JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12011

Coexistence and origin of trophic ecotypes of pygmy whitefish, *Prosopium coulterii*, in a south-western Alaskan lake

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Keywords:

Coregoninae;
diet analysis;
fish;
morphometrics;
phylogeography;
population genetics;
sympatric populations.

Abstract

Ecologically, morphologically and genetically distinct populations within single taxa often coexist in postglacial lakes and have provided important model systems with which to investigate ecological and evolutionary processes such as niche partitioning and ecological speciation. Within the Salmonidae, these species complexes have been well studied, particularly within the *Coregonus clupeaformis–C. laveratus* (lake and European whitefish, respectively) group, but the phenomenon has been less well documented in the other whitefish genera, *Prosopium* and *Stenodus*. Here, we examined the morphology, feeding biology and genetic structure of three putative forms of the pygmy whitefish, *Prosopium coulterii* (Eigenmann & Eigenmann, 1892), first reported from Chignik Lake, south-western Alaska, over 40 years ago. Field collections and morphological analyses resolved a shallow water (< 5 m depth) low gill raker count form (< 15 first arch gill rakers), a deepwater (> 30 m), low gill raker form and a deepwater, high gill raker count (> 15 gill rakers) form. The two low gill raker count forms fed almost exclusively on benthic invertebrates (mostly chironomids), while the deepwater, high gill raker count form fed almost exclusively on zooplankton; differences in diet were also reflected in differences both in δ^{13}C and δ^{15}N stable isotopes. All three forms were characterized by the same major mitochondrial DNA clade that has been associated with persistence in, and postglacial dispersal from, a Beringian glacial refugium. Analysis of variation at nine microsatellite DNA loci indicated low, but significant differentiation among forms, especially between the two low gill raker count forms and the high gill raker count form. The extent of differentiation along phenotypic (considerable) and genetic (subtle) axes among the Chignik Lake forms is similar to that found among distinct taxa of *Prosopium* found in pre-glacial Bear Lake (Utah–Idaho, USA) which is probably at least ten times older than Chignik Lake. Our analyses illustrate the potential for the postglacial differentiation in traits subject to divergent natural selection across variable environments.

Introduction

Many vertebrate species consist of sympatric forms that are phenotypically, ecologically and sometimes genetically distinct, and a number of these sympatric populations have been closely studied in several groups of fishes (Schluter, 1996; Smith & Skúlason, 1996; Taylor, 1999). These sympatric populations have been denoted by various terms such as eco-phenotypes, ecotypes, morphs, forms, species pairs, sibling species and, when occurring across a variety of lakes, species complexes (reviewed by Schluter, 1996; Taylor, 1999). A key process that typically promotes divergence between forms is trophically based ecological interaction (McPhail,

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1984; Lu & Bernatchez, 1999). Indeed, the creation of lakes in the Northern Hemisphere postglacially provided opportunities for colonizing fishes to exploit a variety of trophic niches based on variation in available habitat (e.g. benthic and limnetic, littoral and profundal) and diet (zooplankton, benthic invertebrates, fishes). Such ecological opportunity in novel habitats is thought to be an important factor in driving ecological speciation via sympatric (Taylor & Bentzen, 1993; Barluenga *et al.*, 2006) or allopatric divergence and subsequent sympatry (Svärdson, 1979; Bernatchez & Dodson, 1990; Taylor & McPhail, 2000) or by a combination of these processes (Pigeon *et al.*, 1997; Ostberg *et al.*, 2009; McKeown *et al.*, 2010). Morphological features in general and especially the jaws and feeding structures of fishes are, however, subject to phenotypic plasticity driven by diet and habitat use (Wimberger, 1994; Mittlebach *et al.*, 1999; Olsson & Eklöv, 2005). The combination of alternative evolutionary pathways, links among behaviour, habitat, diet and morphology and phenotypic plasticity make the study of population divergence intriguing but complex.

Three groups of fishes have proven to be especially fruitful for studies of sympatric populations in postglacial lakes: the threespine stickleback, *Gasterosteus aculeatus*, various salmon, trout and char (Salmonidae: subfamily Salmoninae), and whitefishes (subfamily Coregoninae; Schluter, 1996; Bernatchez *et al.*, 1999; Taylor, 1999). A lake may contain from two to as many as four distinct morphs, distinguished by food habits, growth rate and size at maturation, habitat use, morphological features associated with feeding and armour for defence, colour, body shape or some combination of features (Schluter, 1996; Smith & Skúlason, 1996).

Among the whitefishes, the lake whitefish (*Coregonus clupeaformis*) and European whitefish (*C. laveratus*) have been the subject of numerous studies of intra-lacustrine divergence (Lindsey, 1981; Sendek, 2004; Østbye *et al.*, 2005; Bernatchez *et al.*, 2010). By contrast, other members of the subfamily have been less well studied but may also exhibit sympatric populations, including within a primarily riverine species, the mountain whitefish, *Prosopium williamsoni* and the pygmy whitefish, *P. coulterii* (McCart, 1970; Whiteley, 2007). The pygmy whitefish is a small-bodied whitefish that exists in north-eastern Russia and has a highly disjunct distribution in North America, where it is found in western Lake Superior and at least one inland lake in north-western Ontario, a few localities in western Alberta, and in interior British Columbia, south-western Yukon and south-western Alaska (McPhail & Lindsey, 1970; Witt *et al.*, 2011). Witt *et al.* (2011) used mitochondrial and nuclear DNA sequences to demonstrate the existence of two major phylogenetic lineages of pygmy whitefish in North America that differed from each other by about 3.3% in terms of their mtDNA: one found in south-western Alaska and Yukon, and the other east and south of these areas in Yukon, British Columbia, Washington and western Lake Superior. Coalescent analyses suggested that the current distribution of these distinct pygmy whitefish lineages in North America, which originated perhaps 1.6–3 million years ago, stems from isolation in, and postglacial dispersal from, at least two Pleistocene glacial refugia, Beringia and Cascadia (and perhaps the Mississippi; Witt *et al.*, 2011). Further, McCart (1970) reported the existence of multiple morphs of pygmy whitefish in many south-western Alaskan lakes including Aleknagik, Naknek and Chignik lakes. Typically, the morphs were distinguishable by various meristic counts (e.g. caudal peduncle scale counts, lateral line scale counts, dorsal fin rays), especially gill raker counts that have special significance in taxonomy and feeding ecology of coregonid fishes (Lindsey, 1981). McCart (1970) defined a high gill raker count type (gill rakers $\geq$ 16) and a low gill raker count type (gill rakers $\leq$ 15). 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 'south-eastern' and 'north-western' groups, respectively (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 north-western group evolved in ice-free areas somewhere in the Yukon River–Bering Sea region and that the south-eastern group evolved in the vicinity of the lower Columbia River south of the Wisconsinan ice sheet (cf. Lindsey & Franzin, 1972; Bird & Roberson, 1979). The presence of two major DNA clades of pygmy whitefish in North America originating from isolation in distinct refugia (Witt *et al.*, 2011) signals potential source lineages for such multiple colonizations, but the phylogenetic analysis of Witt *et al.* (2011) included fish from only two Alaskan lakes whose morphology was known: all fish sampled from Black and Chignik lakes were of the low gill raker type (McCart, 1970; P. Kong and E. Taylor, unpublished data) and were characterized by the north-western or 'Beringian' DNA lineage. Further, McCart (1970) reported that in addition to the high gill raker count form in Chignik Lake, the low gill raker count form actually consisted of two morphological types, differing from each other in head shape and body depth. Thus, there appeared to be three sympatric populations in Chignik Lake. In summary, although we have recently gained a better understanding of the evolutionary origins and biogeography of pygmy whitefish on a continental scale (Taylor *et al.*, 2011; Witt *et al.*, 2011), the ecological and genetic relationships between distinctive morphological types within localities and their evolutionary origins are poorly understood and McCart's (1970) 'two refuge' hypothesis has not been tested.

The purpose of this study was to better understand the origin, spatial context and basis for coexistence of phenotypes of pygmy whitefish in the Chignik Lake system by testing for ecological and genetic differentiation among them. Specifically, we collected fish from nearshore and deepwater habitats, determined their trophic position from diet and stable isotope analysis, and their morphology (gill raker number and body shape) to test for and quantify differences in morphology and trophic ecology and to test for an association between the two. In addition, we performed mtDNA and microsatellite DNA analyses to test for genetic divergence and the presence of one or more phylogeographic lineages within the lake (*sensu* Witt *et al.*, 2011). The presence of two phylogeographic lineages with Chignik Lake and their association with morphological/ecological form would be consistent with the idea that the current biodiversity of pygmy whitefish had arisen, at least in part, from multiple invasions rather than strictly from intra-lacustrine divergence.

