuclear DNA sequence analysis

The incongruence length difference test (Farris *et al.*, 1994) was used to determine if discordant phylogenetic signal existed among the ATPase subunit VI, and both ITS partitions in PAUP* 4.0b10. The test was conducted using 10,000 replicates, heuristic searches with the starting trees obtained by simple stepwise addition, and TBR branch swapping. The program MODELTEST 3.7 (Posada & Crandall, 1998) was used to estimate the best-fit model of 56 models of sequence evolution for the combined mitochondrial and nuclear data set using the Akaike information criterion (Posada & Buckley, 2004). A maximum likelihood (ML) analysis was subsequently conducted in PAUP* 4.0b10 with the model determined by MODELTEST implemented. This analysis consisted of one heuristic search replicate with the starting tree obtained by NJ and TBR branch swapping, followed by 20 replicates with the starting trees obtained randomly. Confidence was assessed with 500 bootstrap pseudo-replicates, with the starting trees obtained by NJ and TBR branch swapping. An NJ analysis was conducted on the combined data set with the Tamura–Nei distance model, and confidence assessed by 1000 bootstrap pseudo-replicates, as well as the interior branch test (1000 replicates). An MP analysis was also conducted on the combined data set using the same methods as described for the ATPase subunit VI gene.

Hypothesis tests of refugial origins

We applied the gene tree–population tree approach of Knowles (2001) to assess alternative hypotheses of the historical demographic causes of the mtDNA variation resolved. The alternative historical models assessed were the 'single refuge-fragmentation model' and the 'multiple glacial refuge model'. The former is the null model and in it the extant population structure of mtDNA haplotypes was hypothesized to result from post-glacial fragmentation and subsequent lineage sorting within a single and widespread ancestral lineage that dispersed from a single glacial refugium. In the alternative multiple-refuge model, the extant structure is hypothesized to have resulted from isolation and divergence within two or more glacial refugia. In this approach, the different historical models are represented by different population trees (e.g. see Fig. 3 of Knowles, 2001). The observed mtDNA gene tree is contained within the population trees, and the degree of concordance between the two trees is assessed using the *s*-statistic of Slatkin & Maddison (1989). The *s*-statistic measures the number of parsimony steps in the mtDNA data under the various population models, and low *s*-values represent high concordance between the gene trees and the hypothesized population trees. Higher values of *s* in the observed gene tree indicate that the haplotypes are widely scattered across populations. This observed value of *s* is then compared with the distribution of *s*-values expected under the null (fragmentation) and multiple-refuge models. The distributions of *s* under each model are generated by simulating gene trees ($N = 1000$) by neutral coalescence and containing them within the population tree represented by each model. If the observed gene tree *s*-value falls outside the 95% confidence interval of the distribution of *s* associated with any population model then that model can be rejected (Knowles, 2001). This approach has been applied to a number of studies assessing alternative historical models of population divergence within species (e.g. DeChaine & Martin, 2005; Steele & Storfer, 2006; Carstens & Knowles, 2007; Shepard & Burbrink, 2008). The coalescent simulations and population tree fitting procedures were conducted in MESQUITE 2.6 (Maddison & Maddison, 2009). The fragmentation model was represented by a tree of 30 localities (Table 1) diverging from a single polytomy. Multiple-refuge models were represented by two population trees. The first consisted of two assemblages (hypothesized refugia) of the 30 populations described above, pooled as suggested by the gene tree of mtDNA haplotypes and their geographic distributions (see below): (1) south-west Alaska and Yukon; and (2) British Columbia, western Washington and Lake Superior (Table 1). The second refuge model consisted of three assemblages: (1) south-western Alaska; (2) British Columbia and western Washington; and (3) Lake Superior, and represented the three major range disjunctions. As recommended by Maddison & Maddison (2009), we performed these tests under a range of effective population sizes (10,000 to 250,000) and divergence times associated with the approximate timing and durations of late to

mid-Pleistocene glacial events (75,000 years, most recent glaciation; 250,000 years; 500,000 years; earlier Pleistocene glacial periods: see, for example, Paillard, 1998).

