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ARTICLE

The origin and genetic divergence of "black" kokanee, a novel reproductive ecotype of *Oncorhynchus nerka*

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Abstract: Kokanee and sockeye salmon are the freshwater-resident and anadromous forms, respectively, of *Oncorhynchus nerka*. Unique populations of "black" kokanee are found in Lake Saiko, Japan, and in Anderson and Seton lakes in the southwestern interior of British Columbia. They are distinct from other populations of *O. nerka* in that black kokanee display black nuptial colouration and they spawn between 20 to 70 m below the surface of lakes in the winter or early spring. Analysis of mitochondrial DNA and nine microsatellite loci supported the hypothesis that black kokanee in Lake Saiko and in Anderson and Seton lakes have had a diphyletic origin resulting from at least two episodes of divergence in the North Pacific basin. Further, black kokanee in the Anderson and Seton lakes system were genetically distinct from sympatric populations of sockeye salmon in Gates and Portage creeks (inlets to Anderson and Seton lakes, respectively) and were distinct from one another. Anderson and Seton lake black kokanee differed dramatically from one another in standard length at maturity, but no differences were found between the two populations in size-adjusted maximum body depth or in gill raker numbers. Independent origins of black kokanee represent novel diversity within *O. nerka*, are consistent with the importance of parallel evolution in the origin of biodiversity, and suggest that independent management regimes are required for the persistence of black kokanee biodiversity within a physically interconnected lake system.

Résumé : Le kokani et le saumon sockeye sont les formes résidente en eau douce et anadrome, respectivement, d'*Oncorhynchus nerka*. Des populations singulières de kokanis « noirs » sont présentes dans le lac Saiko (Japon) et dans les lacs Anderson et Seton, dans le sud-ouest de l'intérieur de la Colombie-Britannique. Elles se distinguent d'autres populations d'*O. nerka* en cela que les kokanis noirs présentent une coloration nuptiale noire et qu'ils fraient entre 20 et 70 m sous la surface des lacs en hiver et au début du printemps. L'analyse d'ADN mitochondrial et de neuf microsatellites appuie l'hypothèse voulant que les kokanis noirs du lac Saiko et des lacs Anderson et Seton aient une origine diphylétique résultant d'au moins deux épisodes de divergence dans le bassin du Pacifique Nord. En outre, les kokanis noirs du réseau des lacs Anderson et Seton sont génétiquement distincts de populations sympatriques de saumons sockeyes dans les ruisseaux Gates et Portage (qui se déversent dans les lacs Anderson et Seton, respectivement) et sont distincts les uns des autres. Les kokanis noirs des lacs Anderson et Seton présentent d'énormes différences sur le plan de la longueur standard à maturité, mais aucune différence n'a été notée entre les deux populations pour ce qui est de la profondeur du corps maximum ajustée selon la taille ou du nombre de branchicténies. Des origines indépendantes des kokanis noirs constituent une nouvelle forme de diversité chez *O. nerka*, appuient l'importance de l'évolution parallèle en ce qui concerne l'origine de la biodiversité et donnent à penser que des régimes de gestion indépendants sont nécessaires pour assurer la persistance de la biodiversité des kokanis noirs au sein d'un réseau de lacs interconnectés. [Traduit par la Rédaction]

Introduction

One of the major challenges in conservation biology is identifying and prioritizing intraspecific variation for conservation action (Moritz 2002; Wood and Gross 2008) especially when strategies to conserve populations conflict with prioritization based on species diversity (e.g., Vasconcelos et al. 2012). Phenotypic specialization and genetic structure, within and among populations, provide information regarding the potential for local adaptation and the demographic history and degree of independence of populations. Such information can generate insights into the persistence of individual populations across diverse environments and how the recovery of declining populations can be facilitated (Willi and Hoffmann 2009; Suk and Neff 2009). Additionally, understanding the evolutionary origin of distinct phenotypes in terms of whether they may have arisen independently and in what geographic areas or under what environmental conditions can greatly aid in conservation decision-making (Behnke 1972; Wood and Gross 2008). For instance, conservation of freshwater fishes, which typically exist across a broad range of often naturally fragmented habitats, has been greatly aided by an understanding of genetic and phenotypic intraspecific biodiversity (e.g., Berst and Simon 1981; COSEWIC 2007; Taylor et al. 2011).

The taxon *Oncorhynchus nerka* includes two main life-history forms: sockeye salmon, the anadromous (or sea-going) form, and kokanee, the nonanadromous (or freshwater-resident) form and has been the focus of many studies of intraspecific diversity and the evolutionary processes that have shaped this biodiversity in nature (e.g., Nelson 1968; Burgner 1991; Lemay and Russello 2015). The migratory life-history differences between sockeye salmon and kokanee are accompanied by divergence in numerous behavioral, morphological, developmental, and physiological traits that appear to be specializations to the different life histories (Nelson 1968; Wood and Foote 1990; Taylor and Foote 1991; Foote et al. 1992, 1994, 1999; Wood and Foote 1996). Extant populations of kokanee appear to be the result of multiple, independent, postglacial divergences from sockeye salmon throughout the range of *O. nerka* (Ricker 1940; Foote et al. 1992; Taylor et al. 1996).