Materials and methods

Study site

The Chignik watershed (Fig. 1), located in south-western Alaska, is comprised of two lakes. Black Lake is shallow (mean depth = 1.5 m) and mesotrophic, with a surface area of 36–41 km^2 (depending on lake level), and flows via the 15-km-long Black River into Chignik Lake, a deeper (mean = 64 m) oligotrophic lake with a surface area of approximately 22 km^2 (Westley *et al.*, 2008, 2010). Chignik Lake is subject to strong winds, and the shoreline is primarily gravel, cobble and rocks and is fed by several low–moderate gradient streams of varying size. The lake drains via the short (2 km) Chignik River into Chignik Lagoon, a large brackish body of water where salinities gradually increase with distance from the river. The lagoon is about 41.4 km^2 at high tide (Phinney, 1968), experiences a tidal range of about 4 m and is separated by a large sand spit from Chignik Bay, which opens into the Gulf of Alaska.

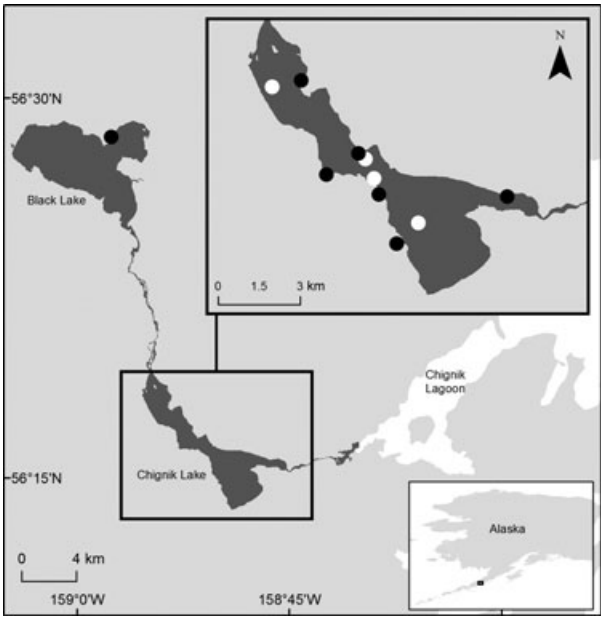

Fig. 1 Map of Chignik Lake basin Alaska, with insets showing the location of the basin in south-western Alaska, and the beach seine (closed symbols) and gill net (open symbols) sites within Chignik Lake where samples of pygmy whitefish, *Prosopium coulterii* were obtained.

Water temperatures in the upper 10 m of Chignik Lake reach about 9 °C in summer, and the lake is ice-covered from about December to May. The plankton community is dominated by calanoid and cyclopoid copepods and, late in the season, by the cladoceran *Bosmina*. Pygmy whitefish are part of a fully native fish community that includes several potential competitors in the littoral and limnetic zones (Westley *et al.*, 2010), including pond smelt (*Hypomesus olidus*), ninespine stickleback (*Pungitius pungitius*), threespine stickleback (*Gasterosteus aculeatus*), coastrange sculpin (*Cottus aleuticus*), slimy sculpin (*C. cognatus*), juvenile sockeye salmon (*Oncorhynchus nerka*), juvenile coho salmon (*O. kisutch*) and Dolly Varden char (*Salvelinus malma*) (Roos, 1959). The numerically dominant species are juvenile sockeye salmon and threespine stickleback. The only large piscivore, Dolly Varden, is found in the lagoon and large rivers, but Chignik Lake itself primarily contains small individuals (mean fork length = 112 mm, SD = 42 mm, $N > 100$) except for a brief period when adults migrate through the lake to riverine spawning areas.

Fish sampling

Fish were captured by beach seine at six sites in the littoral zone and by sinking gillnet at four sites in the offshore waters of Chignik Lake on multiple days between June and September of 2008–2009. The beach seine was 32 m long, tapering from 5 m deep in the 6-m-wide centre section with 6-mm mesh to 13-mm mesh in the 2.5-m side panels and 1 m deep at the wing ends with 30-mm mesh. It was deployed during the day from shore by boat in a semi-circle reaching approximately 15 m offshore. Two gillnets were used, both 30 m long by 2 m high, one with 30 mm and the other with 25-mm stretched mesh. They were set from a boat for 2–24 h in water 28.0–54.3 m deep, where nets were suspended above the bottom by floats.

Upon capture, fish were given unique identification numbers, photographed for geometric morphometric analysis, measured (fork length, FL in mm) and weighed, and the gill rakers on the first gill arch from the left side of each specimen were counted. Dorsal

muscle tissue was removed and frozen for isotopic analysis, and the right pelvic fin was preserved in 95% ethanol for genetic analysis. Sex was determined by direct observation of gonads. Stomach contents were taken from the foregut of freshly sampled specimens, examined using a dissecting microscope and classified as zooplankton, aquatic insects (all life forms, primarily chironomids, other larval or adult dipterans and plecopterans), fish eggs, pelecypods, diatoms, algae, mollusks, nematodes, arachnids and undetermined items. Percentage composition for each diet item and overall fullness were also recorded.

Stable isotope analysis

Longer-term diet characterization was inferred using stable isotope analysis (Post, 2002). Frozen fish tissue (dorsal muscle plugs) was freeze-dried and then homogenized using a mortar and pestle. Samples were then weighted out to 1 mg (± 0.2) and analysed for $\delta^{13}C$ and $\delta^{15}N$ using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer at the University of California (UC) Davis Stable Isotope Facility. Ratios were expressed as parts per thousand of difference relative to the international standard: $\delta = 1000 * (R_{sample} - R_{standard})/R_{standard}$, where R is the ratio of the heavier, more rare isotope (^{13}C or ^{15}N) over the common isotope (^{12}C or ^{14}N). The international standard ratios based on V-PDB (Vienna Pee-Dee belemnite) carbon and atmospheric nitrogen were used to derive $R_{standard}$. Nylon, peach leaf, glutamic acid and enriched glutamic acid standards were used to calibrate the spectrophotometer against National Institute of Standards and Technology Reference Materials and established that the standard deviation for measurements using the apparatus was < 0.20 for $\delta^{13}C$ and < 0.50 for $\delta^{15}N$.

Morphology

Digital photographs were taken of the left side of each fish using a Canon Rebel Xti digital camera with a Canon 100-mm macro lens, chosen to minimize distortion on the edges of the photographs to ensure accurate representation of each specimen. Images were imported into the program TPSDIG (vers. 2.16, Rohlf, 2012, http://life.bio.sunysb.edu/morph/) where coordinates of nine landmarks were obtained (Fig. 2) and imported into PAST (vers. 1.93), a general spreadsheet-based statistical software package (Hammer *et al.*, 2001). In PAST, we used the Geomet utility to calculate the Euclidean distance between landmark 1 and all other landmarks for each specimen resulting in a total of eight linear measurements per fish. These distances were adjusted for differences in body size among all specimens using standard length as the covariate following the method of Elliott *et al.* (1995) as implemented in PAST's Transform menu.

Statistical analysis of gill raker, morphometric, diet and table isotope data

The shallow water and deepwater low-rakered whitefish did not differ in body length or in gill raker counts between 2008 and 2009 ($t_{24–28} = 0.09–1.8$, all $P > 0.15$), so all data were pooled within forms across years in subsequent analyses. We used Mixture analysis in PAST to test for two or more normal distributions of gill raker counts within all samples pooled. We employed the corrected Akaike Information Criterion (AIC_c) to quantitatively assess the support for models invoking unimodal vs. bimodal or trimodal gill raker count distributions. The difference in AIC_c between competing models is used to evaluate the strength of support for each model by calculating $\Delta_i = AIC_{ci} - AIC_{cmin}$ where AIC_{cmin} is the minimum AIC_c among all models. Models having Δ_i values ≤ 2 have 'substantial support', those where $4 \leq \Delta_i \leq 7$ have 'considerably less support' and models with $\Delta_i > 10$ have 'essentially no support' (Burnham & Anderson, 2004).

We employed a model-based clustering method using the program mclust (Fraley & Raftery, 2002, 2007; implemented in R version 2.9.1 using the MCLUST package; R Development Core Team, 2009) to determine how many clusters best explained the morphological and gill raker count data. The method fits the observed frequency distribution of any attribute to a series of alternative clustering models consisting of one or more mixtures each following a Gaussian distribution. The model with the highest (least negative) Bayesian Information Criterion (BIC) is selected as the most likely. This analysis was conducted on the multivariate scores that resulted from a principal components analysis on the correlation matrix of the gill raker/linear distance data using PAST. We used a combination of the Jolliffe (1986) eigenvalue cut-off criterion and Scree

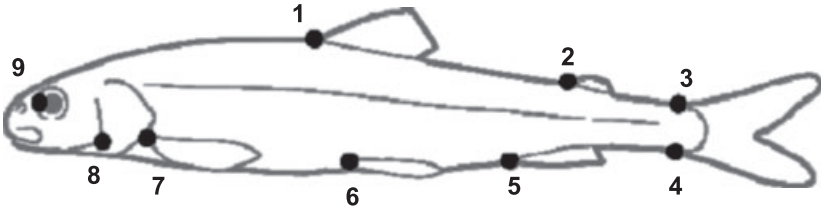

Fig. 2 Position of landmarks used in morphological analysis of pygmy whitefish, *Prosopium coulterii*.

plots (Hammer *et al.*, 2001) to determine the number of principal components to retain for subsequent analyses. The importance of each variable to explaining variation along each PC was assessed via the significance of the correlation between the original variables and the PC scores along each axis.

