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Genetic analysis of sympatric char populations in western Alaska: Arctic char (*Salvelinus alpinus*) and Dolly Varden (*Salvelinus malma*) are not two sides of the same coin

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Abstract

The North Pacific Ocean has been of great significance to understanding biogeography and speciation in temperate faunas, including for two species of char (Salmonidae: *Salvelinus*) whose evolutionary relationship has been controversial. We examined the morphology and genetics (microsatellite and mitochondrial DNA) of Arctic char (*Salvelinus alpinus*) and Dolly Varden char (*Salvelinus malma*) in lake systems in western Alaska, the eastern and western Arctic, and south of the Alaskan Peninsula. Morphologically, each lake system contained two forms: one (Arctic char) largely confined to lake habitats and characterized by greater numbers of pyloric caeca, gill rakers, and shallower bodies, and another (Dolly Varden) predominated in adjacent stream habitats and was characterized by fewer pyloric caeca, gill rakers, and deeper bodies. MtDNA partial (550 bp) d-loop sequences of both taxa were interspersed with each other within a single 'Bering' clade and demographic inferences suggested historical gene flow from Dolly Varden to Arctic char had occurred. By contrast, the taxa were strongly differentiated in sympatry across nine microsatellite loci in both lakes. Our data show that the two taxa are highly genetically distinct in sympatry, supporting their status as valid biological species, despite occasional hybridization. The interaction between these species highlights the importance of the North Pacific, and Beringia in particular, as an evolutionary wellspring of biodiversity.

Introduction

The nature of species (their definition, origin, biogeography, and interrelationships) is central to evolutionary biology. The biogeography of taxa has been of great interest to evolutionary biologists through documenting and understanding the evolution of regional biotas and as a way to resolve the distinction between, and origin of, species (e.g., Mayr, 1963). For instance, the biological species concept (*sensu* Mayr, 1963) has been criticized because the criterion of reproductive isolation cannot be applied directly to allopatric taxa. By contrast, putative species that come into sympatry can provide an 'acid test' of the validity of their status as distinct biological species (Avise 1994). In addition, simultaneous examination of allopatric and sympatric populations of putative biological species can provide critical insights into the geographic mode of speciation, i.e., whether speciation has proceeded largely in allopatry or sympatry (e.g., Lynch, 1989; Bernatchez & Dodson, 1990). Such an approach to understanding species and speciation is manifested in emerging paradigms such as de Queiroz's (2005) attempted reconciliation of various species concepts under the framework of a 'metapopulation lineage concept of species'. In de Queiroz's model, reproductive isolation in sympatry (a so-called 'contingent property' of species) is no longer necessarily required to establish

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species identity. Genetic and ecological isolation in sympatry as measures of reproductive isolation, however, are still important in establishing the evolutionary independence among metapopulation lineages ('species') and to identify different kinds of species such as 'reproductively isolated species' or 'diagnosible species' (de Queiroz, 2005).

Fishes in the family Salmonidae constitute about 66 Holarctic species of salmon, trout, char, grayling, and whitefish (Nelson, 2006). Salmonids have long been of interest to evolutionary biologists (e.g. Mayr, 1963, p. 39; Hendry & Stearns, 2004) because they typically form spatially (and sometimes temporally) discrete breeding units that exhibit pronounced phenotypic and genetic differentiation. Such differentiation occurs in a variety of geographic and taxonomic contexts; from distinct taxa hybridizing along a contact zone to ecotypes within a taxon that are genetically distinct in sympatry (see Behnke, 1972 and Taylor, 2004 for reviews). Consequently, salmonids have provided rich material with which to study the ecology, behaviour, genetics, and biogeography of evolutionary divergence, adaptive radiation and their relevance to speciation (e.g. Foote & Larkin, 1988; Jonsson & Jonsson, 2001; Redenbach & Taylor, 2002; Rogers & Bernatchez, 2006). Arguably, char (*Salvelinus*) have been the focus of some of the most intense and controversial studies both of the patterns (i.e. systematics and taxonomy) and processes in the evolution of biological diversity (Behnke, 1980; Savvaitova, 1980; Coyne & Orr, 2004). Char are found in freshwater, estuarine, and marine environments, but breed exclusively in freshwater. Across this range, there are seven well-accepted species (Behnke 1980). One of these species, the Arctic char (*Salvelinus alpinus*) has been described at many times as a 'species complex', i.e. a collection of closely related forms each of whose status as distinct species has at one time or another been controversial often owing to incomplete reproductive isolation among forms, variable levels of current and historical introgression, and the presence of morphological intermediates (e.g. McPhail, 1961; Behnke, 1980; Savvaitova, 1980). Recent ecological and genetic studies have, however, indicated that within the *S. alpinus* complex, the distinction between Arctic char/Dolly Varden (*S. malma*) and bull trout (*Salvelinus confluentus*) has been well established (Phillips *et al.*, 1995; Redenbach & Taylor, 2002, 2003). By contrast, the distinction between Dolly Varden and Arctic char has remained somewhat more clouded, especially because multiple (up to 23 in the case of Arctic char) taxa have been described within the putative *S. alpinus* and *S. malma* lineages (Behnke, 1980; Phillips *et al.*, 1999; Adams & Maitland, 2007).

The distribution of Arctic char encompasses circumpolar nearshore marine waters and adjacent freshwater habitats (McPhail, 1961; Behnke, 1980; Fig. 1). By contrast, the distribution of Dolly Varden encompasses coastal marine and freshwaters from Korea in the western Pacific to the Olympic Peninsula in northwestern Washington State (USA) in the eastern Pacific (McPhail, 1961; Behnke, 1980; Fig. 1). Brunner *et al.* (2001) conducted an mtDNA-based phylogeographic survey of Arctic char and found five distinct mtDNA lineages across the Holarctic. The origin of these lineages was suggested to stem from isolation within, and subsequent dispersal from, distinct Pleistocene glacial refugia. All Dolly Varden samples, diagnosed *a priori* using morphological and biogeographic criteria, comprised the 'Bering' lineage found from Kamchatka north to the Bering Strait and east to southeastern Alaska (Brunner *et al.*, 2001). Dolly Varden and Arctic char mtDNA were paraphyletic; European, eastern North American, and central Siberian Arctic char were more closely related to Dolly Varden (Beringian) mtDNA than they were to the ancestral *S. alpinus* lineage from the Arctic (Canada, Greenland, and northeastern Russia) (Brunner *et al.*, 2001; see also Oleinik *et al.*, 2007). This result led Brunner *et al.* (2001) to conclude that the status of *S. malma* as a distinct species and one sister to *S. alpinus* (cf. Phillips *et al.*, 1995) could not be supported.

In the Canadian Arctic and adjacent portions of northern Alaska, Arctic char have been diagnosed as those lacustrine and anadromous individuals living east of the Mackenzie River, as well as most of those lacustrine char found west of the Mackenzie River and in Alaska (Reist *et al.*, 1996). By contrast, Dolly Varden are usually identified as anadromous and stream-resident fish west of the Mackenzie River and in Alaska (Reist *et al.*, 1996). McPhail (1961) examined the morphology of sympatric char in southwestern and central Alaska and found consistent differences between putative Arctic char and Dolly Varden, but some populations were morphologically ambiguous. The genetic status of these sympatric populations, however, has not been examined. Morphological distinctions between Arctic char and Dolly Varden are strongly tied to each putative taxon's distinct life-histories and habitat use (lakes vs. streams, freshwater resident vs. anadromous). It is possible, therefore, that they represent a purely phenotypic response to environmental differences even within a given body of water – a phenomenon for which char are well known (Savvaitova, 1980; Behnke, 1980; Nordeng, 1983; Sandlund *et al.*, 1992; Skúlason *et al.*, 1996). Alternatively, genetic distinction between sympatric char would provide the most direct test of reproductive isolation and distinct biological species status for Arctic char and Dolly Varden.

Directly assessing the biological species status of Arctic char and Dolly Varden has important implications for our understanding of the evolution of Arctic biodiversity and phylogeography, the relationship between and causes of phenotypic and genetic divergence, and processes of speciation. First, there is clearly spectacular phenotypic diversity with the Arctic char species complex (Behnke, 1980; Klemetsen *et al.*, 2003),