In addition to the divergence within *O. nerka* represented by sockeye salmon and kokanee, there is also considerable variation

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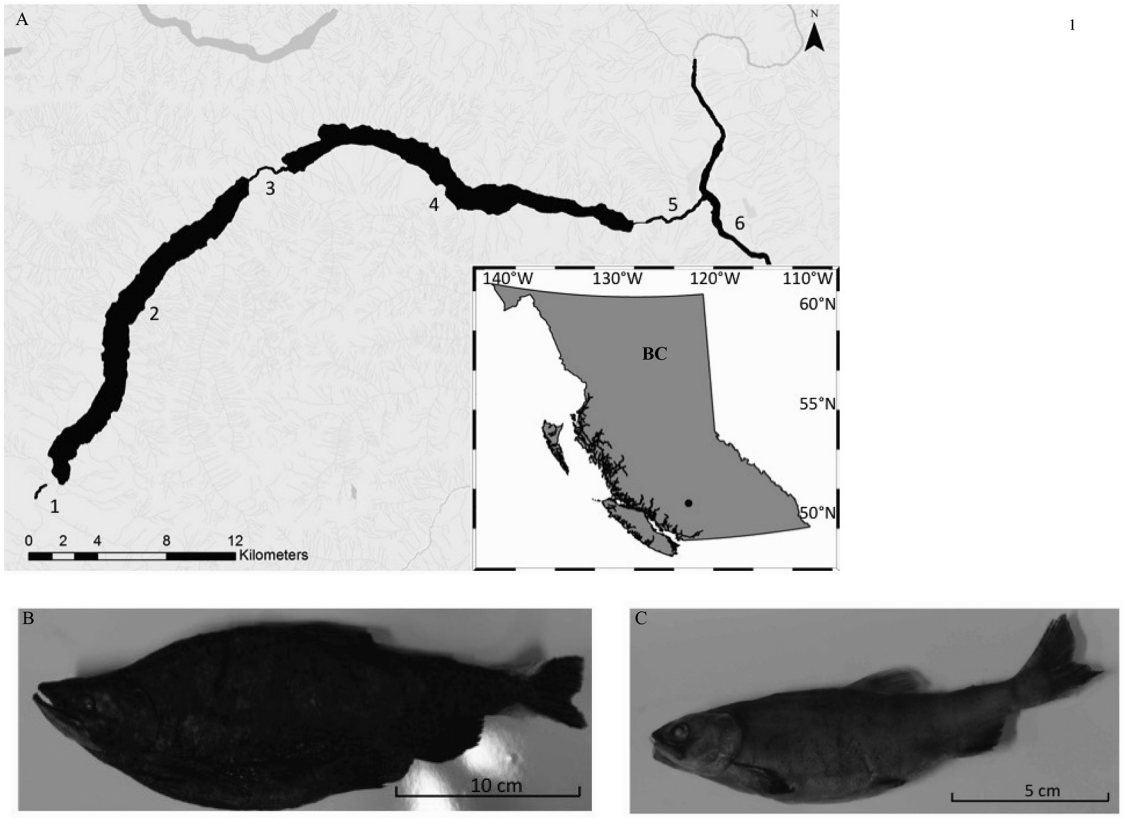
Fig. 1. (A) The Anderson–Seton watershed in the Bridge–Seton system of British Columbia (BC) southwestern interior. 1: Gates Creek, 2: Anderson Lake (50.63°N, 122.38°W), 3: Portage Creek, 4: Seton Lake (50.70°N, 122.15°W), 5: Seton River, and 6: Fraser River. Seton Lake is 24.3 km² with a mean depth of 85 m. Anderson Lake is 28.3 km² with a mean depth of 140 m. (B) Mature black kokanee (*Oncorhynchus nerka*) collected from Anderson Lake in January 2014 during peak spawning season. (C) Mature black kokanee collected from Seton Lake in November 2013 during peak spawning season. Inset shows the position of the Anderson–Seton lakes system (black dot) in BC.



within both of these forms (e.g., Blair et al. 1993; Lemay and Russello 2015). One particularly remarkable and understudied example of such diversity is found within two lakes in the Bridge–Seton river system of the Fraser River drainage in the southwestern interior of British Columbia (BC), Canada. In the Bridge–Seton river system, Anderson and Seton lakes contain populations of kokanee that are black at maturity and have spawning behaviour that is distinct within *Oncorhynchus* (Fig. 1). These so-called "black" kokanee spawn late in the season, typically starting in November in Seton Lake and in January in Anderson Lake (St'át'imc Government 2012). In both lakes, black kokanee spawn on bottom substrates at depths of 20 to 70 m (typically greater than 50 m) below the surface of the lake (A.R. Morris, BC Conservation Foundation, and A. Caverly, BC Ministry of Environment, Kamloops, BC, unpublished data; St'át'imc Government 2012). After spawning, they float to the lake's surface owing to their distended swim bladders (St'át'imc Government 2012). In the Anderson and Seton lakes system, there are no known "regular" kokanee, but there are spawning populations of sockeye salmon in Gates Creek (spawning in July to September) and Portage Creek (spawning in September to November) (Beacham et al. 2004; St'át'imc Government 2012). While sockeye salmon and black kokanee are broadly sympatric in both lakes, the sockeye salmon juveniles feed almost exclusively in Seton Lake, whereas kokanee reside in both lakes as juveniles (K.S. Shortreed, K.F. Morton, K. Malange, J.M.B. Hume, Fisheries and Oceans Canada, Cultus Lake, BC, unpublished data).

Remarkably, and despite the broad distribution of kokanee in watersheds across the North Pacific, the only other known occurrence of black kokanee is in Lake Saiko, Japan. Black kokanee in Lake Saiko resulted from transplantation of black kokanee from their native Lake Tazawa, in northern Japan (Nakabo et al. 2011). Additionally, in Japan, native regular kokanee from Lake Akan were introduced into Lake Saiko. Consistent with most North American regular kokanee, these populations spawn in streams in the fall and have the regular nuptial colouration of green heads and red bodies (Nakabo et al. 2011). By contrast, in addition to being black at maturity, Japanese black kokanee spawn at mean depths of 30–40 m in lakes from March to April and also appear to float to the surface upon senescence (Akitaken Suisanshikenjo 1907, cited in Nakabo et al. 2011). The black kokanee in Japan are so distinctive that they are recognized as a separate species, *Oncorhynchus kawamurae*, based on morphological and genetic differences from sympatric regular kokanee (Jordan and MacGregor 1925, cited in Jordan and Hubbs 1925; Nakabo et al. 2011, 2014; Muto et al. 2013). Hybridization between the two types of kokanee in Lake Saiko has been found to occur only rarely (Muto et al. 2013). The apparently widely disjunct distribution of black kokanee in the North Pacific, their distribution among lakes, and their sympatric occurrence with sockeye salmon in Anderson and Seton lakes raise several interesting questions. Have black kokanee evolved more than once across the North Pacific? Are populations of black kokanee in physically interconnected lakes distinct populations, and what is the degree of distinction between black kokanee and sympatric sockeye salmon?