The extent of diet overlap between ecotypes was expressed using Horn's (1966) index of dietary overlap following Power *et al.* (2005). Horn's index (R_o) varies from 0 (no overlap) to 1 (complete overlap) and demonstrates high performance in the face of potential biases in sample size and resource evenness (Power *et al.*, 2005). Stable isotope data were analysed within forms between years to assess inter-annual variation in diet using two-sample *t*-tests. These tests indicated that three of four comparisons within shallow water and deepwater low gill raker count fish differed between years for $\delta^{13}C$ and $\delta^{15}N$ (all $P < 0.001$) so subsequent comparisons between forms were analysed separately by year using *t*-tests, or analysis of variance. When variances were significantly heterogenous (Leven's test) we used the unequal variance *t*-test or Welch's *F*-test in PAST.

Mitochondrial and microsatellite DNA analyses

The general laboratory methods for DNA extraction and quantification are detailed in Taylor *et al.* (2011) and Witt *et al.* (2011). Briefly, we extracted DNA from the ethanol-stored fin clips using the QiaQuick DNA extraction kit (Qiagen Inc., Valencia, CA, USA) and stored DNA at −20 °C. To assay mitochondrial DNA variation we used the polymerase chain reaction (PCR) to amplify a 650 bp portion of the ATPase VI gene using primers and PCR conditions as outlined by Witt *et al.* (2011). This fragment was separated from PCR reagents and sequenced with a 37390S sequencer using dGTP BigDye® Applied Biosystems (ABI) BigDye chemistry (Witt *et al.*, 2011).

Variation across nine microsatellite DNA loci was assayed with PCR using fluorescently labelled primers and a Beckman-Coulter CEQ 8000 laser detection system as detailed in Taylor *et al.* (2011). We scored individuals at nine microsatellite loci that had been isolated from lake whitefish, *Coregonus clupeaformis* (*Cocl-Lav49b*; *49b*; Rogers *et al.*, 2004), European whitefish, *C. lavaretus* (*ClaTet1*, *9* and *12*; Winkler & Weiss, 2008), Arctic cisco, *C. autumnalis* (*Aut134*, *139* and *151*; Ramey *et al.*, 2008), broad whitefish, *C. nasus* (*LGL-BWF1*; Patton *et al.*, 1997) and Atlantic salmon, *Salmo salar* (*Ssa456*, Slettan *et al.*, 1995).

Analyses of genetic data

The ATPase VI sequences were aligned using BIOEDIT (vers. 7.0.5.3; Hall, 1999) and novel sequences have been deposited in GENBANK under accession numbers JX033600–JX033604. We also used seven sequences from Witt *et al.* (2011); accession numbers HQ616436 and HQ616455, HQ616456, HQ616457, HQ616458, HQ616459 and HQ616460, HQ616493 and HQ616495 and one sequence from mountain whitefish (*Prosopium williamsoni*, GENBANK JX033605) as an outgroup. Following sequence alignment, the program JMODELTEST (Posada, 2009) was used to estimate the best fit model of 88 models of sequence evolution using the AIC_c (Posada & Buckley, 2004). A phylogenetic analysis was subsequently conducted on 13 haplotypes using the Neighbor-Joining (NJ) distance method (Saitou & Nei, 1987) used on a matrix of nucleotide difference numbers between all pairwise combinations of sequences based on the Tamura-Nei substitution model in MEGA 4.0 (Tamura *et al.*, 2007). We included a novel haplotype from Iliamna Lake (located about 485 km north-east of Chignik Lake) and two haplotypes representative of the eastern clade resolved in Witt *et al.* (2011) in our analyses. Confidence for the NJ analysis was estimated using 1000 bootstrap pseudo-replicates.

Microsatellite data were first processed using MICRO-CHECKER (van Oosterhoust *et al.*, 2004) to identify possible scoring errors and/or the presence of null alleles. Thereafter, basic descriptive statistics of sample size (N), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosity were compiled using FSTAT ver 2.9 (Goudet, 2001). The following tests were performed using GENEPOP ver. 3.3 (Raymond & Rousset, 2001). 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.

We used the model-based Bayesian clustering analysis within STRUCTURE (Pritchard *et al.*, 2000) to assess population structure spatially. We used the admixture model with correlated allele frequencies and a burn-in of 50 000 iterations followed by an additional 100 000 iterations, replicated five times. We ran simulations with hypothesized numbers of populations (K) ranging from $K = 1$ to $K = s + 2$ were s = the total number of samples (three sample areas × years plus Black Lake sample). We used STRUCTURE HARVESTER to process the results from multiple runs of STRUCTURE (Earl & vonHoldt, 2012). Our initial analyses indicated that the signal of population differentiation was weak and failed to detect $K > 1$ despite indications of significant differentiation using other analyses (see below). Consequently, we employed the LOCPRIOR option in STRUCTURE to enhance the probability of detecting $K > 1$ when population structure likely exists, but when the signal is too weak for the standard model in STRUCTURE to function sufficiently, as detailed by Hubisz *et al.* (2009). In this analysis, we used a combination of habitat, gill

raker count and diet to identify three groups of fish (see below) which served as the prior in the STRUCTURE analysis.

Pairwise genetic differentiation between samples was expressed as F_{ST} estimated by calculating θ (Weir & Cockerham, 1984) and pairwise values were tested for significance using permutation analyses ($N = 1000$) using GENETIX (ver 4.05, Belkhir *et al.*, 2001). To summarize microsatellite differentiation among all samples, a factorial correspondence analysis (FCA) was conducted on allele frequency data using GENETIX. All statistical tests of differences between samples were adjusted for multiple comparisons following Narum (2006).

Results

Fish captures and gill raker counts

In the 2 years, 332 pygmy whitefish were captured and sampled, including 156 in beach seines from the littoral zone and 176 in gillnets from deep water (Table 1). Those in shallow water (mean length = 111.0 mm, SD = 22.8; mass = 12.3 g, SD = 6.9 g) had 9–16 gill rakers (mean = 12.2, SD = 1.7, $N = 94$, Fig. 3a) and a model of a single normal distribution of gill raker counts had the highest support ($AIC_c = 82.1$ vs. 84.2 for two groups). The gill raker counts of fish captured at $\geq$ 28 m depth in gill nets (mean length = 134.1 mm, SD = 13.4; mass = 23.5 g, SD = 8.0 g) had a bimodal distribution, with peaks at 13 and 17 (Fig. 3b). A model invoking two normal distributions of gill raker counts had the greatest support ($AIC_c = 233.1$ vs. 262.4 for one group). Over all samples, the distribution of gill raker counts was bimodal; a model invoking two groups had greater support than a one-group model (AIC_c two modes = 444.8 vs. 490.1 for a single group). The overall distribution of gill raker counts had two modes of 12 and 17 gill rakers. The sex ratio of sampled fish was biased in favour of females, 0.73 female, 0.27 male. We used the combination of habitat and gill raker number to categorize the fish for subsequent analyses: fish caught in the littoral zone had between 9 and 16 gill rakers and were called 'shallow water low-rakered', those caught in deep water having 9–15 gill rakers were 'deepwater low-rakered' and fish from deep water having 16–20 gill rakers were 'deepwater high-rakered'. Within each of these categories females tended to be more common: 0.66 : 0.34, 0.69 : 0.31 and 0.88 : 0.12 in the shallow water low-rakered, deepwater low-rakered and deepwater high-rakered samples, respectively. In no case, however, was there significant sexual dimorphism in gill raker counts ($t_{10-32} = -0.2$ to -0.6, minimum $P > 0.50$).

Stomach contents analysis

Examination of the stomach contents of fish caught in shallow water revealed exclusively benthic items, primarily chironomids and other larval insects, some snails and nematodes and one fish was recorded with an empty stomach both in 2008 and 2009 (Figs 3 and 4). The predominance of chironomids was slightly greater in 2009 than in 2008, but in neither year were any zooplankton detected in the stomachs (data not shown). The fish caught offshore contained either exclusively, or almost exclusively, zooplankton in the high-rakered fish, or predominantly benthos such as chironomids, some nematodes and algal matter in the low-rakered fish. These differences were consistent between samples collected in 2008 and 2009 and only 1–2 fish per year in either group had an empty stomach (data not shown). Horn's dietary overlap index was below 0.35 for all comparisons and was lowest (i.e. least overlap) between the deepwater high-rakered and deepwater low-rakered fish (0.187) and greatest (most overlap) between the deepwater and shallow water low-rakered fish (0.322) and intermediate between shallow water low-rakered and the deepwater high-rakered fish (0.225).