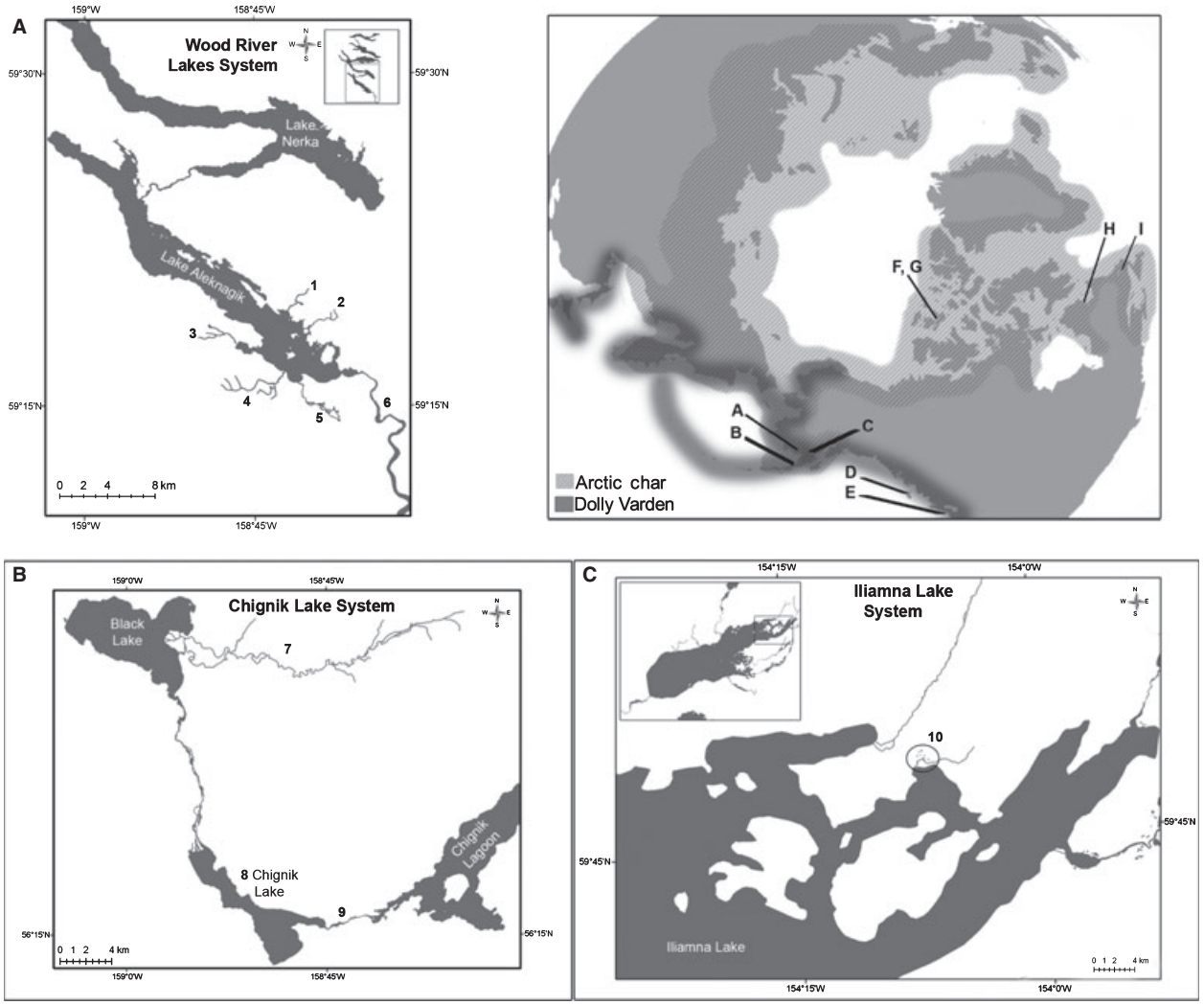

Fig. 1 Global distributions of Arctic char (*Salvelinus alpinus*) and Dolly Varden. (*Salvelinus malma*) and collection localities in western Alaska, northern British Columbia, and the central and eastern Canadian Arctic. A = Lake Aleknagik, B = Chignik Lake, C = Iliamna, Lake, D = Chache Creek, E = Zeballos Lake, F = Char Lake, G = Resolute Lake, H = Lac Duquet, Weir, I = Ikadlivik Brook. Maps A, B, and C show collection localities in Lake Aleknagik, Chignik, Lake, and Iliamna Lake, respectively. 1 = Hansen Creek, 2 = Eagle Creek, 3 = Bear Creek, 4 = Yako Creek, 5 = Whitefish Creek, 6 = Wood River, 7 = Alec River, 8 = Chignik Lake, 9 = Chignik River, 10 = Pedro Ponds.

including co-existence of distinct Icelandic morphs that appear to have diverged sympatrically (Gíslason *et al.*, 1999). Such diversity has been accounted for by the hypothesis of a single polymorphic species that has repeatedly given rise to similar phenotypes in different geographic areas without necessarily invoking the possibility of reproductive isolation and speciation (e.g., Savvaitova, 1995; Jonsson & Jonsson, 2001). Alternatively, reproductive isolation and morphological and ecological divergence in sympatry have been considered evidence of speciation within the *S. alpinus* complex (Behnke, 1980; Jonsson & Jonsson, 2001). Resolution of these alternatives in *Salvelinus,* therefore, has implications for the relative importance of polymorphism within genetic lineages versus divergence associated with speciation in explaining diversity (Magurran & May, 1999; Turner *et al.*, 2001). Second, Brunner *et al.* (2001) suggested that the lack of concordance between ecological/morphological differentiation and molecular divergence in the *S. alpinus* complex may result from a more rapid pace of evolution for the former traits. Alternatively, it is well established that mtDNA may cross species boundaries owing to introgression even when nuclear loci remain strongly differentiated, particularly in char (e.g. Bernatchez *et al.*, 1995; Arnold, 1997; Redenbach & Taylor, 2002). Consequently, an alternative explanation for strong similarity of mtDNA between *S. alpinus* and *S. malma* reported by Brunner *et al.* (2001) involves

historical and current hybridization between them. Finding strong differentiation at nuclear loci in the face of similarity in mtDNA would implicate historical hybridization in the paraphyly of mtDNA between the species (cf. Redenbach & Taylor, 2002). Third, we have an incomplete understanding of the evolution of the geographic ranges of species (Brown *et al.*, 1996; Ree *et al.*, 2006). Where the two putative taxa broadly co-occur, Dolly Varden are associated with stream habitats and, if those habitats have access to the sea, with anadromy (sea-going life history) whereas Arctic char are associated with lake-dwelling (lacustrine) life histories (e.g. McPhail, 1961; Reist *et al.*, 1996). Morphological/ecological differences associated with habitat and life history driven by environmentally induced plasticity or by genetic divergence are alternatives both with precedents in northern fish faunas (e.g. Taylor, 1999). Consequently, if these 'ecotypes' of char represent environmentally driven polymorphisms it is possible that they may have developed following post-glacial colonization by a polymorphic ancestor from a single glacial refuge. Alternatively, if sympatric Dolly Varden and Arctic char represent distinct genetic lineages, then they may have originated from an entirely different biogeographic scenario – secondary contact between distinct refugial isolates or from post-glacial divergence in sympatry or parapatry.

To help address these uncertainties, we examined microsatellite and mitochondrial DNA to assess genetic distinction and relationships between several sympatric and allopatric populations of Arctic char and Dolly Varden. Specifically, we tested for evidence of reproductive isolation between sympatric char populations collected from three drainages in western Alaska. Populations from two of these drainages were also examined morphologically before genetic analysis to provide putative species identifications based on pre-existing criteria (McPhail, 1961).

Materials and methods

Study area

The main area of study involves three lake systems in western Alaska in the vicinity of Bristol Bay (Iliamna Lake and Lake Aleknagik) and the southeastern margin of the Alaskan Peninsula (Chignik Lake, Fig. 1). Iliamna Lake (59°32′12″N; 155°1′28″W) is the second largest lake in the United States (2600 km^2) and empties into southeastern Bristol Bay via the Kvichak River (Fig. 1). The specimens for this study were collected from two proximate, but discrete habitats in the eastern region of the lake: (i) the shores of low-lying, rocky islands (herein referred to as 'island beaches'), and (ii) a series of small, spring-fed ponds that are interconnected with the lake and each other by intervening streams near the village of Pedro Bay (herein referred to as 'Pedro Ponds'). Lake Aleknagik (832 km^2, 59°17′0″N; 158°37′0″W) is located at the northern edge of Bristol Bay, near Dillingham, Alaska, and is one in a series of interconnected lakes that drains via the Wood River into the Nushagak River. Within the Lake Aleknagik system we collected char from the lake proper and from five streams: Whitefish Creek, Hansen Creek, Yako Creek, Eagle Creek, and Bear Creek. (Fig. 1). Chignik Lake (24.9 km^2, 56°16′10″N, 158°46′54″W) is located on the southeastern margin of the Alaskan Peninsula and drains to the Gulf of Alaska. It is one of the two interconnected lakes (the other being Black Lake and the two lakes constituting 'Chignik Lakes'). Our samples came from Chignik Lake, the Chignik River (the lake's outlet stream) and from the Alec River, a tributary of Black Lake that is immediately upstream of Chignik Lake. Black Lake and Chignik Lake are interconnected by the Black River (Fig. 1). All these lakes have large populations of various salmonids including char and sockeye salmon (*Oncorhynchus nerka*), the numerically dominant salmonid, and other native fishes including sticklebacks (*Gasterosteus*) sculpins (*Cottus*), and whitefish (*Prosopium*).

Char collections

Samples for DNA analysis were collected in August 2002, 2004 and 2005 by a variety of methods including angling, beach seines, and minnow traps. We also obtained samples from allopatric populations of *S. alpinus* (eastern Canadian Arctic and northwestern Quebec) and *S. malma* (Queen Charlotte Islands and Vancouver Island, British Columbia) to serve as reference samples. Specimens for morphological analyses consisted largely of char collected during July and August 2004, augmented with several fish collected in August 2005 (Table 1). All fish were fixed in a solution of 10% formalin and stored in 70% ethanol before morphological analysis.