RESULTS

Mitochondrial and nuclear DNA sequence analysis

A total of 155 mitochondrial ATPase subunit VI sequences were obtained from individuals across the 30 localities. The sequence alignment was unambiguous, and there were no gaps or stop codons identified in the alignment. The alignment was 627 bp in length, and the preliminary UPGMA analysis identified 26 haplotypes (Table 1). Thirty-six nucleotide positions are variable among the 26 haplotypes, and 28 positions are phylogenetically informative using the parsimony criterion. The MP analysis recovered 104 equally parsimonious trees 123 steps in length (consistency index = 0.686, retention index = 0.874). Both the MP and the NJ analysis identified two well-supported clades (Fig. 2). Clade 1 consists of all haplotypes derived from individuals in north-western North America in the Chignik and Ugashik river drainages, and also those found in individuals from Aishihik Lake in the Alsek River drainage (Figs 1 & 2; Table 1). Clade 2 consists of all individuals from Cascadia and the Mackenzie (Peace) River drainage. Clade 2, however, also contains the haplotype from individuals in Lake Superior, which we hypothesized would form a phylogroup of their own (LS). The average pairwise percentage sequence divergence within each clade is 0.6 and 0.5% for clades 1 and 2, respectively, and the average pairwise sequence divergence among haplotypes between the two clades is 3.3%. Within clade 1, all haplotypes found in individuals from the Chignik and Ugashik river drainages in Alaska (H22, H23, H24, H25 and H26) form a well-supported subclade, and haplotype 21, which was derived from individuals in Aishihik Lake within the Alsek River drainage, is sister to this clade (Fig. 2). As a consequence of their very close phylogenetic affinities, the relationships among haplotypes within clade 2 could not be resolved by either the MP or the NJ analyses.

The statistical parsimony analysis of all mitochondrial haplotypes (H1–H26, Table 1) indicated that clades 1 and 2 are separated by 14 mutational steps (Fig. 3). Within clade 1, haplotype 21 is separated from haplotypes 22–26 by a minimum of five mutational steps, supporting the subclade identified in the phylogenetic analysis. Most of the haplotypes in clade 2 are derived from either haplotype 6 or 16 by a single mutational step (Fig. 3). These two haplotypes were the most geographically widespread and commonly occurring in the data set (Fig. 2). Haplotype 20, which was present in all individuals sequenced from Lake Superior (Table 1), is distantly related to most haplotypes within clade 2. This analysis identified two loops involving haplotypes 14, 15 and 16, as well as 16, 17 and 18, which represent uncertainty (Fig. 3).

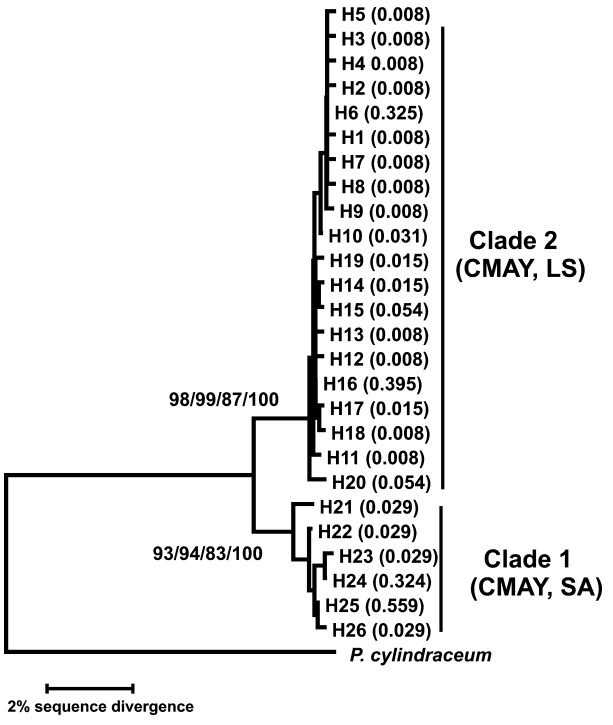

Figure 2 Neighbour-joining (NJ) phenogram showing relationships among 26 pygmy whitefish (*Prosopium coulterii*) ATPase subunit VI haplotypes (Table 1). Numbers above the nodes respectively give NJ bootstrap percentages, maximum parsimony bootstrap percentages, interior branch test percentages, and partition percentages among the 104 equally parsimonious trees. Numbers in parentheses give clade-specific haplotype frequencies.