Table 1 Summary of pygmy whitefish (*Prosopium coulterii*) captured from Chignik Lake, Alaska, by habitat, mean standard lengths, and gill raker counts (GRC, first arch, mean and range). Sample sizes refer to the numbers of fish used in comparisons of standard length, gill raker counts, morphology, stomach contents, stable isotopes, mitochondrial, and microsatellite DNA, respectively.

Sample	Year	Habitat	Standard length	GRC	N	N	N	N	N	N	N
Shallow	2008	Littoral seine	108.1 (25.6)	12.5 (2.01)	116	54	32	36	32	26	78
Deep*	2008	Deep gill net[1]	138.8 (11.64)	13.0 (1.80)	41	27	33	17	19	17	45
Shallow	2009	Littoral seine	107.9 (11.38)	11.9 (1.00)	40	40	40	40	40	–	40
Deep†	2009	Deep gill net[2]	112.1 (4.00)	12.1 (1.50)	74	29	26	27	29	–	29
Deep†	2009	Deep gill net[2]	125.9 (8.24)	16.9 (1.46)	60	56	55	47	58	20	60
Black Lake‡	2008	Littoral seine	80.3 (3.12)	13.6 (0.98)	20	20	–	–	–	–	42

*28–55 m.

†40–55 m.

‡Kong and Taylor (unpublished data).

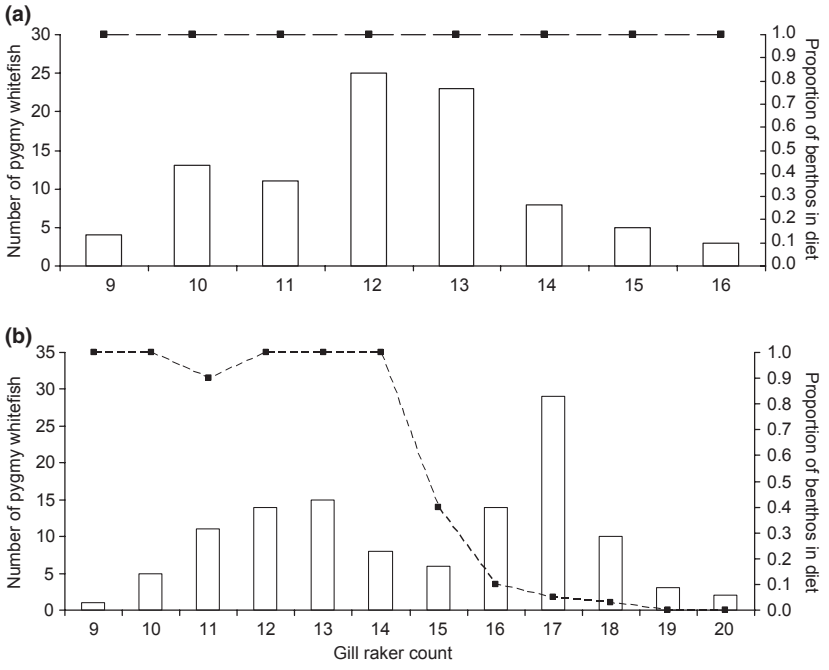

Fig. 3 Distributions of gill raker counts (open bars) and mean proportion of benthic food items in stomachs (closed squares) for pygmy whitefish (*Prosopium coulterii*) collected from Chignik Lake, Alaska, from (a) shallow water littoral areas sampled by beach seine, (b) deepwater areas sampled by gill nets set at > 28 m depth.

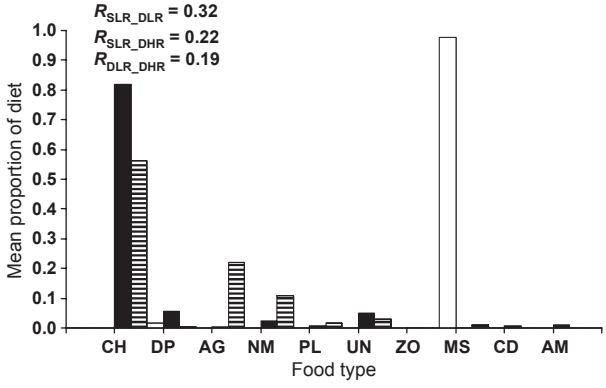

Fig. 4 Average proportion of different food items recorded from the stomachs of pygmy whitefish (*Prosopium coulterii*) samples from Chignik Lake, Alaska. Open bars are deepwater, high-rakered fish (DHR), hatched bars are deepwater, low-rakered fish (DLR), and closed bars are shallow water, low-rakered (SLR) fish. CH, chrionomids; DP, Diptera; AG, algae; NM, nematodes; PL, plecypods; UN, undetermined; ZO, zooplankton; MS, miscellaneous; CD, caddis; AM, amphipods. R = Horn's index of diet overlap.

Stable isotope analysis

Across all samples, stable isotope values were variably associated with gill raker counts. There was a strong positive correlation between gill raker count and δ^{15}N ($r_{192} = 0.69$, $P < 0.001$), but the correlation between δ^{13}C and gill raker count was low, negative and not significant ($r_{192} = -0.068$, $P = 0.34$). Stable isotope analysis indicted that the three groups defined by a combination of depth of catch and gill raker count differed in diet (Fig. 5). There were no significant differences in δ^{15}N between 2008 and 2009 for the low-rakered forms so they were pooled to compare with the high-rakered form collected in 2009 (t_{16-37} = 1.3–1.8, maximum $P = 0.15$). Large differences among all three forms were found in δ^{15}N ($F_{2,170} = 312.9$, $P < 0.001$, all Tukey $P < 0.001$). The deepwater high-rakered fish were enriched in δ^{15}N (mean ± SD = 10.5 ± 0.40) relative to the other groups (maximum Tukey *a posteriori* $P < 0.001$) and the deepwater low-rakered fish were slightly higher in δ^{15}N (8.5 ± 0.72) than the shallow low-rakered individuals (6.5 ± 0.64; Tukey *a posteriori* $P < 0.001$, Fig. 5). Significant differences in δ^{13}C occurred between 2008 and 2009 samples for both the low-rakered forms (t_{16-37} = 7.7–21.7, both $P < 0.001$, δ^{13}C = −22.4 + 1.98 and −30.2 + 1.03 in 2008 and 2009, respectively for the shallow water, low gill raker form; −22.3 + 2.5 and −27.4 + 1.94, respectively, for the deepwater, low-rakered form). In 2008, there was no significant difference in δ^{13}C between shallow water and deepwater, low-rakered fish (t_{16} = −0.15, $P = 0.88$, δ^{13}C = −0.22.4 ± 1.98 and −22.3 ± 2.34, respectively; deepwater, high-rakered fish not sampled). In 2009, the three forms were significantly distinct in δ^{13}C (Welch's $F_{57.9} = 30.2$, $P < 0.001$). In this case, the deepwater low-rakered fish were slightly more enriched in δ^{13}C (−27.4 ± 1.94) than the deepwater high-rakered (−27.8 ± 4.1, Tukey $P = 0.75$) while the shallow water low-rakered pygmy whitefish showed a value significantly less enriched than the two

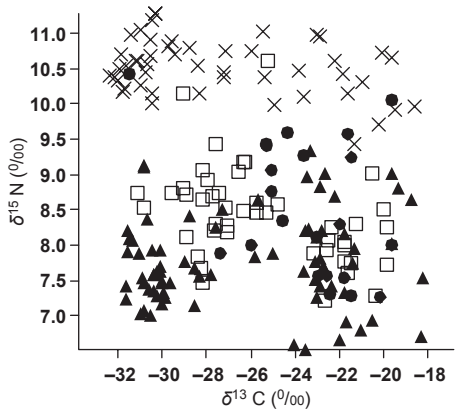

Fig. 5 Biplot of δ^{13}C and δ^{15}N for pygmy whitefish (*Prosopium coulterii*) sampled from Chignik Lake, Alaska. Shallow water low-rakered fish are represented by filled triangles, deepwater low-rakered fish by open squares, and deepwater high-rakered fish by crosses.

deepwater forms (−30.2 ± 1.03; both Tukey $P < 0.002$, Fig. 5).