Meristics and morphometrics

All counts and measurements were conducted on the left side of the specimens following Hubbs & Lagler (1958). We counted dorsal (DRN), anal (ARN), pelvic (PRN) and pectoral (PERN) rays and gill rakers (GR) were counted including rudimentary rakers. Total GR as well as upper limb (UGR) and lower limb (LGR) of the gill arch were counted. Lateral line pores (LLP) were counted by drying the specimens with a paper towel and counting them under a dissecting microscope. Pyloric caeca (PC) were counted after individual caeca were separated from the caecal mass. The following morphometric measurements were taken: standard length (SL), head length (HL), snout length (SNTL), interorbital width (IOW), length of maxilla (ML), head depth (HD), body depth (BD), least caudal peduncle depth (LCPD), length of pectoral fin (PL), length of pelvic fin (PEL), predorsal length (PDL) and post-dorsal length (PODL).

Table 1 List of localities and sample sizes for Arctic char (*Salvelinus alpinus*) and Dolly Varden (*S. malma*) examined for variation in morphology and mitochondrial and microsatellite DNA (see Fig. 1).

Nominal taxon	Locality	N*
Arctic char (*Salvelinus alpinus*); Dolly Varden (*Salvelinus malma*)	Iliamna Lake, Iliamna Lake, Bristol Bay Alaska	31/2/31
	Pedro Ponds, Iliamna Lake, Bristol Bay, Alaska	34/2/34
	Hansen Creek, Lake Aleknagik, Bristol Bay, Alaska	32/2/31
	Whitefish Creek, Lake Aleknagik, Bristol Bay, Alaska	10/2/29
	Bear Creek, Lake Aleknagik, Bristol Bay, Alaska	1/–/1
	Lake Aleknagik, Bristol Bay, Alaska	30/2/36
	Eagle Creek, Bristol Bay, Alaska	4/–/4
	Yako Creek, Bristol Bay, Alaska	4/–/4
	Chignik Lake, Gulf of Alaska, Alaska	–/–/34
	Chignik River, Gulf of Alaska, Alaska	–/–/17
	Alec River, Gulf of Alaska, Alaska	–/–/14
Arctic char (*S. alpinus*)	Char Lake, central Canadian Arctic	–/–/9
	Resolute Lake, central Canadian Arctic	–/–/17
	Lac Duquet, northwestern Québec	–/–/28
	Ikadlivik Brook, Labrador, Canada	–/–/30
Dolly Varden (*S. malma*)	Zeballos Lake, Vancouver Island, British Columbia	–/–/30
	Chache Creek, Queen Charlotte Islands, British Columbia	–/–/15

*Numbers indicate sample sizes for morphology, mitochondrial, and microsatellite DNA, respectively.

Data analysis

In order to determine the general differences within the entire dataset, the meristic and morphometric measurements of all individuals were subjected to a standard Principal Component Analysis (PCA). PCA was used to treat the meristic and size-adjusted morphological variables such that they had no intercorrelations and to reduce the original 22 variables to a smaller summary set of uncorrelated multivariate components (Pimental, 1979). Because body shape and size are typically confounded, we used Burnaby's (1966) method to project our measurements onto a space independent from the first principal component which is usually (as in our case) dominated by overall body size (e.g. McPhail & Taylor, 1999). The resulting values for each trait were all uncorrelated with standard length (all $P > 0.1$). These size-adjusted values were then used in a PCA along with the raw meristic data (which were uncorrelated with length) using the inter-trait correlation matrix. Subsequent genetic analyses (see below) permitted the identification to species by independent means. Fish genetically identified as *S. malma* or *S. alpinus* were contrasted for mean PCA scores using multivariate analysis of variance (MANOVA). Discriminant functions analysis was also used to establish the degree to which char could be identified to species using the individual PCA scores as input data to develop the discriminant functions. Morphological classifications employed the 'jacknife' procedure where the individual being classified was not included in the derivation of the discriminant function. Finally, where some individual meristic traits suggested bimodality in frequency distributions from visual inspection, we tested for deviation from a single normal distribution using the Shapiro-Wilk test (Shapiro & Wilk, 1965). Morphometric data analyses were conducted using the PAST ver 1.43 software package (Hammer *et al.*, 2001; available at http://folk.uio.no/ohammer/past/; last accessed 17 August 2008).

Mitochondrial and microsatellite DNA analyses

Samples (Table 1) consisted of adipose or pelvic fin clips and, occasionally in the case of very small fish (< 50 mm total length) whole organisms, stored in 95% ethanol until DNA extraction using Qiaquick spin columns (Qiagen, Inc., Valencia, CA, USA). DNA was stored at –20 °C until genetic analysis.

Our first objective was to place char within our study area within the pre-existing evolutionary framework presented by Brunner *et al.* (2001). Consequently, a 550 bp portion of the mitochondrial DNA control region homologous with that assayed by those authors was sequenced in a representative subset of our char samples. We sequenced two each of Dolly Varden and Arctic char from each of the lakes that we sampled. Fish were identified *a priori* as either Dolly Varden or Arctic char through the combined use of microsatellite DNA assignment tests and morphology (see Results below). Initial polymerase chain reaction (PCR) amplification of an approximately 1 kbp control region fragment used the primers HN20 and Tpro2 (Brunner *et al.*, 2001). PCR conditions included (final concentrations in 50 μL final volumes): 0.1 mM of each dNTP, 2 mM $MgCl_2$, 0.2 μM of each primer, 1 unit of NEB *Taq* polymerase and 1× of the associated buffer (New England Biolabs, MA, USA). All PCR amplifications were run under the following cycling conditions: 1 × 95 °C for 3 min, 50 °C for 1 min, 72 °C for 1 min, 4 × 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, 30 × 92 °C for 30 s, 50 °C for 30 s, 72°C

for 30 s, and 1 × 72 °C for 10 min. Subsequent purification and automated sequencing of amplified products are outlined in Stamford & Taylor (2004).

We scored individuals using nine microsatellite loci that had been isolated from Dolly Varden (Smm22; Crane *et al.*, 2005), bull trout (Sco106, 204, 206, 215, 216, 218, and 220; DeHaan & Ardren, 2005; S. Young, Wash. Dept. Fish and Wildlife, Olympia, WA, unpublished) and brook trout (Sfo18; Angers *et al.*, 1995). Samples were examined using fluorescently labelled primers and assayed on a CEQ 8000 automated genotyper (Beckman Coulter, Inc., Fullerton, CA, USA). All raw allele frequency data can be obtained from an electronic database (http://www.zoology.ubc.ca/~etaylor/nfrg/akchar/akchardata.html) or by contacting the senior author.

Genetic data analysis

Control region sequences were aligned using BIOEDIT (Hall, 1999; available at: http://www.mbio.ncsu.edu/BioEdit/bioedit.html) with gaps treated as single characters. Sequences obtained were compared with 41 sequences representing haplotypes from Holarctic *Salvelinus* selected to represent a range of divergences within each of the major clades resolved by Brunner *et al.* (2001). We also included several sequences from relevant taxa or geographic areas studied by Redenbach & Taylor (2002) and Elz (2003) (Supporting Information Appendix S1). Subsequent phylogenetic analyses utilized homologous sequences from lake trout (*Salvelinus namaycush*), white-spotted char (*S. leucomaenis*), and brook trout (*S. fontinalis*) as outgroup taxa.

We used MODELTEST version 3.7 (Posada & Crandall, 1998) to identify the most appropriate model of sequence evolution. The model chosen by the Akaike Information Criterion (AIC, Posada & Buckley, 2004) method of model testing was the General Time Reversible (GTR, plus I and Γ) model. We subsequently calculated the measures of sequence divergence using this model and clustered these estimates with Neighbour-Joining using routines in PAUP under 1000 bootstrap replicates (Version 4b10, Swofford, 2002).

We also used the sequence data obtained from our samples and those of Brunner *et al.* (2001) to quantitatively assess the likelihood of historical gene flow between Arctic char and Dolly Varden across the range of samples. The program IM (Hey & Nielsen 2004) was used to fit the 'isolation-with-migration' model with population size changes to the mtDNA dataset. The program uses a coalescent approach to model and provide maximum likelihood estimates of six demographic parameters resulting from the split of an ancestral population into two descendant populations after which gene flow may have occurred between the two descendant populations: ancestral population size (θ_A), population size of species 1 (θ_1), population size of species 2 (θ_2), migration rate from species 2 (Arctic char) to species 1 (Dolly Varden, m_1), probability of migration rate from species 1 to species 2 (m_2), and time of lineage splitting (t), all scaled by the neutral mutation rate, μ. The program uses a Markov Chain Monte Carlo simulation procedure to generate gene genealogies that are initially produced from parameter values based on random selections from a range supplied by the user. These parameter values are continuously updated from further randomizations such that the distribution of recorded parameter values approximates the posterior probability density of those parameters, given the data. Evidence for historical gene flow between Arctic char and Dolly Varden, therefore, was taken as estimated parameter values for m_1 or m_2 that were > 0. We converted the mutation-rate scaled parameter estimates into demographic units using the methods outlined in the IM program documentation and employing a mutation rate range for the dloop segment sequenced of $5.0–10.0 \times 10^{-6}$, i.e. 1–2% per million years (Shedlock *et al.*, 1992; McKay *et al.*, 1996) × 550 bp, and a generation time of 5 years (Scott and Crossman 1973). Given the uncertainty of the actual mutation rate (Arbogast *et al.* 2002) the demographic estimates are clearly provisional and we emphasize the relative differences between species. As the IM analysis works ideally for panmictic populations, we conducted the analysis on a subset of haplotypes comprising fish from Beringia, and adjacent portions of Siberia, and the Canadian high Arctic ($N = 36$ haplotypes in total, Supporting Information Appendix S1). The burn-in period used was 1 000 000 steps and parameter values were estimated after the effective sample sizes were at least 1000 for each parameter for a maximum of 5 000 000 subsequent steps. We applied the Hasegawa-Kishino-Yano substitution model (HKY, Hasegawa *et al.*, 1985), a less specialized form of the GTR model, as recommended for estimating parameter distributions for mtDNA data (Hey & and Nielsen 2004). The total analysis consisted of five distinct runs of each simulation to verify convergence of parameter estimates around similar values.