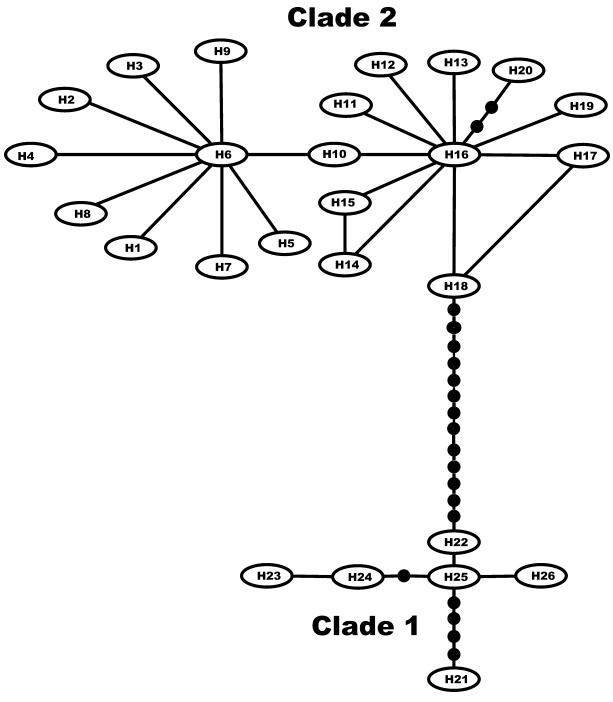

Figure 3 Statistical parsimony network showing relationships among 26 mitochondrial pygmy whitefish (*Prosopium coulterii*) ATPase subunit VI haplotypes (Fig. 2, Table 1). Solid circles indicate mutational steps.

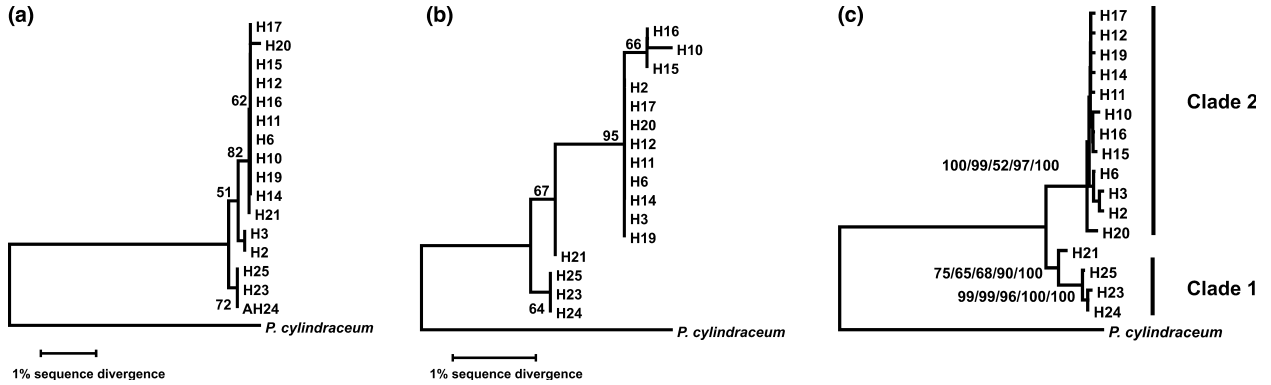

Figure 4 Neighbour-joining (NJ) phenograms showing the relationships among 16 pygmy whitefish (*Prosopium coulterii*) mtDNA haplotypes (Fig. 2) that were also sequenced for (a) ITS-1, (b) ITS-2 and (c) relationships on the basis of three gene regions: ATPase subunit VI, ITS-1 and ITS-2. Numbers above the nodes respectively give NJ bootstrap percentages (a and b), and interior branch test percentages, maximum likelihood bootstrap percentages, maximum parsimony bootstrap percentages, and partition percentages among the 45 equally parsimonious trees (c).

Sequence alignments for both ITS-1 and ITS-2 were unambiguous. Among the 16 ingroup haplotypes, the alignments contained four gaps (ITS-1) and no gaps (ITS-2). Variability was low in both regions, with six and seven variable sites among the ingroup haplotypes for ITS-1 and ITS-2, respectively. The NJ analysis of ITS-2 distinguished all haplotypes belonging to mitochondrial clade 2 (Fig. 4a) as a well-supported monophyletic cluster; however, mitochondrial clade 1 was paraphyletic with respect to clade 2. By contrast, the relationships based on ITS-1 were not well resolved (Fig. 4b).