To address these questions, our study tested the following hypotheses. First, we tested the hypothesis that black kokanee evolved postglacially at least twice, once in the eastern North Pacific and once in the western North Pacific. To test this hypothesis, we used a combination of mitochondrial and microsatellite

DNA to see if black kokanee were mono- or polyphyletic, as would be predicted under single or multiple origins, respectively. Multiple, independent divergences between forms within taxa are common in freshwater fishes (Ryman and Ståhl 1981; McPhail 1984; Hindar et al. 1991; Taylor 1999), including *O. nerka* (Foote et al. 1989; Taylor et al. 1996; Frazer and Russello 2013), but some exceptions are known (e.g., Murphy and Collier 1997; Lampert et al. 2005). Second, we tested the hypothesis that distinctiveness in breeding colouration and behaviour in Anderson and Seton lakes black kokanee would be accompanied by divergence in genetic and morphological traits. Across their geographic range, kokanee exhibit substantial genetic differentiation even within lake systems (Wood and Foote 1996; Taylor et al. 1997; Frazer and Russello 2013). Morphological variation may reflect local adaptation arising from divergent selection associated with differences in spawning environments, from phenotypic plasticity, or some combination thereof. Here, we used microsatellite DNA and morphological analysis to assess the differentiation between black kokanee in Anderson and Seton lakes. Genetic and morphological distinction between the two lakes' kokanee populations would be at least consistent with the idea that they are demographically distinct and probably require independent management in terms of habitat protection and (or) harvest levels (see Palsbøll et al. 2007; Lowe and Allendorf 2010). Further, concordance among a number of traits, including genotypic and phenotypic variation, can provide strong evidence for distinctiveness within a species and result in more nuanced conservation strategies (e.g., Rising and Avise 1993; O'Donnell et al. 2004; Taylor et al. 2011). Third, given the great divergence in body size and life history between sympatric black kokanee and sockeye salmon, we tested the hypothesis that these forms would be genetically distinct from each other to a greater degree than divergence between black kokanee populations from Anderson and Seton lakes.

Materials and methods

Lakes and samples

Seton Lake and Anderson Lake are part of the Fraser River drainage system, located west of the town of Lillooet in the southwestern interior of BC. Anderson Lake has a total surface area of 28.3 km^2 and mean depth of 140 m and is fed by Gates Creek and empties into Seton Lake via Portage Creek. Seton Lake has a surface area of 24.3 km^2 and mean depth of 85 m, and it empties into the Fraser River, via the Seton River, at the town of Lillooet, BC (Fig. 1).

Black kokanee (postspawning moribund and dead fish) were collected from the surface of Seton Lake using dipnets in November 2013. Anderson Lake black kokanee were collected in January 2013 and 2014 by surveying the beach for postspawning adults. Adipose fin clips were taken from all fish on site and stored in 95% ethanol. There are no known regular kokanee populations in either Anderson or Seton lakes. Additional samples from across *O. nerka*'s range (including Japanese black kokanee) were used in the analysis of phylogeographic structure from archival collections of the Beaty Biodiversity Museum's fish collection (Table 1; also see online supplementary material, Table S1[1]).

Table 1. Summary of location, drainage, sample size, and "form" of *Oncorhynchus nerka* (black kokanee, regular kokanee, and sockeye salmon).

Location	Drainage	Sample size	Form
Chedakuz Creek^S	Fraser River, B.C.	29	Regular
Davidson Creek^S	Fraser River, B.C.	31	Regular
Meadow Creek^S	Kootenay River, B.C.[a]	30	Regular
Anderson Lake^S	Fraser River, B.C.	80	Black
Seton Lake^S	Fraser River, B.C.	47	Black
Gates Creek^S	Fraser River, B.C.	41	Sockeye
Portage Creek^S	Fraser River, B.C.	36	Sockeye
Hansen Creek	Bristol Bay, Alaska	20	Sockeye
Lake Saiko	Japan	12	Black

Note: Superscript "S" denotes samples used in the STRUCTURE analysis.
[a]Columbia River system.

Mitochondrial DNA

Qiagen spin columns were used to extract genomic DNA following the animal tissue protocol supplied by the manufacturer. The resulting DNAs were stored at –20 °C until analysis. An approximately 950 base pair (bp) long fragment of the mitochondrial DNA (mtDNA) NADH dehydrogenase-1 (ND1) subunit gene was amplified using the polymerase chain reaction (PCR) and the forward (F) and reverse (R) primers: ND1F (5′-ACCTCGATGTTGGATCAG-3′) and ND1R (5′-TATTCGGCCAGGAAAAACAG-3′). We also amplified an approximately 900 bp fragment of ND2 using the primers ND2F (5′-AGCACTACCAACGCCTGAC-3′) and ND2R (5′-AACAAGGGCTGGGAGATTTT-3′). The ND1 and ND2 regions were selected owing to their relatively high variability in sockeye salmon (see Gharrett et al. 2001), and we designed the primers from sequences in Gharrett et al. (2001) and Churikov et al. (2001). The PCR amplifications were carried out in a final volume of 50 μL using the following reagents (final concentrations): 5 μL 10× New England Biolabs ThermoPol buffer, 4 μL dNTP (5 mmol·L^{-1}), 1 μL of each primer (10 μmol·L^{-1}), 0.3 μL New England Biolabs *Taq* polymerase, 37.2 μL distilled autoclaved water, and 1.5 μL of template DNA (between 50 and 100 ng·μL^{-1}). The PCR conditions were as follows: initial denaturation 95 °C for 3 min, followed by four cycles of 95 °C denaturation (30 s), 55 °C annealing (30 s), 72 °C extension (90 s), 32 cycles of 92 °C denaturation (30 s), 54 °C annealing (30 s), 72 °C extension (90 s), and a final extension step at 72 °C for 10 min. The PCR products were checked for quality on 1.5% agarose gel and purified using QiaQuick columns. The DNA samples were then sequenced in the reverse direction using ND1R and ND2R primers. Eight samples were also sequenced using the ND1F and ND2F primers to verify all substitutions, and only a single base pair difference was found in one of the samples when comparing the 16 sequences (eight samples sequenced in two directions).