The following tests were performed using GENEPOP version 3.3 (Raymond & Rousset, 2001, updated from Raymond & Rousset, 1995) for the microsatellite dataset. Tests for deviations from Hardy–Weinberg equilibrium were performed for each locus-population combination using an exact test in which probability values were estimated using a Markov chain method. Tests for genotypic linkage disequilibrium for all combinations of locus pairs within a population were also made using a Markov chain method with GENEPOP default values. Basic descriptive statistics of sample size (N), number of alleles (N_A), allelic richness (A_R), observed (H_O) and expected (H_E) heterozygosity were compiled using FSTAT ver 2.9 (Goudet, 2001, updated from Goudet, 1995). To summarize microsatellite differentiation among all samples, a factorial correspondence analysis (FCA) was conducted on allele frequency data using GENETIX

(ver 4.05, Belkhir *et al.*, 2004). Factorial Correspondence Analysis is a type of factor analysis that seeks to find the linear combination of variables (in this case, allele frequencies at different loci) that best describe variation between individual observations (fish). In general terms, FCA is best suited for categorical data, such as allele frequency counts, and determines the first K axes of an orthogonal number of axes that describe the most variance from a 'cloud' of observations.

We used the program STRUCTURE (Pritchard *et al.*, 2000) to probabilistically determine the number of genetically distinct populations within each lake. The method clusters individuals, based on their genotypes, into K randomly intrabreeding groups by minimizing departures from Hardy–Weinberg and linkage disequilibrium within groups. Because we sampled discrete localities within each system (i.e. three localities within Lake Aleknagik, two in Iliamna Lake, and three in Chignik Lake) and because between species differentiation could be confounded with within-species inter-locality differentiation, we conducted the STRUCTURE analyses on each locality separately, within lakes. We employed 50 000 replications during the presimulation, 'burn-in' period and estimated q after a subsequent 450 000 replications. We then calculated the posterior probability of each K following Bayes' theorem (Pritchard *et al.*, 2000). In all but one case, we found that $K = 1$ or 2 population was the most likely population structure (see Results). Consequently, we used Bayesian and maximum likelihood-based assignment test procedures to assign fish caught in sympatry to Arctic char or Dolly Varden taxa and possible hybrids between species based on the use of baseline samples from known species collected in allopatry. These analyses make use of multilocus genetic profiles of individual char to assign them to *a priori* groups (i.e. known Arctic char and Dolly Varden from allopatric reference samples) characterized at the same loci (e.g. reviewed by Hansen *et al.*, 2001). First, we used the program HYBRIDLAB (Nielsen *et al.*, 2006) to generate a database of simulated hybrids between our reference samples of Arctic char and Dolly Varden. The program estimates allele frequencies at each locus in each of the parental species. Multilocus F_1 and post-F_1 hybrid genotypes ($N = 100$) were then created through random draws of one allele at each locus, as a function of their calculated frequency distributions, within each species (assuming linkage equilibrium among loci, selective neutrality and random mating). These simulated hybrids and the reference samples of Arctic char and Dolly Varden were used to assign the Alaskan char to parental species and to set the admixture boundaries for identification of parental species, and possibly hybrids, in the Alaskan drainages using STRUCTURE as recommended by Vähä & Primmer (2006). We also used the program GENECLASS (version 2.0, Piry *et al.*, 2004) to classify individual char to one of either Arctic char or Dolly Varden using the Bayesian and frequencies-based likelihood assignment procedures of Rannala & Mountain (1997) and Paetkau *et al.* (2004), respectively.

Results

Morphological variation

In Iliamna Lake, PCA on the combined meristic and size-adjusted morphological traits resulted in five components that together accounted for 55% of the total variation and separated char into two general groups (Fig. 2a). As identified subsequently by DNA analysis, one group (largely Arctic char) was characterized by high numbers of pyloric caeca, gill rakers, deep long heads, and long PODLs. The second group (largely Dolly Varden) consisted of fish with opposite trends in these characters, many LLPs, long and many-rayed anal and paired fins,

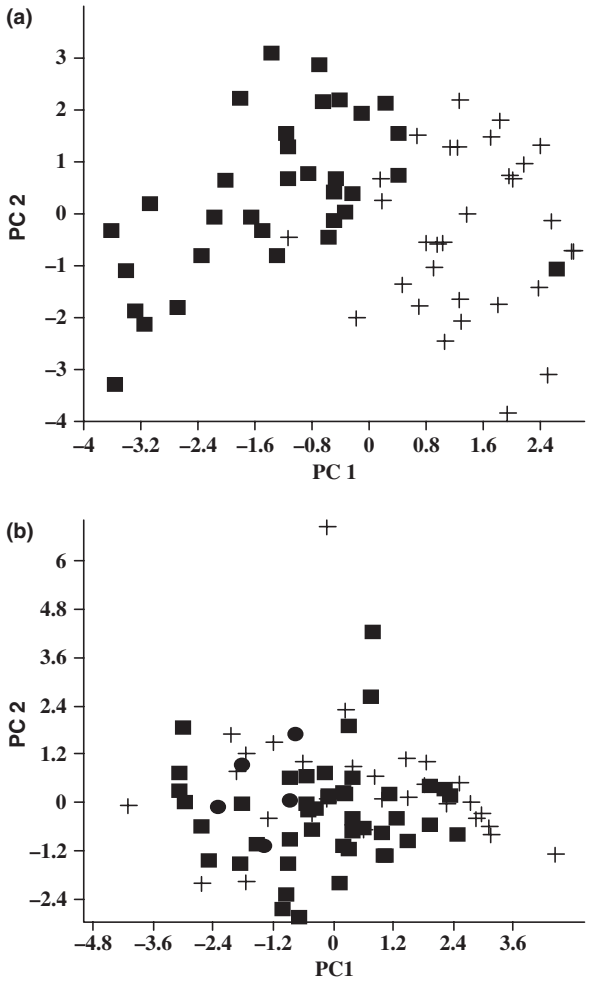

Fig. 2 Plots of scores along the first two principal components axes based on variation of meristic and size-adjusted morphological traits in samples of Arctic char (*Salvelinus alpinus,* squares) and Dolly Varden (*Salvelinus malma,* crosses) in (a) Iliamna Lake and (b) Lake Aleknagik, western Alaska.

and relatively deep bodies. Both groups of char were highly distinct across all five PC (MANOVA, $F_{5,59} = 13.2$, $P < 0.001$, Supporting Information Appendix S2).

In Lake Aleknagik, the first five PCs accounted for 52% of the total meristic and morphological variation, but there was less pronounced separation of char into two distinct morphological groups (Fig. 2b). As in Iliamna Lake, fish identified genetically as Arctic char tended to have more pyloric caeca, more gill rakers on the lower limb of the first arch, and longer heads and snouts relative to Dolly Varden. Arctic char and Dolly Varden were distinct across all five PC axes (MANOVA, $F_{5,66} = 2.45$, $P = 0.010$, Supporting Information Appendix S2).

There were also a few univariate traits that showed clear signs of bimodality in their frequency distributions. In particular, pyloric caeca counts displayed evidence of distinct bimodality (Shapiro-Wilk test of normality, $P = 0.001$) in Iliamna Lake where one mode was at 47 caeca and the other at 27. The separation of the two modes in pyloric caecal count was less striking in Lake Aleknagik, but one mode occurred at 51 caeca and the other occurred at 21 (Shapiro-Wilk $P = 0.001$). These modal pyloric ceaca counts are similar to values of 40–50 and 20–35 for *S. alpinus* and *S. malma*, respectively reported by McPhail (1961).