Combined mitochondrial and nuclear DNA sequence analysis

The incongruence length difference test did not provide evidence for discordant phylogenetic signal among the three gene partitions ($P = 0.14$). The nucleotide base frequencies in the 1625-bp sequence alignment are A: 0.19, C: 0.31, G: 0.27, T: 0.23, and there is no evidence for heterogeneous nucleotide composition among the haplotypes (chi-square homogeneity test, $P > 0.99$). The alignment contained 44 variable characters among the ingroup haplotypes, of which 32 were phylogenetically informative using cladistic criteria. The Akaike information criterion indicated that the data were best explained by the TrN+I model of nucleotide substitution (Tamura–Nei with a proportion of invariant sites), with I = 0.8934. As this model is not implemented in PAUP*, and because TrN+I is a special case of the general time-reversible model, the ML analysis was conducted using the GTR+I model of substitution, with I set to 0.8934. The ML analysis resolved mitochondrial clades 1 and 2, but with lower bootstrap support than the other analyses (Fig. 4c). The MP analysis recovered 45 equally parsimonious trees 193 steps in length (consistency index = 0.92, retention index = 0.86). This analysis recovered mitochondrial clades 1 and 2 with strong support, and converged on the same topology as the NJ analysis, which also strongly supported both clades (Fig. 4c).

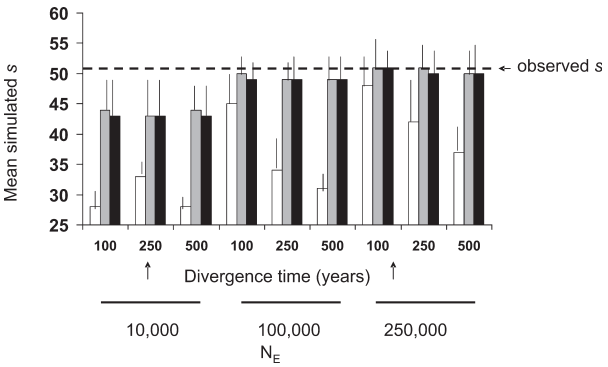

Figure 5 Summary of coalescent analyses of various models of population structure. Each bar represents the mean value of Slatkin & Maddison's (1989) *s*-parameter (from 1000 simulated gene trees), which measures the degree of concordance between the pygmy whitefish (*Prosopium coulterii*) mtDNA gene tree and one of three models of population structure: fragmentation of populations after isolation in a single glacial refugium (white), isolation in two refugia (grey), or isolation in three refugia (black). The thin vertical line on each bar represents the upper 95% confidence level of *s* under each model. The horizontal dashed line represents the observed value of *s* (= 51) for the mtDNA gene tree (see Fig. 2). Simulations were conducted under three values of effective population size (N_E) indicated under the thick horizontal lines and across three divergence times indicated along the horizontal axis (× 1000 years). When the upper 95% confidence level for any particular model falls below the dashed line, that model is rejected at a maximum $P = 0.05$.

Tests of historical hypotheses of divergence

The observed value of *s* for our MP mtDNA tree was 51. The results of the coalescent simulations indicated that all models across three time periods of divergence were rejected when employing an effective population size of 10,000 (Fig. 5). Using effective population sizes of 100,000 or 250,000 consistently led to rejection of the single refuge-fragmentation model

over all three time periods examined, while the two- (south-western Alaska versus BC/western Washington/Lake Superior) and the three- (south-western Alaska versus BC/western Washington versus Lake Superior) refuge models could not be rejected nor differentiated from each other (Fig. 5). The null, single refuge-fragmentation model was rejected consistently except under a single set of conditions – a time frame of 100,000 years and an effective population size of 250,000 (Fig. 5).