Morphology

A principal components (PC) analysis of the gill raker count and eight linear measurements accounted for 82.5% of the total morphological variance across three PC axes (Table 2). Principal component axis 1 reflected differences between fish elongated along the dorsal surface and towards the head (i.e. large distances from the anterior insertion of the dorsal fin (landmark 1) to the caudal fin and to the head (landmarks 3, 7–9) and with high gill raker counts compared to fish with deeper posterior regions (greater distances from landmark 1 to landmarks 4–5), and fewer gill rakers. Principal component axis 2 was largely a reflection of differences between fish that were elongated between the dorsal fin and the pelvic and anal fins and that had high gill raker counts and those with average values for all traits. Principal component axis 3 reflected differences between fish with high gill raker counts and shallow bodies towards the head and those with fewer gill rakers and deeper bodies (Table 2). In general, shallow water low-rakered fish had the lowest (most negative) PC scores along PC1 and PC2 and intermediate scores along PC3. Deepwater, high-rakered fish had consistently the highest scores along all three axes and the deepwater, low-rakered fish had intermediate scores along PCs 1 and 2 and the most negative scores along PC3.

When the PC scores for these three PC axes were subject to analysis with mclust a model invoking two morphological clusters, both ellipsoidal in shape with equal variance received the greatest support (BIC = −1710, Fig. S1). A model invoking a single morphological cluster (BIC = −1760) ranked fifth in terms of support and was less supported than models invoking 3–4 morphological clusters, Fig. S1). Models with ΔBIC of > 10 are considered to lack support (Raftery, 1996). Projections of the two morphogroups resolved by mclust based on PC1 and 2 scores indicated that the deepwater high-rakered fish were the most divergent and largely separated from the shallow and deepwater low-rakered fish (Fig. 6). Despite some overlap in the PC scores of low-rakered and high-rakered fish, the model-based discriminant analyses employed in mclust classified 98.2% of the deepwater, high-rakered fish into one cluster and 96.9% of the shallow water and deepwater, low-rakered fish into the other (Fig. 6). Most of the misclassified low-rakered fish (three of four) were deepwater low-rakered fish that were misclassified into the group in which all but one deepwater, high-rakered fish were classified (misclassified fish are shown as '*' in Fig. 6).

Table 2 Principal component analysis of eight landmark distances (Fig. 2) and gill raker counts (GRC) for pygmy whitefish (*Prosopium coulterii*).

Trait	PC1	PC2	PC3
GRC	0.2398*	0.1392*	0.8980*
LD1-2	−0.3615*	0.0575	0.0382
LD1-3	0.3999*	0.0078	−0.0003
LD1-4	−0.3872*	0.0318	−0.0404
LD1-5	−0.1987*	0.6178*	−0.1536
LD1-6	0.1498*	0.7417*	−0.1160
LD1-7	0.3896*	0.1252*	−0.0964
LD1-8	0.3861*	−0.0032	−0.3404*
LD1-9	0.3965*	−0.1043	−0.1685*
Cumulative variance	59.6	74.0	82.5

*Characters that were significantly correlated with PC scores for individual fish across each PC axis.

There was a strong and positive correlation between δ^{15}N and score along PC1 ($r_{157} = 0.64$, $P < 0.001$) which reflected a separation between the deepwater, high-rakered whitefish, which had high δ^{15}N and PC1 scores, from the low-rakered whitefish (Fig. S2a). By contrast, δ^{13}C and PC1 score had a lower and negative correlation ($r_{157} = -0.19$, $P = 0.014$). Although differences were less striking, a larger fraction of the shallow water, low-rakered fish were associated with enriched δ^{13}C and lower PC1 scores than either the deepwater, low-rakered or deepwater high-rakered forms (solid triangles in Fig. S2b).

Mitochondrial DNA variation

A total of 512 base pairs were analysed in 74 pygmy whitefish, including 63 from Chignik Lake. A total of 13 haplotypes were resolved and the best model of molecular evolution was found to be the Tamura-Nei model with a Gamma distribution ($G = 0.34$) for site variation in evolutionary rates (TrN + G model, $AIC_c = 2249.4$ vs.

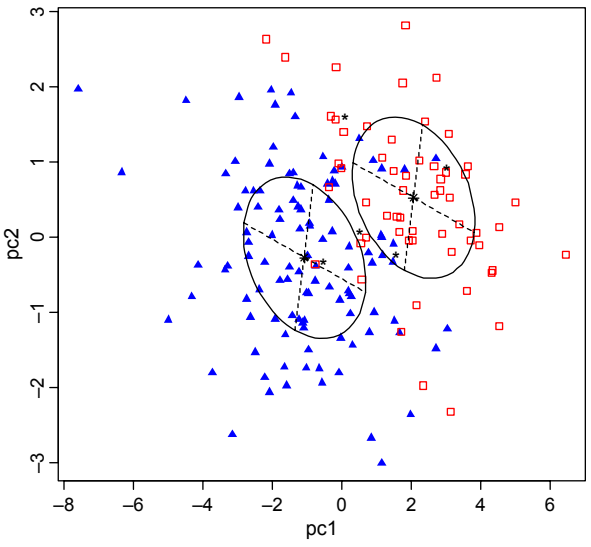

Fig. 6 Identification of two morphoclusters by mclust analysis from PC scores of landmark distances and gill raker counts in pygmy whitefish (*Prosopium coulterii*) sampled from Chignik Lake, Alaska. Each symbol indicates the position of an individual fish along the first two principal components of morphospace. The ellipses represent the multivariate standard deviations for each morphocluster. Individuals are coded by symbol shape and shading on the basis of a discriminant analysis generated by mclust. All but one of the fish symbolized by the closed triangles are either shallow water or deepwater, low-rakered fish and all but four of the fish symbolized by the open squares are deepwater, high-rakered fish (such misclassified fish are denoted by an '*').

2250.1 for next best TiM + 3 + G model). Among the 13 haplotypes, the eastern and western clades differed from each other by a net sequence divergence of 2.1%. The two eastern haplotypes differed by 1.2% [they were selected to represent the maximum divergence reported by Witt *et al.* (2011)], while the western (Alaskan) haplotypes differed by an average of only 0.4% sequence divergence. Pygmy whitefish differed from mountain whitefish by an average 23.4% sequence divergence. Within Chignik Lake itself, eight haplotypes were resolved which differed from one another by an average of 0.3%. All analyses supported the monophyly of pygmy whitefish mtDNA and the subdivision of eastern and western clades with all Chignik Lake haplotypes falling within the western clade (Fig. S3). The two most common haplotypes were widely shared among the deepwater and shallow water low-rakered fish and the deep high-rakered fish and there was no detectable difference in haplotype frequencies among the three kinds of pygmy whitefish (contingency test, $P > 0.25$, 18 d.f., Fig. S4).

Microsatellite DNA variation within and among forms

MICRO-CHECKER Reported no unusual observations in most samples except for a higher than expected number of homozygotes, perhaps associated with the presence of one or more null alleles, at *ClaTet1* (two samples) and *ClaTet12* (one sample). Of the 90 tests of linkage disequilibrium (nine loci within each of Black Lake, shallow water low-rakered 2008 and 2009, deepwater low-rakered 2008 and 2009, deepwater high-rakered 2009), eight were significant at the nominal level of $P \leq 0.05$; two samples (Chignik shallow water low-rakered 2008 (four tests of 15) and Chignik deepwater low-rakered 2009 (two of 15 tests) exhibited more than one instance of linkage disequilibrium, There were 54 tests of departures from Hardy–Weinburg Equilibrium (HWE, nine loci × six samples) 10 of which were significant $P \leq 0.05$. Three samples (Chignik shallow water low-rakered 2008, deepwater low-rakered 2008, deepwater high-rakered 2009) exhibited more than one locus that was out of HWE (Table S1). In only two cases (*BWF1* and *ClaTet12*) was the same locus out of HWE in as many as three samples.

The number of alleles resolved ranged from 7 (*ClaTet9*) to 44 (*BWF1*). The highest average (over loci and populations) expected heterozygosity was observed at *ClaTet1* (0.95) with the lowest at *ClaTet9* (0.41). Averaged across loci, the greatest number of alleles (and allelic richness) was in 17.2 (13.0) in the Chignik deepwater high-rakered fish while the lowest was 11.2 (6.8) in the Chignik shallow water low-rakered 2009. Similarly, expected heterozygosity averaged across loci was highest, 0.79, for the Chignik deepwater high-rakered 2009 and least for the Chignik shallow water low-rakered 2008 fish, 0.74 (Table S1).