MtDNA phylogeography and historical demography

Integration of our data from two localities where Dolly Varden and Arctic char are sympatric (Iliamna Lake and Lake Aleknagik) and one in which Dolly Varden live in allopatry (Chignik Lake) with those of Brunner *et al.* (2001) indicated that there is no consistent clustering of mtDNA haplotype by taxon (Fig. 3); Dolly Varden and Arctic char haplotypes are interspersed with each other in samples from eastern Siberia, Kamchatka, western, central, and southeastern Alaska and BC (i.e. the 'Bering' lineage of Brunner *et al.*, 2001). Consistent with results of Brunner *et al.* (2001), this clade was sister to another group of haplotypes consisting of 'high Arctic' *S. alpinus* from Canada, Alaska, Greenland and portions of Siberia (63%). The 'Bering'/'high Arctic' clade was sister to a final grouping consisting of 'Atlantic', 'Siberian', and 'Acadian' *S. alpinus* (63% support), with the latter forming a strongly supported subgroup (85%). The

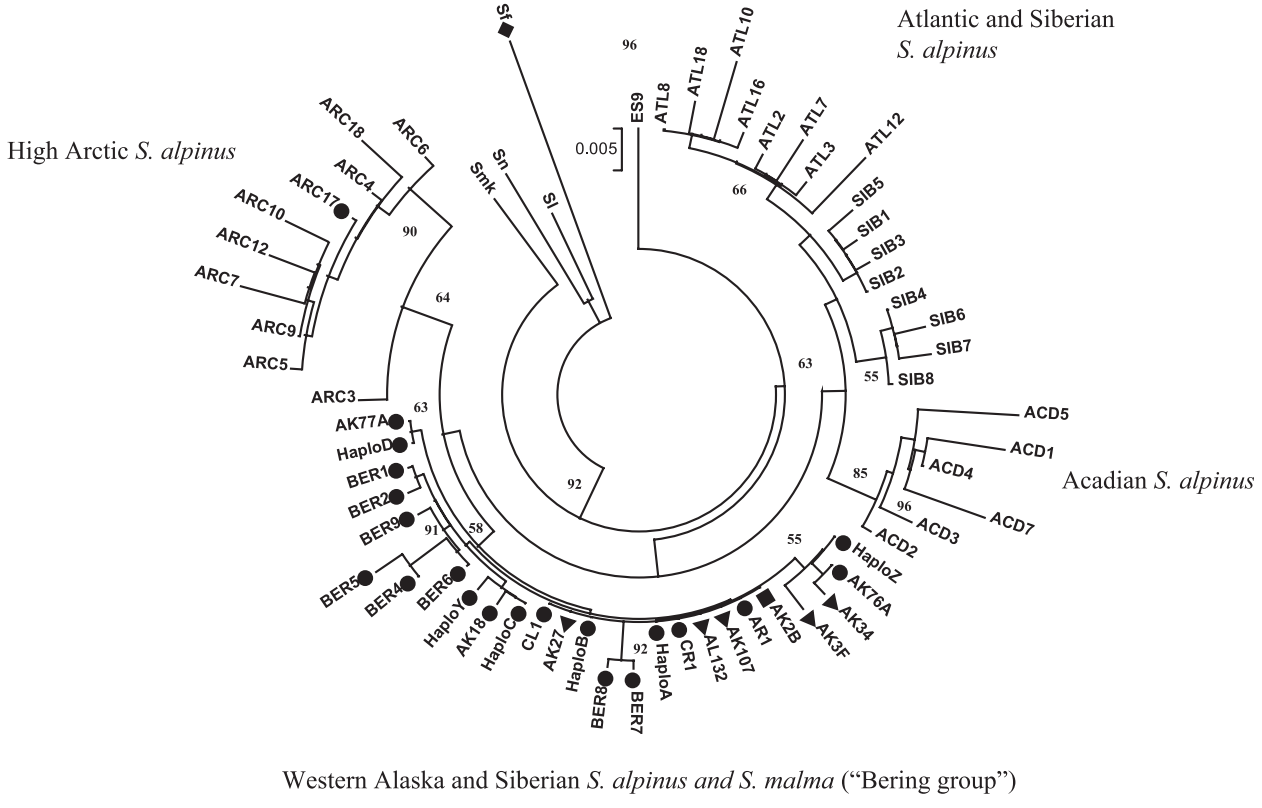

Fig. 3 Phylogeny of mitochondrial DNA haplotypes among samples of *Salvelinus*. Phylogeny is based on Neighbor-Joining clustering of GTR pairwise genetic distances among haplotypes derived from 550 bp of the control region. Bootstrap scores (> 50%) are indicated at branch points. Haplotypes names 'SIB', 'ACD', 'ATL', 'BER', and 'ARC' represent haplotypes from the Siberian, Acadian, Atlantic, Bering, and Arctic lineages taken from Brunner *et al.* (2001). Arctic char are indicated by ▲, Dolly Varden by ●, haplotypes found both in Arctic char and Dolly Varden by ■, and the outgroup (Sf) by a ◆. Identity and locality of each haplotype are given in the Supporting Information Appendix S1.

Table 2 Maximum-likelihood estimates (MLE) and the 90% highest posterior density (HPD) intervals of demographic parameters for Dolly Varden (1) and Arctic char (2) mtDNA sequences.

	θ_1	θ_2	θ_A	t	m_1	m_2	N_1	N_2	N_A	t (years)	$2N_1m_1$	$2N_2m_2$
MLE	71.1	62.1	25.1	1.67	0.001	0.15	710,500	620,500	250,500	330,000	0.0355	4.657
Lower 90% HPD	48.1	42.3	0.05	0.95	0.000	0.05	480,500	400,500	500	217,500		
Upper 90% HPD	99.9	100.0	69.9	8.53	0.009	0.43	983,500	999,500	699,250	458,500		

The parameters θ_1, θ_2, θ_A, m_1, m_2, and t are scaled by the neutral mutation rate which was used for the conversions to N_1, N_2, N_A, $2N_1m_1$, $2N_2m_2$, and t (years). The 90% HPD represent the high and low boundaries for the interval that contains 90% of the estimates for the various parameters.

N_1, effective population size of contemporary Dolly Varden; N_2, effective population size of contemporary Arctic char; N_A, effective population size of ancestral population; t, time of divergence between species; $2N_1m_1$, population migration rate of Arctic char mtDNA into Dolly Varden; $2N_2m_2$, population migration rate of Dolly Varden mtDNA into Arctic char.

phylogenetic reconstruction also suggested basal positions in the phylogeny of a subspecies of Dolly Varden, *S. m. krascheninnikovi,* and a distinct taxon from a lake on the Chukotka Peninsula, *Salvethymus svetovidovi.*

Coalescent analysis of 36 haplotypes (22 Dolly Varden and 14 Arctic char) from the 'Bering' and 'High Arctic' clades using IM resulted in maximum likelihood estimates of mitochondrial DNA migration rate and other demographic parameters (Table 2). The replicate analyses resulted in general convergence around similar values although some parameters never reached sharp peaks around single estimates resulting in some broad parameter estimate distributions. The data suggested divergence times between Dolly Varden and Arctic char of between 215 000–458 000 years ago (Table 2). Migration estimates were asymmetrical with the peak probability (0.91) associated with 0 migration events from Arctic char to Dolly Varden. By contrast, the highest probability values were associated with from 1–6 migration events from Dolly Varden to Arctic char (total probability of 0.85, Table 2) occurring between 1100–10 900 years ago. The consistently most likely scenario resolved 3–4 migration events ($P = 0.72$) occurring between 6300 and 8100 years ago.

Evidence for distinctive gene pools within lakes

Microsatellite variation among all the char assayed was extensive. The total number of alleles ranged from 5 (Sfo18) to 97 (Sco220). Unbiased expected heterozygosity ranged from 0.0 (Sfo 18 in the Pedro Ponds (Iliamna Lake), Chignik watershed, and BC Dolly Varden samples; Sco215 in one BC Dolly Varden population) to over 0.90 at several loci in many populations (Supporting information Appendix S3). Multilocus heterozygosity ranged from 0.67 in the Pedro Ponds sample to 0.86 in the Hansen Creek sample. There were a number of deviations from Hardy–Weinberg Equilibrium within samples; after sequentially adjusting for multiple tests (Rice 1989) there were up to five loci out of nine that were out of equilibrium within some samples and these deviations all involved heterozygote deficiencies (Supporting Information Appendix S3). There were also several deviations from linkage equilibrium dependent on population. For instance, Pedro Ponds (Iliamna Lake), Iliamna Lake island beach samples, and all the Chiknik Lake watershed samples showed no deviations. By contrast, in Lake Aleknagik, the samples from Whitefish Creek and Hansen Creek showed one (Sfo18 and Sco202) and several deviations (Sfo18 and Sco202; Sco202 and Sco218; Sfo18 and Sco215), respectively.

In the Lake Aleknagik drainage, STRUCTURE analysis indicated that the most likely number of genetic populations both within Hansen and Whitefish creeks was 2 [i.e. each creek had two populations; all $P(K = 2) > 0.99$] whereas in the lake samples, the most likely number of populations was 1 (Table 3). By contrast, in the Iliamna Lake system the most likely K

Table 3 Results of STRUCTURE analysis for the number of genetically distinct populations within Lake Aleknagik, Iliamna Lake, and Chignik Lake, western Alaska.

	log likelihood K (no. of populations)		
Locality	1	2	3
Iliamna Lake, Iliamna Lake, Bristol Bay Alaska	**−1290.6**	−1294.5	−1338.6
Pedro Ponds, Iliamna Lake, Bristol Bay, Alaska	**−1343.4**	−1350.9	−1355.2
Hansen Creek, Lake Aleknagik, Bristol Bay, Alaska	−1486.4	**−1465.1**	−2269.7
Whitefish Creek, Lake Aleknagik, Bristol Bay, Alaska	−1273.5	**−1269.6**	−1442.7
Lake Aleknagik, Lake Aleknagik, Bristol Bay, Alaska	**−1700.4**	−1720.6	−1859.1
Chignik Lake, Gulf of Alaska, Alaska	**−3153.5**	−3197.2	−3875.2
Chignik River, Gulf of Alaska, Alaska	**−704.8**	−710.4	−743.1
Alec River, Gulf of Alaska, Alaska	**−543.6**	−583.8	−590.2
Chignik Lake (pooled samples)*	−1625.5	−1622.2	**−1616.7**

The values shown are the mean log likelihoods supporting each hypothesized K number of populations within each locality given the allele frequencies observed at those localities. Bold values indicate the most likely K value. Each likelihood is the mean from five different runs of the STRUCTURE analysis.