DISCUSSION

Phylogeographic divergence within pygmy whitefish

Intraspecific mitochondrial sequence differences resolved in pygmy whitefish (3.3% divergence between clades, 14 mutational steps) were substantial, and 'standard' mitochondrial sequence divergence rates of 1–2% per million years (Avise, 2000, 2004) suggest that clades 1 and 2 diverged from each other some 1.65 to 3.3 million years ago (Ma). A whitefish-specific molecular clock calibration for the ATPase subunit VI mitochondrial gene is not available, and the use of non-lineage-specific molecular clock calibrations is problematic (Ho *et al.*, 2005; Smith & Dowling, 2008). In addition, when levels of genetic divergence between clades are in the range resolved here, it is difficult to distinguish between mutational differences that accrued after a vicariance event and the partitioning of ancestral variation (Ho *et al.*, 2005; Witt *et al.*, 2008). It is, however, unlikely that the divergence observed between mitochondrial clades 1 and 2, as well as that between subclades within clade 1, originated during the most recent Wisconsinan glaciation. Instead, these divergences probably pre-date the Wisconsinan and may have occurred during early Pleistocene (e.g. Nebraskan) glaciations or perhaps into the late Pliocene (*c.* 2.5–3.0 Ma). This inference is supported by the coalescent simulations, which failed to reject two- or three-refuge models dating to at least 500,000 years ago. Furthermore, despite the lower resolution expected at nuclear loci in phylogeographic analyses (Hare, 2001; Palumbi *et al.*, 2001; Zhang & Hewitt, 2003), ITS-2 clearly discriminated members of the two mitochondrial clades from each other. Although clade 1 was paraphyletic with respect to clade 2 in the nuclear analyses, the combined mitochondrial/nuclear gene analysis resolved both mitochondrial clades (cf. Presa *et al.*, 2002).

Origins of pygmy whitefish disjunct distributions in western North America

Following the Wisconsinan glaciations, which reached their maximal extent 18,000 years ago (Dyke & Prest, 1987; Dyke *et al.*, 2003), North American freshwater fishes re-colonized the northern half of the continent from up to six glacial refugia as the Laurentide and Cordilleran ice sheets receded: the Atlantic Refuge, which consisted of ice-free regions proximal to the eastern seaboard; the Beringian Refuge, located in the ice-free expanses of Alaska and parts of the Yukon Territory; the Missouri River Valley to the south of the ice sheets on the Great Plains; the Mississippi River Valley; the Nahanni Refuge, which was located in an ice-free corridor extending from the extreme south-west corner of the Canadian Northwest Territories and up through the Mackenzie Mountains; and the Pacific Refuge, which consisted principally of the unglaciated portions of the Columbia River drainage (Bailey & Smith, 1981; Legendre & Legendre, 1984; Crossman & McAllister, 1986; Lindsey & McPhail, 1986; McPhail & Lindsey, 1986; Underhill, 1986; Pielou, 1991; Foote *et al.*, 1992; Bernatchez & Wilson, 1998; Sheldon *et al.*, 2008). Within this context, the refugial origins and post-glacial dispersal of pygmy whitefish have been a source of considerable interest. McCart (1970) recognized two gill-raker forms: a 'high-raker' form present among populations in Alaska and a 'low-raker' form distributed throughout Cascadia, and suggested that they represented two glacial races, with Beringian and Pacific refugial origins, respectively, that occurred in sympatry in some Alaskan lakes (McCart, 1970; Lindsey & Franzin, 1972). Subsequent investigations indicated a more complex history of post-glacial dispersal and suggested that *P. coulterii* was isolated in three refugia during the Wisconsinan glaciations: a Beringian Refuge (Alaskan and possibly Yukon populations); a Pacific Refuge (Cascadian, Peace/Mackenzie and possibly Yukon populations); and a Mississippi Refuge (Lake Superior population) (Lindsey & Franzin, 1972; Bailey & Smith, 1981; Lindsey & McPhail, 1986; McPhail & Lindsey, 1986; Underhill, 1986). Consequently, we predicted that the three disjunct pygmy whitefish range fragments (SA, CMAY, LS) would each be discriminated as distinct phylogroups, stemming from isolation and dispersal from Beringian (SA), Pacific (CMAY) and Mississippi (LS) refugia. Our analyses, however, discriminated only two highly divergent clades: (1) haplotypes derived from populations in Alaska and the population in Aishihik Lake (clade 1), and (2) haplotypes derived from populations in Cascadia, the Peace River drainage and Lake Superior (clade 2), which is consistent with the hypothesis that western North American pygmy whitefish originated post-glacially from two refugia: Beringia (Alaskan and some Yukon pygmy whitefish) and the Pacific Refuge (all other western pygmy whitefish).