The factorial correspondence analysis indicated a marked differentiation between fish from Black Lake and Chignik Lake as well as a distinction between the Chignik deepwater high-rakered and both the shallow water and deepwater low-rakered forms, but with relatively little separation between the latter two ecotypes (Fig. S5). Analysis of the microsatellite DNA data using STRUCTURE, however, suggested the presence of only a single genetic population among the six samples (mean log likelihood of −9508.2 for $K = 1$ vs. −9669.7 for $K = 2$ as next most likely model). By contrast, when the groups were defined on the basis of sample locations (shallow or deep water) and gill raker count and these groups used as priors in the STRUCTURE analysis, $K = 3$ was the most likely model of population structure among the six samples; mean log likelihood = −9360.5 vs. mean log likelihood = −9425.1 for $K = 2$). The Chignik shallow water and deepwater, low-rakered fish (dominated by genetic group 1) were distinct from the deepwater high-rakered fish and from the Black Lake samples (dominated by genetic groups 2 and 3, respectively) (Fig. 7). In the $K = 3$ model, the level of admixture (Q) was greatest between the Chignik deepwater low-rakered fish and the Chignik deepwater high-rakered fish (Fig. 7); genetic group 2, which predominated in the Chignik deepwater high-rakered 2009

sample (average $Q = 0.669$), was also found at a modestly high frequency in the Chignik deepwater low-rakered 2008 sample (average $Q = 0.175$), but was below $Q = 0.05$ for all other samples. There was a moderate, but highly significant positive correlation ($r = 0.66$, d. f. = 156, $P < 0.0001$) between PC1 morphological score and proportional contribution of genetic group 2 illustrating that both in terms of morphology and genetic grouping, the deepwater, high-rakered fish were distinguishable from the shallow and deepwater, low-rakered fish.

Pairwise F_{ST} ranged from −0.0006 between temporal samples of Chignik low-rakered fish ($P > 0.5$) to 0.053 between Chignik deepwater high-rakered fish and Black Lake fish ($P < 0.001$, Table 3). Again, the Chignik deepwater high-rakered fish appeared to be the most distinct; pairwise F_{ST} between these fish and the Chignik shallow and deepwater low-rakered fish averaged 0.022 which was comparable to the average pairwise F_{ST} (0.021) between all Chignik ecotypes and fish from Black Lake.

Table 3 Pairwise F_{ST} (θ) estimated by variation across nine microsatellite DNA loci in pygmy whitefish (*Prosopium coulterii*) from Chignik and Black lakes, south-west Alaska. Bold values are significantly > 0 (significant if $P \leq 0.01507$, Narum, 2006). SLR2008 = Chignik Lake shallow water low-rakered form-2008, DLR2008 = Chignik Lake deepwater low-rakered form-2008, DHR2009 = Chignik Lake deepwater high-rakered form-2009, SLR2009 = Chignik Lake shallow water low-rakered form-2009, DLR2009 = Chignik Lake deepwater, low-rakered form-2009.

	DLR2008	DHR2009	SLR2009	DLR2009	Black lake
SLR2008	0.00435	**0.03003**	**0.01210**	−0.00045	**0.02205**
DLR2008		**0.01044**	0.00637	0.00141	**0.03084**
DHR2009			**0.02226**	**0.02549**	**0.05348**
SLR2009				−0.00062	**0.02961**
DLR2009					**0.02334**

Discussion

Sympatric ecotypes in north temperate freshwater fishes

The existence of ecologically and morphologically different coexisting forms of fishes within a taxon has long been considered a common feature of many north temperate freshwaters (reviewed by Behnke, 1972; Robinson & Wilson, 1994; Taylor, 1999). Over the last 20 years, our understanding of this phenomenon has been extended to observations of coexisting forms in southern latitude lakes, some marine systems and, with the use of genetic methods to test for reproductive isolation between forms, the resolution of cryptic species and to inferring the geographic processes involved in their evolution (Bernatchez & Dodson, 1990; Colborn *et al.*, 2001; Ruzzante *et al.*, 2003; Barluenga *et al.*, 2006; Langerhans *et al.*, 2007). One of the best studied examples of sympatric forms in the salmonid fishes are the so-called benthic and limnetic forms of lake whitefish (*Coregonus clupeaformis*) which have become a key system with which to study ecological speciation and the genetics of speciation (Bernatchez *et al.*, 1999; Rogers & Bernatchez, 2007). Similar divergence occurs in the closely related European whitefish (*C. laveratus*, Østbye *et al.*, 2005), but such divergence within other members of the subfamily Coregoninae appears less well developed. A possible exception is *Prosopium* where sympatric morphological forms of mountain (*P. williamsoni*) and pygmy whitefish (*P. coulterii*), have been reported (McCart, 1970; McPhail & Lindsey, 1970; Whiteley, 2007). Whiteley (2007) detected no molecular genetic differences between coexisting morphological and ecological types of mountain whitefish sampled from two localities, and the level of genetic differentiation between forms of pygmy whitefish has remained unknown since McCart's (1970) description of sympatric forms in Chignik Lake, Alaska. Further,

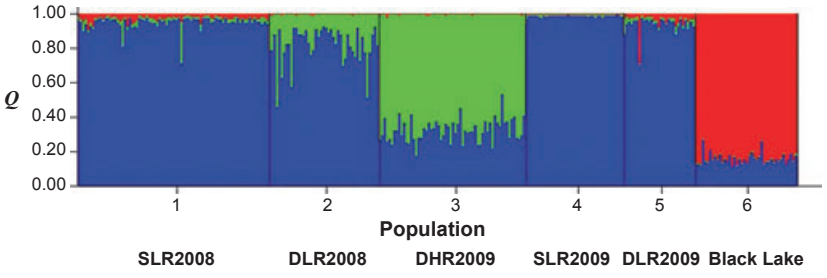

Fig. 7 Results of STRUCTURE analysis (with locality information used, Hubisz *et al.*, 2009) for pygmy whitefish (*Prosopium coulterii*) sampled from Chignik and Black lakes, south-western Alaska, and assayed at nine microsatellite DNA loci. Each fish is represented by a thin vertical line in which the height of each coloured portion indicates the proportional genetic contribution (Q) from each of the three genetic groups (red, green, and blue portions): 1 = Chignik shallow water low-rakered 2008, 2 = Chignik deepwater low-rakered 2008, 3 = Chignik deepwater high-rakered 2009, 4 = Chignik shallow water low-rakered 2009, 5 = Chignik deepwater low-rakered 2009, 6 = Black Lake.

the forms in Chignik Lake matched 'low' and 'high-rakered' forms of pygmy whitefish from across North America, leading to competing but untested hypotheses to explain the origin of such variation (Lindsey & Franzin, 1972). Our integrated analysis of phenotype and genotype indicated the continued existence of these forms in Chignik Lake since McCart's original description, provided a better understanding of their ecological relationships, demonstrated a measure of genetic differentiation between forms and can now provide a plausible scenario for their evolution.

Morphological differentiation

McCart (1970) primarily described differences among Chignik Lake pygmy whitefish in terms of gill raker counts (and dorsal fin ray and caudal peduncle scale counts which we did not assess), the 'bluntness' of the snout and degree of body compression. The deepwater low- and high-rakered forms tended to have less blunt snouts and less compressed bodies than the shallow water low-rakered form and, in general, the two low-rakered forms tended to be more similar to one another morphologically than either was to the deepwater, high-rakered form (McCart, 1970). Our more quantitative morphological results were in broad agreement with McCart's (1970) conclusions; we found separation among groups of pygmy whitefish especially between the deepwater, high-rakered form and the two lower-rakered forms. Similarly, McCart reported two modes of gill raker counts: one at 14–15 gill rakers in fish caught both in deep and shallow water and one at 20 in fish caught only in deep water. We found a similar biomodal distribution of gill raker counts, although our counts were, on average, lower than those reported by McCart (i.e. modal counts of 12–13 and 17, respectively). The difference in total gill raker counts could be a function of differences in counting technique (i.e. sometimes the smallest gill rakers can be missed) or owing to some environmental change in the lake over the last 40 years since McCart's (1970) work. For instance, Crowder (1984) reported a change in mean gill raker count from 44.0 to 41.9 in samples of the bloater, *Coregonus hoyi*, in Lake Michigan between 1960 and 1979. The decline in gill raker counts (and length) were associated with a greater presence of bloater in bottom catches and a greater degree of exploitation of benthic prey that was associated in time with the appearance of the alewife, *Alosa pseudoharengus* – a specialist planktivore and invasive species in Lake Michigan (Crowder, 1984). By contrast, Siwertsson *et al.* (2008) reported stability in gill raker counts for up to 24 years across seven populations of European whitefish despite variable levels of human disturbance across lakes. In addition, given that our data indicated that Chignik Lake whitefish occupy the same habitats and exploit the same food resources as reported by McCart (1970), differences in gill raker counting techniques are the more likely explanation for the small differences in counts from McCart (1970).