Mitochondrial DNA data analysis

The ND1 and ND2 sequences (GenBank accession Nos. KR867664 to KR867675 and KR909029 to KR909046, respectively) were combined to increase the resolution in detecting distinct haplotypes. Sequences were aligned using the multiple sequence alignment ClustalW in Bioedit version 7.2.5 (Hall 1999). The best evolutionary model of nucleotide substitution was estimated using Bayesian information criterion (BIC) as implemented in MEGA (version 6.0.5; Tamura et al. 2013). Subsequently, a maximum likelihood tree was constructed based on the Tamura–Nei sequence evolution model (including a Gamma distribution of among-site variation in substitution rate) and bootstrapped with 2000 replicates using MEGA. Chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*) were used as outgroups (GenBank acc. Nos. AF392054.1 and EF126369.1, respectively). To test the idea of a single origin for black kokanee, we constrained trees to monophyly of black kokanee haplotypes and tested whether or not they were significantly worse than the derived maximum likelihood tree given the sequence data. As a measure of confidence in these alternative trees, we calculated the expected likelihood weights (ELWs) following Strimmer and Rambaut (2001) using TREE-PUZZLE (Schmidt et al. 2002). The ELW method is a

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfas-2015-0145.

form of model selection procedure where the ELWs can be interpreted as direct measures of the relative confidence of alternative models of relationships (Strimmer and Rambaut 2001). Further, the ELW method appears to be most suitable for datasets with modest sample sizes and sequence information (i.e., alternative procedures are overly conservative; Strimmer and Rambaut 2001). There are a number of tree topologies that could represent alternative scenarios of black kokanee monophyly. We constructed alternative trees depicting black kokanee monophyly such that these alternative trees invoked the minimum (conservative) number of tree branch rearrangements relative to the maximum likelihood tree (three).

Microsatellite DNA

For a subset of populations (Table 1), nine microsatellite loci were assayed to assess the hypothesized independent origin of western and eastern Pacific black kokanee populations using a data set independent from mtDNA and also to investigate population structure at a finer geographic scale. We assayed polymorphism at *Omy*77 (Morris et al. 1996); *Ots*100, *Ots*103, and *Ots*108 (Nelson and Beacham 1999); *Oki*10 and *Oki*29 (Smith et al. 1998); and *One*103, *One*108, and *One*110 (Olsen et al. 2000). The PCRs were carried out using fluorescent dye-labeled forward primers in 20 μL volumes using the Qiagen PCR Multiplex Kit following the manufacturer's protocol. The PCR products were assayed using a Beckman-Coulter Ceq. 8000 Genetic Analysis system.

Microsatellite DNA data analysis

The program MICRO-CHECKER version 2.2.3 (van Oosterhout et al. 2004) was used to test for evidence of scoring errors and the presence of nonamplifying alleles (null alleles). Tests for deviations from Hardy–Weinberg equilibrium for each locus within each population were performed using an exact test, with P values estimated using a Markov chain method implemented in GENEPOP version 4.2 (Raymond and Rousset 1995). Genotypic linkage disequilibrium between all pairs of loci within a population was also performed in GENEPOP using a Markov chain method. To evaluate within-population variation, basic population genetic summary statistics (number of alleles per locus, allelic richness, observed heterozygosity, and expected heterozygosity) were performed in FSTAT version 2.9.3.2 (Goudet 1995) and GENEPOP.

To assess the single or multiple origin hypotheses for black kokanee, we constructed a neighbor-joining tree (NJ; Saitou and Nei 1987) using Cavalli-Sforza and Edwards' (1967) chord distance (D_C) as a measure of genetic distance between populations. The analysis incorporated 5000 bootstrap replications using POPULATIONS version 1.2.31 (Langella 2000), and the tree was visualized in TREEVIEW version 1.6.6 (Page 1996). The use of the Cavalli-Sforza and Edwards' genetic distance assumes that genetic drift is the predominant factor organizing variation in allele frequencies, which is likely most realistic for the postglacial populations that we sampled and therefore requires no assumptions about models or rates of mutation. The use of alternative genetic distances (e.g., Nei's standard genetic distance, which incorporates drift and mutation) produced identical results. Under the single origin hypothesis, we expected all black kokanee samples to cluster together and separately from all regular kokanee and sockeye salmon samples.

Among-population variation (F_{ST}) was evaluated using pairwise population differentiation estimated as θ (Weir and Cockerham 1984) in FSTAT. Means and 95% confidence intervals of F_{ST} were calculated by jackknifing over populations and significance levels determined in FSTAT. We also used GENEPOP to conduct G tests of allele frequency differences because G tests have been shown to be more powerful than F_{ST}-based analyses at low levels of divergence (Balloux and Lugon-Moulin 2002). Microsatellite allele frequency variation of Anderson–Seton lakes' *O. nerka* was partitioned hierarchically into components that represented variation within individual populations, variation among populations within black kokanee and within sockeye salmon, and variation between black kokanee and sockeye salmon. To perform this hierarchical analysis and to test the statistical significance of each variance component, we used an analysis of molecular variance (AMOVA) performed in GenAlEx version 6.5 (Peakall and Smouse 2012).