Populations of several other fishes have been suggested to have survived the Wisconsinan glaciations in a Beringian Refuge: lake whitefish (*Coregonus clupeaformis*, Bernatchez & Dodson, 1991), lake trout (*Salvelinus namaycush*, Wilson & Hebert, 1998), Arctic char (*Salvelinus alpinus*, Brunner *et al.*, 2001), Arctic grayling (*Thymallus arcticus*, Stamford & Taylor, 2004), burbot (*Lota lota*, Van Houdt *et al.*, 2005), as well as several crustaceans including *Sida* (Cox & Hebert, 2001), *Eubosmina* (Haney & Taylor, 2003), *Daphnia* (Weider & Hobæk, 2003) and *Mysis* (Dooh *et al.*, 2006). The mitochondrial haplotypes from Alaska (H22–H26) formed a well-supported subclade within clade 1, and differed by a minimum of five mutational steps from haplotype 21. In fact, *P. coulterii* has a very limited distribution in Alaska, and the populations that we sampled almost certainly lie within the refugial area itself. These populations occur in watersheds that drain to the

North Pacific/Bristol Bay, and given that *P. coulterii* is intolerant of salinities greater than 10 ppt, the peninsular nature of the location of the populations, and that there is no evidence to suggest that these watersheds drained towards the east at any time during the Pleistocene, they probably would have had limited opportunities for dispersal (cf. Bernatchez & Dodson, 1991, for lake whitefish).

Mitochondrial haplotype 21, found only in Aishihik Lake, Alsek River drainage, was the most distantly related to all other clade 1 haplotypes and was also distinguished from other clade 1 haplotypes by the ITS-2 analysis, hinting at its antiquity. The absence of pygmy whitefish in the middle and lower reaches of the Yukon River drainage, as well as anywhere on the Alaska–Yukon North Slope argues strongly against the hypothesis that haplotype 21 originated from a second central or northern Beringian Refuge. Alternatively, the origin of this divergent haplotype could be from unglaciated portions of the upper Yukon River, or from a refuge in unglaciated regions within the Northwest Territories, proximal to Yukon – the Nahanni Refuge. We favour an upper Yukon River (Beringian) origin for three reasons. First, Aishihik Lake forms the headwaters of the Alsek River, and could not have been colonized by a downstream population owing to the presence of an impassable waterfall. As there are pygmy whitefish in several upper Yukon River tributaries (Scott & Crossman, 1973), the Aishihik Lake fish may have originated via headwater transfer between the Yukon and Alsek river drainages. Lindsey (1975) argued that Aishihik Lake obtained its present fish fauna *c.* 10,500 years ago via dispersal from the Yukon River when the lake drained northwards, prior to the formation of its present-day southern outlet to the Alsek River. Furthermore, Bird & Roberson (1979) suggested that pygmy whitefish from the Copper River area of south-central Alaska originated from the Yukon River and not from a Bristol Bay area refugium, while Wiedmer *et al.* (2010) suggested that a refugium existed in a large proglacial lake (Lake Atna) in south-central Alaska. Second, the round whitefish has a similar Alsek–upper Yukon River distribution, but (like the pygmy whitefish) is absent from the Nahanni River, and probably also colonized the Alsek River from the upper Yukon River (Lindsey, 1975). Third, although some Arctic grayling survived in the Nahanni Refuge, Pacific north coast populations (adjacent to the Alsek River) and those in the upper Liard River appear to have originated from a southern Beringian refuge and not from a Nahanni refuge (Stamford & Taylor, 2004). Further sampling, particularly from the Liard, Peel or Yukon rivers, would prove informative in distinguishing these alternative scenarios more fully.

Clade 2 is the most broadly distributed group of haplotypes, occurring in Cascadia, the Peace River drainage, as well as Lake Superior, and our data strongly support the hypothesis that pygmy whitefish of clade 2 survived the Wisconsinan glaciations in the Pacific Refuge, particularly in ice-free segments of the Columbia River drainage, 'the master river of Cascadia' (McPhail & Lindsey, 1986), and subsequently dispersed widely northwards. Extensive northward dispersal from the Columbia River drainage has been inferred for additional freshwater taxa: bull trout (*Salvelinus confluentus*, Taylor *et al.*, 1999; Costello *et al.*, 2003), mountain whitefish (*Prosopium williamsoni*, Whiteley *et al.*, 2006) and *Sida* (Cox & Hebert, 2001). Pygmy whitefish could have tracked the receding ice sheets into the northern reaches of this watershed, and subsequently accessed the Fraser River system through a series of proglacial lakes that formed in the Okanagan Valley: lakes Oliver, Quilchena, Coldstream and Penticton (McPhail & Lindsey, 1986). Access to the Skeena River system was possible via temporary connections between the Fraser River and two major Skeena River tributaries, the Babine and Bulkey rivers (McPhail & Lindsey, 1986). Pygmy whitefish probably accessed the Peace River drainage from the Fraser River system, probably via proglacial lakes in the Prince George, British Columbia area (Lindsey & McPhail, 1986).