*Includes samples from Alec River ($N = 14$) and Chignik River ($N = 17$).

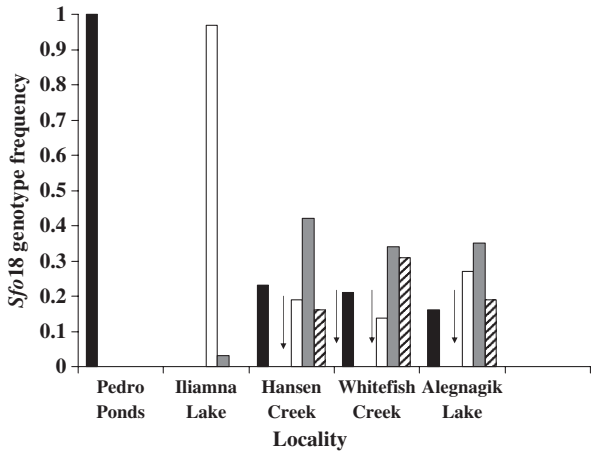

Fig. 4 Distribution of genotypes (in base pairs) at the microsatellite locus Sfo18 in samples of Arctic char (*Salvelinus alpinus*) and Dolly Varden (*Salvelinus malma*) in Iliamna Lake and its tributary stream complex Pedro Ponds, and Lake Aleknagik and its tributaries Hansen Creek and Whitefish Creek, western Alaska. Genotypes are 152152 (solid black), 158158 (open white), 158162 (solid grey), 162162 (diagonal black bars). The genotypes 152158 and 152162 were not observed (indicated by arrows).

was 1 both within the island beach samples and with the stream habitats of Pedro Pond (both $P\,(K = 1) > 0.99$). In the Chignik Lake system, in each of the three localities sampled (Chignik Lake, Chignik River, and Alec River) a single genetic population was inferred to exist ($K = 1$). When all localities were pooled into a single 'Chignik Lake' sample, $K = 3$ was the most likely population structure (Table 3).

The most striking evidence of distinct gene pools in sympatry in Aleknagik and Iliamna lakes was found in the allelic distribution at one locus, Sfo18. Throughout the entire range of samples examined, four alleles were resolved: 152, 158, 160, and 162 bp alleles. The 152 bp allele was fixed in all allopatric Dolly Varden samples that we assayed (and in several hundred other Dolly Varden in other studies, E.B. Taylor, unpublished), whereas Arctic char samples exhibited each of the other three alleles. In no sample, however, did we find genotypes consisting of a 152 bp Dolly Varden allele in combination with any of the Arctic char alleles (158, 160, or 162, Fig. 4).

Admixture analysis and assignment of char

We used Arctic char collected from three localities in the eastern/central Canadian Arctic and northern Québec, and Dolly Varden collected from the Queen Charlotte Islands and northern Vancouver Island as baseline samples with which to assign char from the Alaskan systems (Iliamna, Aleknagik and Chignik lakes) as either Dolly Varden or Arctic char. Using STRUCTURE and constraining $K = 2$, indicated that the admixture coefficient (q_{AC}) for known Arctic char averaged 0.988 (range 0.908–0.998) while the corresponding admixture coefficient for Dolly Varden (q_{DV}) averaged 0.990 (range 0.945–1.00). The average admixture value for simulated hybrids (q_H) was 0.510 (range 0.232–0.785). From these samples, we established 'zone of hybridity', expressed relative to the proportion of the genome representing Dolly Varden (q_{DV}) that ranged from 0.232 to 0.785 within which fish were considered to be possible hybrids between Arctic char and Dolly Varden (Fig. 5). Admixture analysis for the Illiamna Lake and Lake Aleknagik samples (Fig. 5) indicated that the vast majority of fish fell either within the Arctic char admixture zone (i.e. a q-value of < 0.232) or the Dolly Varden admixture zone (i.e. a $q > 0.785$). A total of 18 fish (10.6%), however, fell within the zone of hybridity and half of these were found in Iliamna Lake and the remaining putative hybrids were found scattered in Pedro Ponds, Hansen Creek, and Aleknagik Lake (Fig. 5).

Generally, the results of the assignment tests using GENECLASS were similar to results from the STRUCTURE analysis. The baseline samples were correctly self-assigned to either Dolly Varden or Arctic char with 100% accuracy (Table 4). Chignik Lake watershed samples were all assigned to Dolly Varden using the baseline samples. We then added Chignik Lake char to the baseline sample for Dolly Varden with which to classify fish from Iliamna Lake and Lake Aleknagik. For instance, within the Iliamna Lake system, 100% of the fish sampled from Pedro Ponds were assigned to Dolly Varden [average P(Dolly Varden) = 0.99], but 100% were assigned to Arctic char within the lake sample itself [Table 4, average P(Arctic char) = 0.99]. Similarly, within the Lake Aleknagik system, the stream habitats tended to be dominated by Dolly Varden char; 59% were classified as Dolly Varden and 41% as Arctic char in Hansen Creek. In Whitefish Creek 72% were assigned as Dolly Varden and 28% as Arctic char. In the other fish sampled from streams tributary to Lake Aleknagik, two of four (Yako Creek), three of four (Eagle Creek) and one fish (Bear Creek) were assigned as Arctic char (Table 4). Within the Lake Aleknagik sample, the tests assigned 78% of the fish to Arctic char and 22% to Dolly Varden (Table 4). By contrast to the results from the Iliamna Lake watershed, there were a number of fish with probabilities of being one species or the other of < 0.90. From each of Hansen and Whitefish creeks and from Lake Aleknagik itself, four fish had probabilities of being assigned as either Dolly Varden or Arctic char of between 0.60 and 0.89. In the lake, the tendency (3/4 fish) was for these 'intermediate' fish to be assigned as Arctic char [although at P(Arctic char) < 0.90], while in each of these streams two were assigned as Dolly Varden and two as Arctic char.

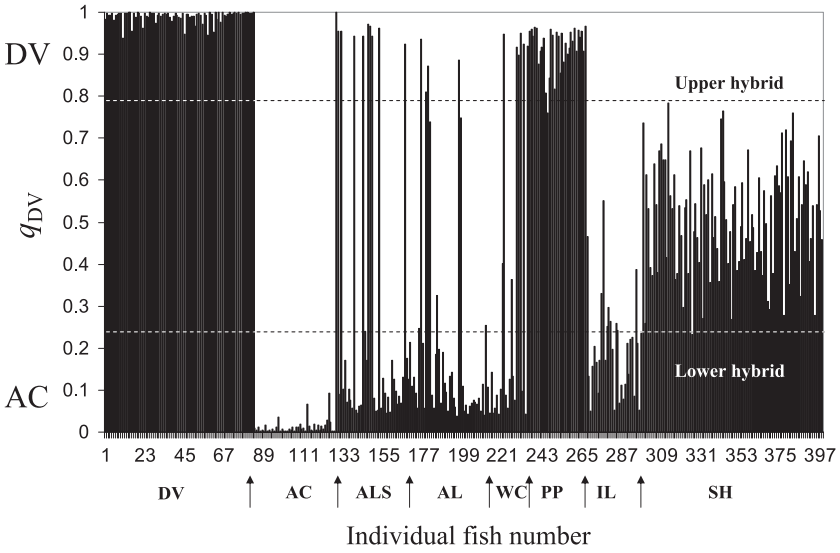

Fig. 5 Admixture coefficients (q_{DV}) for individual char assayed at nine microsatellite DNA loci. Each fish is represented by a thin vertical black line the height of which represents *q*, the proportion of the genome representative of that of Dolly Varden (*Salvelinus malma*). Samples 1–86 are reference Dolly Varden ('DV'), samples 87–128 reference Arctic char ('AC'), samples 129–168 Aleknagik Lake streams ('ALS', Yako, Bear, Eagle, and Hansen creeks), samples 169–205 Alegnagik Lake ('AL'), 206–234 Aleknagik Lake streams ('WC', Whitefish Creek), 235–267 Pedro Ponds ('PP'), 68–299 Iliamna Lake ('LL'), 300–400 simulated hybrids ('SH'). The dashed horizontal lines at (q_{DV}) = 0.232 and 0.785 represent the lower and upper, respectively, limits of admixture values for the simulated hybrids.

The differentiation of char within Lake Aleknagik and Iliamna Lake and the genetic association of each type of char within lakes with Dolly Varden and Arctic char sampled in allopatry were also clearly evident in the factorial correspondence analysis (FCA, Fig. 6). The char identified both by STRUCTURE and the assignment tests as Dolly Varden were found in FCA space nearest the known Dolly Varden from British Columbia. Similarly, the fish identified as Arctic char genetically were associated spatially with known Arctic char from the Canadian eastern Arctic and northwestern Quebec (Fig. 6). The grouping of fish identified as Arctic char, however, were genetically more diverse than the more homogenous Dolly Varden; allopatric Arctic char tended to be more distinct from Alaskan Arctic char along FCA 2 (Fig. 6).