The model-based Bayesian clustering program STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to resolve population structure across all populations of kokanee, sockeye salmon, and black kokanee. The STRUCTURE analysis used the correlated allele frequencies and admixture models with a burn-in period of 100 000 iterations followed by an additional 200 000 iterations. This was replicated ten times with K (hypothesized number of genetic populations characterized by a set of allele frequencies at each locus) ranging from one to eight. This range takes into account each locality sampled and any potentially additional substructure; preliminary analyses using a K value larger than eight resulted in population structure with very low likelihoods. The analysis was run with the location prior option invoked used to assist clustering given the relatively small genetic distances observed (Hubisz et al. 2009). The STRUCTURE analysis was performed on the entire microsatellite data set as well as with only the four populations from the Anderson and Seton lakes system with the same parameter set. The model of K with the highest log-likelihood value was used to evaluate the most likely number of populations. Although we also report the Evanno et al. (2005) ΔK criterion for evaluating the best value of K, it was de-emphasized because its performance appears to be compromised at low levels of population structure (Waples and Gaggiotti 2006).

Discriminant analysis of principal components (DAPC) was also used to visualize the variation among populations within the Anderson and Seton lakes system. The DAPC analysis is a multivariate clustering method that produces synthetic variables to maximize the among-population variation and minimize the within-population variation (Jombart et al. 2010). The DAPC analysis was conducted in R (R Development Core Team 2012) using the ADEGENET package (Jombart et al. 2010). All tests accounted for multiple simultaneous tests as per Narum (2006).

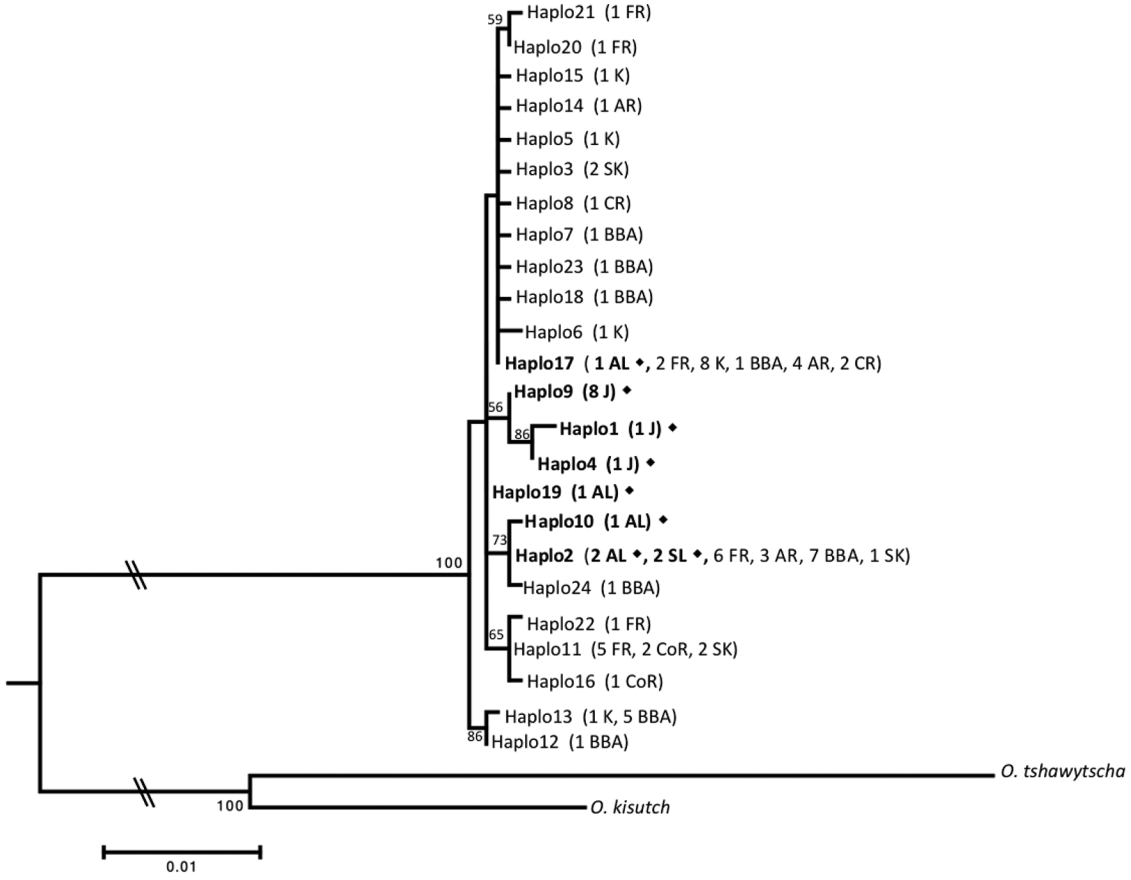
Morphological analysis

As another measure of distinctiveness between black kokanee from Anderson and Seton lakes, we assayed morphological variation. Black kokanee from Anderson Lake (sampled in 2013, $N = 30$; and 2014, $N = 19$) and Seton Lake (sampled in 2013, $N = 30$) were preserved in 10% formalin for a minimum of 4 days (Seton Lake samples) to a maximum of 1 week (Anderson Lake samples owing to their larger size). Fish were then rinsed and soaked in water for 24 h before being measured and then stored in 45% isopropyl alcohol. We measured standard length and the distance from the insertion of the pectoral fin to the insertion of the dorsal fin as a measure of maximum body depth. We measured body depth because this has been shown to vary among *O. nerka* populations from different spawning environments in a potentially adaptive manner (e.g., Blair et al. 1993). Measurements were taken on the left side of the fish, and the sex was determined by internal inspection of gonads. We also counted the number of gill rakers on the first gill arch by removing the first gill arch on the left side and staining each arch in 1% potassium hydroxide with Alizarin red for 2 h. Gill arches were rinsed and then soaked in distilled water for approximately 12 h before gill rakers were counted using a dissecting microscope. Fish were then preserved in 45% isopropyl alcohol for long-term storage.