The observation of two morphological types of whitefish in Chignik Lake and the greater morphological similarity between low-rakered deep and shallow water whitefish compared to the deepwater, high-rakered fish evident in our data is consistent with McCart's (1970) description. Our morphological results cannot, however, be precisely compared with McCart's because he provided diagrams and descriptions, but few detailed measurements of body proportions. In general, however, we also found that deepwater high-rakered fish had shallower bodies, with more elongate heads than shallow and deepwater low-rakered fish. McCart (1970) also described the deepwater high-rakered fish as having a less cylindrical, more compressed body form, but we did not measure body width and so could not make such comparisons. Interestingly, pelagic, high-rakered forms of lake whitefish tend to have deeper bodies and shallower heads than low-rakered, littoral forms (Lindsey, 1963). In other cases of so-called 'dwarf' and 'normal' lake whitefish which differ in size at maturity and have less well understood differences in habitat, high-rakered normal forms tend to have deeper heads and shallower bodies than the low-rakered forms (Vuorinen *et al.*, 1993). In other freshwater fish taxa, pelagic forms are often associated with shallower bodies, more gill rakers or both (McPhail, 1984; Dynes *et al.*, 1999). Consequently, while our results are consistent with the morphological and gill raker differences reported by McCart (1970) it appears that combinations of traits associated with habitat use differences are more variable across taxa.

McCart (1970) reported that the two low-rakered forms in Chignik Lake appeared to differ in that the shallow water form had a more rounded body, a distinct nuchal hump, and a blunt snout, while the deepwater, low-rakered from had a more compressed body and more pointed snout. Although our mclust analysis indicated the best model was one invoking two morphological clusters (shallow and deepwater low-rakered fish and deepwater high-rakered fish), shallow water, low-rakered fish tended to have blunter heads and less elongate bodies anteriorly than deepwater, low-rakered fish, consistent with McCart's (1970) observations as evidenced by generally lower scores along PC1 and higher scores along PC2.

Ecological differentiation

McCart (1970) reported that only low-rakered pygmy whitefish were captured while seining along the margins of Chignik Lake and that high-rakered fish comprised < 2% of catches in gill nets set at depths of 10 m or less. High-rakered fish were relatively rare within the entire lake, but comprised about 36% of fish when gill nets were set at depths exceeding 30 m

(McCart, 1970). These distributional observations are consistent with our results as we never observed high-rakered fish in the beach seines and only one of 19 fish sampled with gill nets set at between 30 and 40 m was a high-rakered fish; all other high-rakered fish were captured with gill nets set at depths of 50 m or more. Therefore, the pygmy whitefish of Chignik Lake appear to partition the available lake habitat in two ways: a littoral – offshore component, and a depth component in offshore areas. The association of high-rakered coregonids with offshore deepwater habitats is not unusual (Amundsen *et al.*, 2004), although exceptions do exist (Østbye *et al.*, 2005). For instance, Lindsey (1963) reported high-rakered lake whitefish in offshore, surface waters, whereas low-rakered fish were found in benthic areas of offshore and inshore habitats.

Our analysis of the diets of Chignik Lake whitefish was also consistent with McCart's observations; high-rakered fish were almost exclusively planktivorous whereas low-rakered fish had diets dominated by bottom-dwelling, larger-bodied prey. McCart (1970) reported that 85% of high-rakered fish stomachs contained plankton whereas about 75% of low-rakered fish contained bottom fauna. McCart (1970) also reported that the deepwater low and high-rakered fish had more similar diets than we observed. Between 0% and 2% of low-rakered fish caught in beach seines or in gill nets set at depths of < 10 m had plankton in their stomachs, compared to 12.5% of low-rakered fish and 87% of high-rakered fish captured in gill nets set at > 10 m depth (McCart, 1970), whereas none of our low-rakered fish captured in deep-set gill nets contained zooplankton in their stomachs and Horn's diet overlap index was lowest between the two deepwater pygmy whitefish morphs.

We extended the original gill raker count, habitat, and diet associations reported by McCart (1970) by conducting stable isotope analysis of Chignik Lake pygmy whitefish to obtain a longer-term view of the diet of the sympatric forms. These analyses were consistent with expectations based on the diet data and comparisons to other lake systems with benthic and pelagic fishes. For instance, McIntyre *et al.* (2006) compared carbon and nitrogen isotope levels for a series of pelagic and littoral fishes and their prey in Lake Washington of north-western Washington State, USA. Pelagic fishes were characterized by higher δ^{15}N and lower δ^{13}C (as were their zooplankton prey) than members of the littoral fish community and their benthic prey including chironomids (which dominated the diet of low-rakered whitefish in Chignik Lake; McIntyre *et al.*, 2006). Our sampling of the prey community in Chignik Lake has been limited but the results are consistent with these patterns. Benthic prey showed higher values of δ^{13}C than zooplankton (chironomids: −19.19, snails: −18.92, larval caddisflies: −21.00 vs. −30.74 for zooplankton) and lower values for δ^{15}N (chironomids: 0.26, snails: −1.07, larval caddisflies: −2.36 vs. 5.04 for zooplankton; C. Gowell, M. Bond and T. P. Quinn, unpublished data). Thus, the stable isotope profiles of Chignik Lake pygmy whitefish corroborate inferences about their differences in trophic ecology from differences in gill raker counts, habitat use, and stomach contents, and are consistent with observations in other ecosystems. Further, the isotope differences at δ^{15}N between the shallow water and deepwater, low gill raker count whitefish (and some genetic differences – see below) suggest that these are ecologically distinct populations and not simply a case of a single population moving between habitats.

The emerging picture of pygmy whitefish biodiversity is that Chignik Lake contain a littoral-oriented, low-rakered benthivore, an offshore, deepwater, low-rakered benthivore, and an offshore, deepwater, high-rakered planktivore, which are common axes of ecological differentiation in the Coregoninae. Interestingly, in Bear Lake (Utah/Idaho, USA) there are three endemic species of *Prosopium* that appear to partition the lake's habitat in a very similar way. The Bonneville cisco (*Prosopium gemmifer*), is an open water planktivore characterized by many gill rakers (37–45, Smith & Todd, 1984). The Bonneville whitefish (*P. spilonotus*) and the Bear Lake whitefish (*P. abyssicola*) are benthically oriented with lower gill raker counts (18–23) that tend to be found in relatively shallow (5–30 m) and deepwater (> 40 m) areas, respectively, of the lake (Tolentino & Thompson, 2004; Kennedy *et al.*, 2006). The Bonneville whitefish consumed mostly chironomids while the Bear Lake whitefish consumed mostly ostracods (Tolentino & Thompson, 2004; Kennedy *et al.*, 2006). Both in Chignik Lake and in Bear Lake, the proliferation of *Prosopium* biodiversity is associated with an absence of any other whitefishes. The absence of other members of *Prosopium* (such as the mountain whitefish or the round whitefish, *P. cylindraecium*) or of *Coregonus* species may have increased the ecological opportunity and promoted diversification within Chignik Lake (Lindsey, 1981; Schluter, 2000; Østbye *et al.*, 2006). The availability of both deep and shallow waters may also have been an important factor in promoting ecological diversification. Black Lake, located just upstream of Chignik Lake is larger in surface area, but much shallower (42 km^2, average depth of < 3 m, maximum depth 6 m) with a sand/silt and detritus substrate compared to Chignik Lake (24 km^2, average depth of 30 m, maximum depth 64 m) that has a more complex, boulder, cobble, gravel, sand substrate (Anderson, 2004). Only a shallow water, low-rakered pygmy whitefish has been observed in Black Lake (McCart, 1970; Kong and Taylor, unpublished data). Siwertsson *et al.* (2010) demonstrated that the extent of trophic polymorphism in European whitefish from northern Fennoscandian lakes increased with two correlates of ecological opportunity – lake size and productivity.