Morphology-microsatellite associations

In Iliamna Lake there was a very strong positive association between genetic (microsatellite) classification and that using morphology; 95% (61/64) of individual fish were morphologically classified using discriminant functions analysis into the same taxon (Dolly Varden or Arctic char) as they were genetically. The three fish that were identified morphologically as one species and genetically as another tended to be intermediate in morphology. In Lake Aleknagik, there was a lower degree of concordance between microsatellite and morphological classification; 69.7% (53/76) of all fish were morphologically classified as the same species as they were genetically. As in Iliamna Lake, fish that were assigned genetically as one species, but morphologically as the other tended to have intermediate classification scores for both metrics.

Discussion

Evidence for distinct gene pools in sympatric char

Variability in phenotype is one of the hallmarks of species across their geographic ranges (Mayr, 1963; Futuyma, 1986). Given that morphological variation has been so closely tied to taxonomic distinction, it is no surprise that in *Salvelinus*, a genus well known for extensive geographic variation, up to 29 species have been described in Europe, 15 in North America, and 12 in Siberia and the Far East (Savvaitova, 1995; Kottelat & Freyhof, 2007). A common idea is that the 29 European putative taxa belong to one polytypic species, *S. alpinus* and that the extensive variation is part of the *S. alpinus* 'species complex' (Jonsson & Jonsson, 2001). North American char constitute five recognized taxa and while the situation in Siberia and the Far East remains clouded, the number of valid taxa is probably on the order of 3–4. These three geographic areas are linked by the common uncertainty of whether or not *S. alpinus* should include char populations in North America and the western Pacific described as Dolly Varden, *S. malma*. A number of biogeographic studies have explored this latter issue (e.g. McPhail, 1961; Reist *et al.*, 1996; Brunner *et al.*, 2001), but all have been constrained by either the lack of broad geographic coverage, lack of genetic data, or the lack of both elements applied to sympatric forms. This latter limitation is a critical one because it is only in sympatry that a direct test of species validity, under the biological species concept, can be applied (Jiggins & Mallet, 2000; Coyne & Orr, 2004). We have addressed this limitation directly in the current study and find strong evidence that two lakes in western Alaska contain genetically (and probably ecologically) distinct populations of *Salvelinus* in

Table 4 Results of assignment analyses for samples of char (*Salvelinus*) collected from localities within Iliamna Lake and Lake Aleknagik, western Alaska.

Sample	Assigned as Arctic char	Assigned as Dolly Varden
Reference samples		
Arctic char (*Salvelinus alpinus*)		
Char Lake, central Canadian Arctic	9/9	–
Resolute Lake, central Canadian Arctic	17/17	–
Lac Duquet, northwestern Québec	28/28	–
Ikadlivik Brook, Labrador, Canada	30/30	–
Dolly Varden (*Salvelinus malma*)		
Zeballos Lake, Vancouver Island, BC	–	30/30
Chache Creek, Queen Charlotte Islands, BC	–	15/15
Unknown samples		
Iliamna Lake, Iliamna Lake, Bristol Bay, AK	31/31	–
Pedro Ponds, Iliamna Lake, Bristol Bay, AK	–	34/34
Hansen Creek, Lake Aleknagik, Bristol Bay, AK	13/31	18/31
Whitefish Creek, Lake Aleknagik, Bristol Bay, AK	8/29	21/29
Bear Creek, Lake Aleknagik, Bristol Bay, AK	1/1	–
Lake Aleknagik, Lake Aleknagik, Bristol Bay, AK	28/36	8/36
Eagle Creek, Lake Aleknagik, Bristol Bay, AK	3/4	1/4
Yako Creek, Lake Aleknagik, Bristol Bay, AK	2/4	2/4
Chignik Lake, Gulf of Alaska, AK	–	34/34
Chignik River, Gulf of Alaska, AK	–	17/17
Alec River, Gulf of Alaska, AK	–	14/14

Reference samples of Arctic char (*S. alpinus*) and Dolly Varden (*S. malma*) consisted of fish from the central and eastern Arctic of Canada, and the Gulf of Alaska and northern British Columbia, respectively.

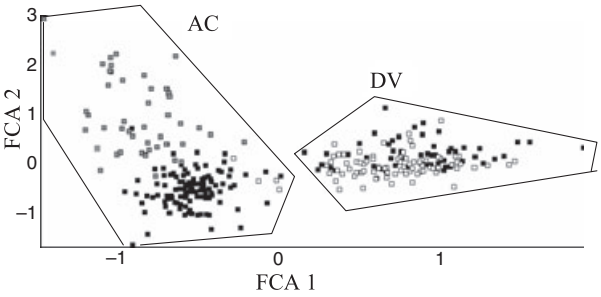

Fig. 6 Plots of scores along the first two factorial correspondence axes based on variation at nine microsatellite DNA loci in Arctic char (*Salvelinus alpinus*) and Dolly Varden (*Salvelinus malma*) from northern British Columbia, Alaska and the central and eastern Canadian Arctic. Allopatric *S. alpinus* individuals are denoted by grey squares (■), allopatric *S. malma* by white squares (□) and sympatric *Salvelinus* from the study populations in Iliamna Lake and Lake Aleknagik by black squares (■).

sympatry and, hence, fulfill the criterion used to recognize distinct biological species. Genetic assignment tests with known baselines and morphological characterization strongly suggest that these sympatric char represent *S. alpinus* and *S. malma*.

First, without designation of individual fish collected from the same habitats (lake areas or stream areas) as one form or the other, our genetic data indicated the existence of at least two genetic 'clusters' both in the Lake Aleknagik and Iliamna Lake watersheds. This was suggested by deviations from Hardy–Weinberg Equilibrium within samples, the results of the STRUCTURE analyses, and, most strikingly, by a complete absence of heterozygotes for alleles at one locus that are diagnostic for Dolly Varden and Arctic char outside the study lakes.

Second, genetic assignment methods and morphological traits associated with Dolly Varden and Arctic char in allopatry indicated the presence of both species both in Lake Aleknagik and Iliamna Lake systems. Our genetic and morphological evidence for two biological species of *Salvelinus* in western Alaska lakes is consistent with earlier suggestions based on morphology and ecology (Delacy & Morton, 1943; McPhail, 1961). The two taxa in the Alaskan lakes possess trait values, especially pyloric ceaca and gill raker counts, that are characteristic of Arctic char and Dolly Varden (McPhail, 1961). In addition, the microsatellite locus Sfo 18 has alleles that are diagnostic for the two taxa and these diagnostic alleles are possessed by fish that are morphologically similar to Arctic char and Dolly Varden. Interestingly, however, the microsatellite DNA variation as summarized in the FCA suggests that there is a high level of divergence between the Arctic char that we sampled from western Alaska and those known Arctic char from more eastern portions of the range in the Canadian High Arctic and Quebec. This is perhaps not surprising given the very large geographic distances between western Alaska and the Canadian High Arctic and northern Quebec relative to the geographically more proximate western Alaskan and BC Dolly Varden samples. Our new microsatellite data are consistent with the mtDNA data in that they both suggest that Arctic char exhibit a high degree of genetic subdivision and that the current taxonomy of Holarctic *S. alpinus* remains uncertain (see also McPhail, 1961).

What is more surprising, however, is that at least in Lake Aleknagik and Iliamna Lake, there appears to be little evidence of contemporary hybridization between these two species consistent with morphological studies in at least some other Alaskan lakes (e.g. McPhail, 1961). Any fish that were potential hybrids in our system appeared to be post-F_1 as we detected no heterozygotes at the apparently diagnostic Sfo18 locus and hybridity values of putative hybrids from admix-

ture and assignment analysis were typically much greater or lower than 0.5. McPhail (1961) collected char from Hansen Creek and found them to be intermediate between Arctic char and Dolly Varden in some traits (gill raker number), Dolly Varden-like in others (spot pattern) and Arctic char-like in still others (caecal counts) that he suggested could have been hybrids. We did detect some fish in this system that were ambiguous in their genetic identification [P(Arctic char or Dolly Varden of < 0.90] and morphological distinctions in this lake were not great as in Iliamna Lake which, combined with McPhail's (1961) findings suggests that some hybrids may be present. There exist many observations of contemporary natural hybridization between other species of sympatric char throughout their ranges, including observations involving *S. malma* and *S. alpinus* when either species is sympatric with other *Salvelinus* (reviewed by Taylor, 2004). The apparently low level of contemporary hybridization is intriguing because *S. alpinus* and *S. malma* are considered sister species (Phillips *et al.*, 1995; Crespi & Fulton, 2004), and are similar morphologically and ecologically, relative to comparisons between other *Salvelinus* species (McPhail, 1961).

Alternatively, it is possible that the complete absence of individuals heterozygous for Sfo18 species-specific alleles might indicate that this locus is under selection or closely linked to a selected locus and could underestimate current hybridization (e.g. Mallet *et al.*, 1985; Kohn *et al.*, 2000). Notwithstanding the lack of direct evidence for selection acting on this locus, two observations argue against this possibility. First, genetically and morphologically intermediate fish that we observed could represent within-species variability in both measures because for some loci, and certainly for morphology, there are no truly 'fixed' differences and there can be broad overlap between species. This will result in some inaccuracy in assignment/classification of individuals when relatively few diagnostic loci or traits are examined. Indeed, all of the genetically intermediate char had assignment probabilities of being one species or the other of at least 0.6 (but <0.9) and thus, no putative F_1 hybrids (expected probabilities of 0.5) were detected. Second, individuals that are heterozygous for specific-specific alleles at Sfo18 have been observed in hybridizing populations of Arctic char and brook trout *Salvelinus fontinalis* (Gross *et al.*, 2004) as well as between bull trout and brook trout (E.B. Taylor, unpublished).