Morphological data analysis

Body measurements typically scale positively with overall body size in fishes (e.g., Reist 1985), and thus assessment of differences in maximum body depth in black kokanee was required to account for the differences in overall body length between fish

Fig. 2. Maximum likelihood tree using the TN93 + G model of evolution demonstrating the phylogenetic relationships among 24 haplotypes resolved from combining ND1 and ND2 mitochondrial DNA sequences of *Oncorhynchus nerka* (*N* = 82) from the Columbia River (CR), Cowichan River (CoR), Alsek River (AR), Fraser River (FR), Skeena River (SK), Bristol Bay Alaska (BBA), and Kamchatka (K) drainages. Bold haplotypes represent black kokanee from Japan (J, ◆) and Anderson Lake (AL, ◆) and Seton Lake (SL, ◆). Number of individuals with each haplotype is shown in parentheses. The percentage of trees in which the associated haplotypes clustered together is shown next to the branches (percentages less than 50% have been removed) from 2000 bootstrap replicates. The double-slash scale break indicated on two lines represents a 33% reduction in branch length to enhance visualization among ingroup haplotypes.



from Anderson Lake and Seton Lake. This was accomplished by using an allometric adjustment to standardize body depth to a common standard body length by first using an analysis of covariance (ANCOVA) to test for homogeneity between the slopes of depth versus length (using $\log_{10}$ values) for both lakes (Reist 1985). Then, we used the adjustment equation to standardized depth to standard length:

$$D_{adj} = D_o(L/L_o)^b$$

where D_{adj} is the adjusted body depth, D_o is the observed body depth of the individual, L is the common standard length among all samples, L_o is the observed standard length of the individual, and b is the allometric coefficient calculated using ANCOVA as implemented in PAST version 3.01 (Hammer et al. 2001). Gill raker numbers were not adjusted for differences in body size because there was no significant correlation between standard length and gill raker count within any of the three samples (Anderson Lake 2013, 2014, and Seton Lake 2013; $r = 0.004$ to 0.116, $P > 0.08$).

Mature salmonids often exhibit substantial sexual dimorphism in body shape (Quinn 2005); therefore, two-sample t tests were performed to test for sexual dimorphism in standard length, size-adjusted maximum body depth, and number of gill rakers for each lake. The means of each measurement between Anderson Lake and Seton Lake as well as between sampling years were compared using t tests for independent samples.

Results

Mitochondrial DNA sequence analysis

After editing the unclear portions of each sequence, an 856 bp fragment of the ND1 subunit gene from 102 individuals was analyzed. The ND1 sequences resolved 12 haplotypes that were then sequenced at ND2 to increase the power to detect distinct haplotypes and resolve their interrelationships. The ND2 subunit gene fragment (823 bp after editing) resolved 18 haplotypes in 82 individuals. After combining the aligned fragments, 24 unique haplotypes were resolved in a 1679 bp fragment from 82 individuals from across *O. nerka*'s geographic range. The 24 combined ND1–ND2 sequences differed from each other by an average of 0.25% sequence divergence. The mean sequence divergence between all *O. nerka* haplotypes and *O. kisutch* and *O. tshawytscha* outgroups was 12.1% and 10.3%, respectively.

There were no phylogenetic trees (ND1 and ND2 analyzed separately or as a combined sequence) that grouped black kokanee from the eastern and western Pacific together (Fig. 2). In fact, Japanese black kokanee grouped separately from all other forms of *O. nerka* from across its range (including Anderson and Seton black kokanee) at about 0.43% sequence divergence, but with

modest bootstrap support. No haplotypes were shared among the black kokanee populations from the eastern and western Pacific, and their haplotypes differed from each other by an average of 0.34% sequence divergence. In contrast, Anderson Lake and Seton Lake black kokanee haplotypes clustered with haplotypes from other populations of *O. nerka* (all "regular" reproductive phenotypes of sockeye salmon and kokanee) from across its range in North America and differed only slightly from other *O. nerka* haplotypes (mean 0.21% sequence divergence). For instance, the most common haplotypes in eastern Pacific *O. nerka* were also found in Anderson and Seton black kokanee (e.g., haplotypes 2 and 17), and these haplotypes were found in individuals ranging from Kamchatka to the Columbia River. Anderson Lake black kokanee sequences included two unique haplotypes (nos. 10 and 19, Table S1[1]).

The ELW for the derived tree (Fig. 2) was 0.936. By contrast, the ELWs for trees constrained to monophyly of black kokanee were much lower and ranged from 0.016 to 0.032. A 95% confidence set of trees, however, included the derived tree (Fig. 2) and one alternative tree that consisted of a monophyletic group containing all black kokanee haplotypes, but this confidence set of two trees also excluded (at $P < 0.05$) the two other trees constrained to black kokanee monophyly.

Microsatellite DNA analyses

Only one locus (*Ots*108) consistently showed evidence of one or more null alleles using MICRO-CHECKER. This locus also had lower than expected heterozygosity in seven out of the eight populations analyzed. The program FreeNA (Chapuis and Estoup 2007) was used to estimate F_{ST} taking the presence of null alleles into account. Adjusting for the possibility of null alleles at *Ots*108 did not change the significance levels of our results using all nine loci or after removing *Ots*108 (Moreira 2014), so we report all results including *Ots*108.