Genetic divergence and origin of Chignik Lake's sympatric whitefish

The presence of divergent gill raker count forms of *P. coulterii* across North America has been suggested to result from isolation and divergence of populations in distinct Pleistocene glacial refugia that encompass its present distribution (McCart, 1970; Lindsey & Franzin, 1972). In particular, McCart (1970) suggested that the existence of sympatric high- and low-rakered pygmy whitefish in several Alaskan lakes, including Chignik Lake, may have resulted from postglacial dispersal of high-rakered whitefish from a Beringian refuge and a low-rakered whitefish from a south-eastern North American refuge. Witt *et al.* (2011) demonstrated the existence of two highly divergent (3.3% sequence mtDNA ATPase VI, and approximately 1% ITS2 divergence) evolutionary lineages of pygmy whitefish across North America. One lineage was restricted to the Bristol Bay area of Alaska and adjacent areas of south-west Yukon Territory and includes samples of the Chignik Lake shallow water, low-rakered form and fish from Black Lake, while the other lineage was widespread from north-western British Columbia to Washington State and included the disjunct population in western Lake Superior (Witt *et al.*, 2011). Coalescent analyses demonstrated that the distribution of the two lineages was consistent with their origin by vicariance in distinct north-western and south-western or south-central North American glacial refuges (Witt *et al.*, 2011). Consequently, if McCart's (1970) 'two refuge hypothesis' for the origin of Chignik Lake sympatric forms was correct, we expected that extending the mtDNA analysis to all three forms in Chignik Lake would reveal the existence of these two genetic lineages within the lake and that there would be a strong association between mtDNA lineage and gill raker count phenotype. All three Chignik Lake forms of pygmy whitefish were, however, characterized by the same lineage 1 clade of haplotypes (*sensu* Witt *et al.*, 2011), and there was no significant differentiation among forms in the distribution of the seven ATPase VI haplotypes we resolved in Chignik Lake. This result is consistent with the origin of all forms from a common glacial refuge and perhaps postglacially within Chignik Lake itself. The alternative, that the forms originated from separate refugia followed by extensive mtDNA gene flow upon secondary contact, seems unlikely given that lineage 2 haplotypes, characteristic of fish that probably had their origin in other North American refugia have yet to be found in Alaskan or western Yukon Territory samples (Witt *et al.*, 2011; E. B. Taylor, unpublished data). In addition, microsatellite DNA variation in Chignik and Black lakes pygmy whitefish suggest little interaction with fish from lineage 2. First, Black Lake pygmy whitefish are highly distinct from lineage 2 samples (cf. Taylor *et al.*, 2011). Second, at one locus, all fish from Chignik and Black lakes share an allele at *SSa456* (156 bp) at an average frequency of 0.143 (range 0.09–0.20), yet this allele has not yet been detected in 255 fish from 14 localities across the western range of lineage 2 (cf. Taylor *et al.*, 2011). The current distribution of Chignik Lake pygmy whitefish was completely glaciated (Lindsey & Franzin, 1972), so our data and those of Witt *et al.* (2011) suggest that their ultimate origin was probably from within the unglaciated portions of Beringia (see also Bird & Roberson, 1979). Wiedmer *et al.* (2010) presented geological data that suggested the existence of a freshwater refuge in the upper Copper River basin, north-west of Cook Inlet, Alaska, which appears to have been the source of extant pygmy whitefish populations in that area (e.g. Klutina Lake). Given, however, the current mountainous glacier-bearing regions between Chignik Lake and lakes of the Copper River basin, it is more likely that the pygmy whitefish in Chignik Lake originated from one or more refugial freshwater habitats further west in Beringia. The Bering Refuge included extensive areas incorporating the Bering Land Bridge and adjacent portions of eastern Siberia that have contemporary populations of *P. coulterii* (Chereshnev & Skopets, 1992).

Our microsatellite data suggest that there are small, but significant differences between both low-rakered forms and the high-rakered form in Chignik Lake; pairwise comparisons of F_{ST} were always significant between low-rakered and high-rakered forms, but not always between the two low-rakered forms. In general, differences within low-rakered forms between years were smaller than between low- and high-rakered forms although we only had data for the high-rakered form for 2009. Analysis using STRUCTURE, however, supported a model of three genetic groups when invoking sample information on habitat/morphotype within Chignik Lake or locality (Chignik Lake or Black Lake). There was variable admixture of the three genetic groups among samples, but the shallow water low-rakered form and the deepwater high-rakered form from Chignik Lake and the fish from Black Lake were each dominated by different genetic groups. Interestingly, the deepwater low-rakered form showed the highest level of admixture of genetic groups that dominated the other two Chignik forms which is similar with their intermediate characterization in terms of morphology and diet as inferred from stable isotopes. McCart (1970) suggested that the deepwater, low-rakered form may result from introgression between the deepwater, high-rakered whitefish and the shallow water, low-rakered form owing to its intermediacy in morphology and diet. While our genetic assessments of admixture were variable between sample years, our results provide some support for McCart's introgression hypothesis. There were few detectable genetic differences between low-rakered fish from shallow or deepwater samples, but the deepwater low-rakered samples displayed

considerable admixture with the deepwater, high-rakered fish. Strong diel offshore-onshore movements have been reported in lakes with single forms of epibenthically oriented pygmy whitefish (Zemlak & McPhail, 2006) which could promote interaction between low-rakered forms in Chignik Lake. Further, the observation that both low-rakered forms from Chignik Lake, and especially the shallow water form sampled in 2008, showed higher levels of admixture of the genetic group that dominated in the Black Lake sample compared to the deep water, high-rakered fish suggests that gene flow might be more frequent between ecologically more similar forms from these interconnected lakes compared to gene flow between low and high-raker forms within Chignik Lake.

Among the *Prosopium* of Bear Lake, Utah-Idaho, whole mtDNA molecule restriction site analysis indicated 0% sequence divergence between *P. abyssicola* and *P. spilonotus* and about 0.33% divergence between either of these species and the morphologically more divergent *Prosopium gemmifer* (Bernatchez *et al.*, 1991). All three Bear Lake taxa were about 2.5% divergent from *P. williamsoni*, their closest relative (Bernatchez *et al.*, 1991). Similarly, *P. gemmifer* and *P. spilonotus* are virtually identical in terms of allozyme divergence (Vuorinen *et al.*, 1998). Bear Lake is located in a region that was not glaciated (although the lake levels changed dramatically as a pluvial lake and during subsequent de-glaciation) and, therefore, is much older (at least 250 000 years old, Bright *et al.*, 2006) than Chignik Lake which was formed only postglacially (i.e. within the last 10 000 years). Although direct comparisons are not possible given the different marker systems used, it is intriguing that despite the greater age of the Bear Lake *Prosopium*, they show similarly low levels of mtDNA and nuclear differentiation among each other as do the forms in Chignik Lake, yet relatively high and comparable levels of morphological and ecological divergence. Although we have no direct evidence of a genetic basis for the morphological and ecological differences we documented and the role of phenotypic plasticity cannot be discounted, many other studies of postglacial fishes have demonstrated inherited differences in trophic morphology and biology (Peichel *et al.*, 2001; Sacotte & Magnan, 2006; Rogers & Bernatchez, 2007). Consequently, our analysis of Chignik Lake pygmy whitefish further illustrates the potential for postglacial differentiation in traits subject to divergent natural selection across variable environments (Clayton, 1981; Østbye *et al.*, 2006; Barrett *et al.*, 2008; Elmer *et al.*, 2010).

Acknowledgments

This study was part of a long-term research program on the ecology of salmon and other fishes in western Alaska, supported by the Chignik Regional Aquaculture Association, and the commercial salmon industry, and recently by the Gordon and Betty Moore Foundation and the National Science Foundation's BioComplexity Program. Support for the genetic analysis was provided by Natural Sciences and Engineering Research Council of Canada operating and equipment grants awarded to EBT. Support was also contributed by the University of Puget Sound chapter of Phi Sigma through the Slater Research Grant. We thank Ryan Simmons, Jennifer Griffiths, Morgan Bond and Lindsy Ciepiela for help in the field, and Pamela Woods and Daniel Hasselman for assistance with data analyses and helpful discussions, and Peter Wimberger for advice and comments. Pamela Woods and two anonymous reviewers also contributed helpful comments on the manuscript.

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Supporting information

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

Figure S1 Values of the Bayesian Information Criterion (BIC) for different models of morphological clusters derived from a mclust analysis of PC scores derived from gill raker counts and eight landmark based morphmetric distances for pygmy whitefish (*Prosopium coulterii*) sampled from Chignik Lake, Alaska.

Figure S2 Plots of (a) δ^{15}N and (b) δ^{13}C vs. score along principal component 1 derived from landmark distance and gill raker counts in pygmy whitefish (*Prosopium coulterii*) collected from Chignik Lake, Alaska

Figure S3 Neighbor-Joining tree of inferred relationships among ATPase VI haplotypes sampled from pygmy whitefish (*Prosopium coulterii*) from Chignik and Black lakes, southwestern Alaska.

Figure S4 Distribution of ATPase VI sequence haplotypes among samples of pygmy whitefish (*Prosopium coulterii*) sampled from Chignik and Black lakes, southwestern Alaska.

Figure S5 Plot of scores for individual pygmy whitefish (*Prosopium coulterii*) sampled from Chignik and Black lakes, southwestern Alaska, in factorial correspondence space based on variation across nine microsatellite DNA loci.

Table S1 Microsatellite DNA variation within samples of pygmy whitefish (*Prosopium coulterii*) sampled from Chignik and Black lakes, southwestern Alaska.

Table S2 Alignment of 512 bp of mitochondial DNA ATPase subunit VI in pygmy whitefish (*Prosopium coulterii* = H1–H31) and mountain whitefish (*P. williamsoni*).

Data deposited at Dryad: doi:10.5061/dryad.7pd82

Received 4 June 2012; revised 26 August 2012; accepted 6 September 2012