Origin of eastern North American pygmy whitefish

The most enigmatic aspect of the distribution of pygmy whitefish is its presence in Lake Superior. While most authors have hypothesized that this population originated from a Mississippi Refuge (McPhail & Lindsey, 1970; Lindsey & Franzin, 1972; Bailey & Smith, 1981; Underhill, 1986), a biogeographic analysis of fish dispersal into Ontario and the Great Lakes could not resolve its origin (Mandrak & Crossman, 1992). Haplotype 20 was found in all individuals analysed from Lake Superior, and it differed from haplotype 16, which is widely distributed in Cascadia, by three mutational steps. Consequently, the apparent restriction of haplotype 20 to Lake Superior could have occurred by dispersal there from the Columbia River drainage, with bottlenecking and lineage sorting generating the differences between haplotypes 16 and 20. Our coalescent analyses using Mesquite were unable to differentiate between a two- (Beringia + Cascadia/Mississippi) and a three- (Bering + Cascadia + Mississippi) refuge model of population structure, but when distributional evidence is considered, the two-refuge hypothesis becomes highly unlikely. For instance, there are no data supporting a Pacific Refuge origin for the *c.* 185 species of fishes known from the Laurentian Great Lakes drainage (Hubbs & Lagler, 1964; Bailey & Smith, 1981; Legendre & Legendre, 1984; Underhill, 1986; Mandrak & Crossman, 1992). A Pacific origin would require a series of time-critical watershed transfers as well as the subsequent extirpation of populations from the many hundreds of water bodies in the proglacial Lake Agassiz basin with seemingly suitable pygmy whitefish habitat. Pygmy whitefish, however, also occur in Waterton Lakes, south-western Alberta, and post-glacial dispersal to Lake Superior from a southern Alberta (Crossman & McAllister, 1986) or Missouri Refuge cannot be ruled out. Waterton Lakes harbour a regionally unique fauna, including pygmy whitefish, as well as 'glacial relict' taxa, including the crustaceans *Mysis* and *Diporeia*, and the deepwater sculpin, *Myoxocephalus thompsonii* (e.g. Dadswell, 1974). The origin of the Waterton Lakes fauna is

uncertain (the lakes sit at an elevation of 1398 m) and their glacial history is poorly resolved (Lindsey & Franzin, 1972; Sheldon *et al.*, 2008). Some aquatic taxa, including lake trout (Wilson & Hebert, 1998), *Sida* (Cox & Hebert, 2001) as well as potentially Arctic grayling (a now extirpated population of grayling occurred in Michigan, Stamford & Taylor, 2004), and burbot (Van Houdt *et al.*, 2005) have dispersed to the Great Lakes from the Beringian Refuge. Haplotype 20, however, showed no close affiliation to haplotypes from the Yukon and Alaska, suggesting that for pygmy whitefish, this hypothesis is unlikely. Again, the hypotheses of Beringian or Missouri origins for Lake Superior pygmy whitefish would need to invoke subsequent extirpation over wide swaths of seemingly suitable habitat. Consequently, distributional and some of our DNA data support the hypothesis of a post-Wisconsinan Mississippi refugial origin of contemporary Lake Superior pygmy whitefish, although the ultimate origin of eastern North American pygmy whitefish was probably in western North America. McPhail & Lindsey (1986) reached a similar conclusion for the round whitefish, which has a wider distribution in eastern North America than the pygmy whitefish (Lee *et al.*, 1980).

Broader implications of pygmy whitefish phylogeography

The Canadian *Species at Risk Act* (SARA) recognizes conservation units below the species level in the form of 'designatable units' (DUs). The large range disjunctions and presence of divergent phylogenetic clades within pygmy whitefish that we have resolved form a strong basis of argument for separate conservation status assessments for several DUs within *P. coulterii* in Canada (COSEWIC, 2010).

Finally, our data underscore the salient roles that Beringian and Cascadian refugia have played, but also suggest that the roles of smaller refugia, such as the Nahanni Refuge, have been more variable among species. As a result of this region's historical complexity, future molecular zoogeographic studies that target taxa that are broadly co-distributed and that possess diverse life histories and dispersal abilities offer the greatest potential to broaden our understanding of this region.

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