Out of 72 tests for Hardy–Weinberg equilibrium for each locus population combination, 18 tests departed from expectations of random mating showing statistically significant heterozygote deficiencies after correcting for multiple comparisons for eight populations ($P < 0.018$; Narum 2006). Of these 18 tests, seven were at *Ots*108; therefore, the deficiencies of heterozygotes are most likely caused by null alleles (Table S2[1]). The remaining 11 tests were not concentrated at one specific locus or population, and expected and observed heterozygosities were generally similar to one another (Table S2[1]). Tests for linkage disequilibrium between loci resulted in statistically significant departures in seven out of 288 tests, but departures were not concentrated on specific locus pairs and were less frequent than expected by chance alone, and therefore, each locus probably represents an independent measure of genetic variation and divergence.

The mean number of alleles per locus corrected for different sample sizes ranged from 6.0 (*Omy*77) to 15.2 (*One*103; Table S2[1]). Averaged across all nine loci and populations, allelic richness was 10.1 per locus per population. Genetic diversity (expected heterozygosity) averaged across loci ranged from 0.63 (Chedakuz Creek) to 0.88 (Seton Lake and Anderson Lake) and ranged from 0.72 (*Omy*77) to 0.93 (*Oki*10) when averaged across populations. Genetic diversity averaged across all seven populations and loci was 0.82. Chedakuz Creek and Gates Creek consistently exhibited the lowest levels of within-population diversity. Genetic diversity averaged across populations within the Anderson–Seton lakes system (excluding the three outgroups) ranged from 0.74 (*Oki*29) to 0.94 (*Oki*10 and *One*103), with the overall mean being 0.85. Black kokanee in both lakes show similar levels of within-population variation to each other as well as to Portage Creek sockeye salmon (Table S2[1]).

The neighbor-joining tree indicated that Anderson Lake and Seton Lake black kokanee clustered separately from Lake Saiko black kokanee, but clustered together with sockeye salmon populations within the same watershed as well as other Fraser River sockeye salmon and kokanee populations (Fig. 3). Apparently unique alleles (i.e., those found only in single population samples) were found in both groups of kokanee (e.g., allele 310 at locus *Oki*10 in eastern Pacific black kokanee and allele 138 at locus *Ots*103 in western Pacific populations; Table S3[1]).

Within the Anderson–Seton system and other North American samples, most unique alleles were found at low frequency and most occurred in Anderson Lake and not in Seton Lake. A majority (68.5%) of unique alleles found in the black kokanee populations were found at *Oki*10 (overall frequency in Anderson–Seton lakes = 0.032), *Oki*29 (0.039), and *One*103 (0.060). Interestingly, when comparing allele frequencies across all populations, alleles found in high frequency in Anderson–Seton lakes black kokanee were typically also shared with Portage Creek sockeye salmon rather than Gates Creek sockeye salmon (Table S3[1]).

Anderson Lake was sampled in 2013 and in 2014 and allele frequencies in these temporal samples differed significantly at two of the nine loci assayed (*Omy*77, $P < 0.005$; and *Oki*10, $P < 0.05$). When comparing allele frequencies between Anderson Lake samples, most differences between years were caused by shifts in the presence or absence of less common alleles or by small changes in the frequency of common alleles from one year to the next. Combining all nine loci, the temporal samples differed significantly in allele frequencies ($F_{ST} = 0.0056$, $P < 0.05$), but temporal variation accounted for only 0.68% of the total variation in allele frequencies. Consequently, we combined the temporal Anderson Lake samples for the subsequent analysis.

Comparing each locus separately, *Ots*108 (F_{ST} mean = 0.155, range = 0.018–0.428), *Ots*100 ($F_{ST} = 0.132$, range = 0.002–0.353), and *Omy*77 ($F_{ST} = 0.106$, range = 0.011–0.417) showed the highest mean levels of divergence among populations (Table 2). The mean level of pairwise divergence among all seven populations and all nine loci was $F_{ST} = 0.080$ (95% confidence interval: 0.051–0.112; Table 2). Chedakuz Creek and Gate Creek consistently had the highest level of mean pairwise divergence from the other populations: $F_{ST} = 0.168$ (range = 0.135–0.200) and 0.106 (range = 0.059–0.200), respectively. When only populations from the Anderson–Seton lakes system were included, the mean F_{ST} among populations was 0.041 (95% confidence interval: 0.029–0.055). Gates Creek sockeye salmon showed the greatest divergence from all other populations: $F_{ST} = 0.071$ (range = 0.059–0.083; Table 2). Anderson and Seton lakes black kokanee and Portage Creek sockeye salmon consistently showed the lowest level of divergence from each other (F_{ST} ranged from 0.012 to 0.035, all $P < 0.020$; Table 2). The Anderson and Seton lakes populations of black kokanee were also significantly distinct from one another at three of nine loci when they were examined individually and over all loci when using *G* tests (*Omy*77, *Oki*29, and *One*108; $P < 0.05$; all loci, $P < 0.05$).

The STRUCTURE analysis using seven populations from the Fraser and Columbia rivers and nine loci suggested that $K = 6$ was the most likely number of genetic groups and Anderson Lake and Seton Lake black kokanee were dominated by a single genetic group (Table S4[1]; Fig. 4). Portage Creek sockeye salmon (and to a lesser extent Gates Creek) showed signs of admixture with the black kokanee populations from Anderson and Seton lakes, but were largely composed of distinct genetic groups. The Chedakuz Creek and Davidson Creek kokanee populations (Tatlekuz Lake system) were characterized by high proportions of a genetic group distinct from that of the Anderson–Seton fish, but showed a small amount of admixture with one another. The Meadow Creek (Columbia River) kokanee also formed a distinct genetic cluster. When the Anderson and Seton lakes system sockeye salmon and kokanee were analyzed alone using all nine loci, a $K = 3$ was best supported, again grouping black kokanee together, although a $K = 4$ indicated some differentiation between black kokanee in Anderson and Seton lakes was also supported (Table S4; Figs. S1, S2[1]).