Sampling of a different range of habitat types between Ilimana and Aleknagik lakes may account for the apparent low level of contemporary hybridization between these two species observed in our study. In the Iliamna Lake watershed, the two species were collected from distinctly different habitats, although they were only a few km apart. Most *S. malma* were collected from a spring-fed pond complex that drains into Iliamna Lake. By contrast, no Arctic char were collected in this pond complex, even though it is fully accessible from the lake, and all were found in the lake itself. Delacy & Morton (1943) indicated that in the Karluk River system (also located in Alaska just southeast of our study area), *S. malma* reproduction was limited to stream habitats while *S. alpinus* spawned in the lakes. While there is some degree of habitat overlap between juveniles of the species in some areas (e.g. Lake Aleknagik system), a high degree of reproductive habitat segregation probably limits opportunities for interbreeding (McPhail, 1961). It is possible, however, that F_1 hybrids might be found in the many habitats in both systems that we did not sample. The greater range of habitats that we sampled in Aleknagik Lake (several streams and beach areas in the lake) may also account for the lower degree of morphological distinction between fish identified genetically as either *S. alpinus* or *S. malma* given the tendency for habitat variation to promote morphological variation within species (e.g. Jonsson & Jonsson, 2001).

There are precedents for strong genetic isolation in sympatry between *S. malma* and another *alpinus*-like taxon, the so-called 'Taranetz char', *S. taranetzi* in lakes from the Chukotka Peninsula (Salmenkova *et al.*, 2000; see discussion in Oleinik *et al.*, 2007). As appears to be common when different *Salvelinus* taxa come into contact, the Taranetz char tends to be a lake specialist while Dolly Varden are stream specialists (Gudkov, 1995; Hagen & Taylor, 2001). While the Taranetz char is considered a full species by some (e.g. Oleinik *et al.*, 2007), mtDNA and allozyme data both demonstrate variation within this taxon that is nested well within that described across the geographic range of *S. alpinus* (e.g. Behnke, 1980; Salmenkova *et al.*, 2000; Brunner *et al.*, 2001). Consequently, a genetically and geographically comprehensive systematic analysis of *Salvelinus,* including examination of putative *S. taranetzi* and *S. alpinus* in sympatry, is needed to better resolve taxa in the genus and whether or not *S. tarantzi* = *S. alpinus* (Oleinik *et al.*, 2005).

Biogeography and evolution of *Salvelinus* in the North Pacific

Brunner *et al.* (2001) based their conclusion that the separate species status of *S. malma* was 'questionable' on the observation that *S. malma* and *S. alpinus* mtDNAs were not reciprocally monophyletic. Presumably because the *S. malma* mtDNA was nested within a clade of *S. alpinus* mtDNA in the analysis by Brunner *et al.* (2001), and because *S. alpinus* has a wider geographic distribution and was described before *S. malma*, these results and conclusions prompted the suggestion that *S. malma* should be considered a subspecies or, less formally, one of the many geographic isolates of *S. alpinus*. When our sequence data were combined with some of that of Brunner *et al.* (2001), Elz (2003), and Taylor *et al.*

(unpublished), char from western Alaska all fell within the 'Bering' lineage identified by Brunner *et al.* (2001) and support the idea of lack of reciprocal mtDNA monophly for Dolly Varden and Arctic char. Our microsatellite DNA results, however, strongly support the idea that *S. malma* and *S. alpinus* are separate biological species. Why then do the species show a lack of reciprocal monophyly of mtDNA?

The attributes of mtDNA and its inheritance, and the many examples in nature of mtDNA introgression between divergent nuclear genomes in *Salvelinus* (reviewed by Taylor, 2004) and in many other taxa (Ballard & Whitlock, 2004; Coyne & Orr, 2004) indicate that designation of species status based on mtDNA alone is problematic. The similarity of mtDNA between *S. malma* and *S. alpinus* then could be a function of widespread contemporary mtDNA introgression between the species as has been suggested for *S. malma* and *S. confluentus* in some areas (Redenbach & Taylor, 2002). This, however, is inconsistent with the apparent low level of contemporary hybridization between these species suggested by our microsatellite DNA data. Lack of reciprocal mtDNA monophyly could also result from historical hybridization between species. Localized and historical hybridization is not uncommon in many animal groups, and has been suggested to occur between species of *Salvelinus* [including *S. alpinus* and *S. malma* - Phillips *et al.* (1999)] even where populations of one of the species are no longer present (e.g. Bernatchez *et al.*, 1995; Redenbach & Taylor, 2002). Indeed, our IM-based coalescent analysis of the mtDNAs of both species gave a consistent signal of mtDNA gene flow between the species. In the present case, however, both species are currently sympatric and it is striking that there appears to be little evidence of contemporary hybridization in our study systems. Finally, lack of reciprocal monophyly in mtDNA could stem from incomplete lineage sorting after recent divergence from a common ancestor. Under this hypothesis, we would expect to see no geographic pattern to the mtDNA phylogeny; *S. malma* and *S. alpinus* haplotypes should be dispersed across all localities in the mtDNA tree (Redenbach & Taylor, 2002; McGuire *et al.*, 2007). By contrast, the data of Brunner *et al.* (2001) and our more limited mtDNA data indicate that *S. malma* haplotypes are most closely related to geographically proximate *S. alpinus* haplotypes from Siberia, Kamchatka, and western Alaska. In addition, a diversity of molecular data suggest that *S. m. krascheninnikovi* is basal to all other *S. malma* and *S. alpinus* (cf. Behnke, 1980; Osinov, 2001; Redenbach & Taylor, 2002; Elz, 2003; Oleinik *et al.*, 2004, 2005, 2007; Shedko *et al.*, 2007) which implies that the divergence of *S. alpinus* and *S. malma* was more widespread and occurred prior to or near the beginning of the Pleistocene.

Alternatively, our coalescent analysis of mtDNA suggested that mtDNA gene flow had occurred primarily from *S. malma* into *S. alpinus*, yet the mtDNA phylogeny is more consistent with a pattern suggestive of capture of *S. alpinus* mtDNA by *S. malma*. This inconsistency could stem from an alternative hypothesis of the systematics of *Salvelinus*, i.e., if *S. malma and S. alpinus,* as currently conceived, are not actually sister species, but rather that *S. alpinus* consists of a number of geographically contiguous species, and *S. malma* has evolved in the North Pacific relatively recently after divergence from a local (Bering) clade representing an undescribed species within the *S. alpinus* group. As hinted at both by Phillips *et al.* (1999) and Oleinik *et al.* (2005), a comparable situation may apply to *S. malma* across its geographic range, i.e. the currently accepted subspecies arrangement within *S. malma* may obscure the true depth of divergence within this group. Regardless of the continuing uncertainties surrounding *Salvelinus* systematics, our results demonstrate the existence of two biological species of char closely resembling *S. alpinus* and *S. malma* in western Alaska.

Our phylogeny of *S. malma* and *S. alpinus* mtDNA indicates a close relationship between haplotypes located on either side of the North Pacific (e.g. Brunner *et al.*, 2001; Elz, 2003). This pattern of relatedness has been observed in a variety of taxa that span 'Beringia'– that area in and around the Yukon River valley and adjacent portions of Siberia and Kamchatka (e.g. Elias *et al.*, 2000; Van Houdt *et al.*, 2003; Galbreath & Cook, 2004; Stamford & Taylor, 2004) and adjacent areas (Ilves & Taylor, 2008). Our analysis, therefore, further highlights the historical interactions between biota from these regions and the importance of the formation and dissolution of the dispersal corridors across this region in influencing such interactions and promoting diversity within the North Pacific (e.g. Marincovich & Gladenkov, 1999; Briggs, 2003; Ilves & Taylor, 2008).

Finally, our study has centred on the biological species status of *S. alpinus* and S. *malma* in sympatry within a relatively small area of contact. Notwithstanding their current interactions in sympatry, a larger question that is still outstanding concerns the geographic origin of the two taxa (as well as the divergent lineages within each taxon – Brunner *et al.*, 2001; Oleinik *et al.*, 2005). The generally wider distribution and taxonomic precedence of *S. alpinus* has favoured the idea that it might provide the best clues to the evolution of the *S. alpinus – S. malma* species group, particularly with regard to vicariant events in the Arctic regions. Data within Elz (2003) and Oleinik *et al.* (2005), however, suggest that *S. m. krascheninnikovi,* which is found in the southwestern North Pacific in and around the Sea of Japan, represents the first and most ancient divergence within the group. Consequently, clues to the origin of *S. malma* and *S. alpinus* might be better sought in the western Pacific and Sea of Japan – areas that have been implicated in the origin of biodiversity for other northern Pacific fishes (reviewed in Ilves & Taylor, 2008).

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1 Mitochondrial control region haplotype designations, taxa sampled, and localities for Arctic char (*Salvelinus alpinus*) and Dolly Varden (*Salvelinus malma*) used in phylogenetic analysis.

Appendix S2 Loadings for morphological and meristic traits for char collected from Iliamna Lake and Lake Aleknagik.

Appendix S3 Microsatellite DNA variation in samples of *Salvelinus alpinus* and *Salvelinus malma* samples from western Alaska.

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