Fig. 3. Neighbor-joining tree constructed using Cavalli-Sforza and Edwards (1967) chord distance (D_C) inferred from variation at nine microsatellite loci in nine populations of *Oncorhynchus nerka*, sockeye salmon (S) and kokanee (K) from the Fraser River drainage (Gates Creek, Portage Creek, Anderson Lake, Seton Lake, Chedakuz Creek, Davidson Creek), Columbia River drainage (Meadow Creek), Bristol Bay, Alaska (Hansen Creek), and Lake Saiko (Japan). The black diamonds (◆) represent black kokanee. Numbers represent percentage of 5000 bootstrap replicates (percentages less than 50% have been removed).

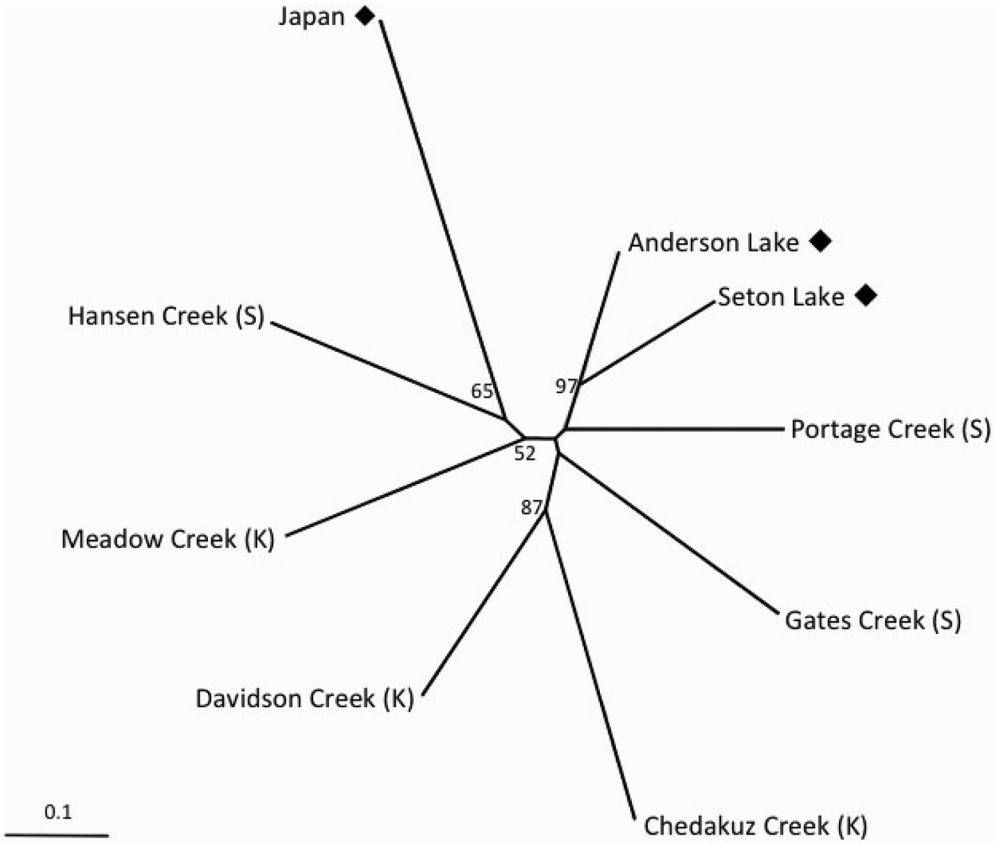


Table 2. Pairwise F_{ST} comparisons between Anderson–Seton lakes (Fraser River) black kokanee (◆) and sockeye salmon and Tatelkuz Lake (Fraser River) kokanee and Kootenay Lake (Columbia River) kokanee using nine microsatellite loci.

	DV	MC	AL◆	SL◆	GC	PC
CC	0.135	0.197	0.162	0.149	0.200	0.167
DV		0.100	0.061	0.067	0.123	0.054
MC			0.040	0.050	0.100	0.055
AL◆				0.012	0.070	0.019
SL◆					0.059	0.035
GC						0.083

Note: Chedakuz Creek (CC), Davidson Creek (DV), Meadow Creek (MC), Anderson Lake (AL), Seton Lake (SL), Gates Creek (GC), and Portage Creek (PC). All comparisons are significant ($P < 0.0137$).

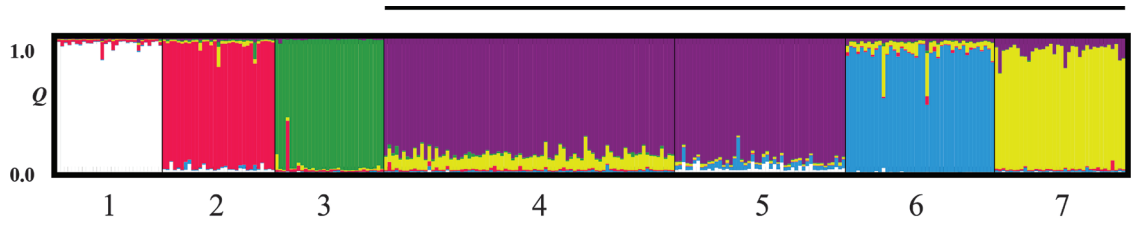
The DAPC analysis using all nine loci also demonstrated significant divergence among populations within Anderson–Seton lakes (Fig. S3[1]). The DAPC retained 95 principal components (PCs) representing 90% of the total variation in the data set across six discriminant functions (see Moreira 2014). Using only Anderson–Seton lakes samples, 68 PCs representing 85% of the total variation across three discriminant functions were retained, and this highlighted the distinctiveness of black kokanee from sympatric sockeye salmon (Fig. S3[1]). Consistent with the F_{ST} and STRUCTURE analysis, the Portage Creek sockeye salmon population was genetically more similar to black kokanee than were Gates Creek sockeye salmon. The relatively close clustering of Anderson Lake and Seton Lake black kokanee together within the DAPC plots was consistent with the genetic similarity between these two populations suggested by all other analyses.

When the variation in microsatellite allele frequencies was partitioned hierarchically using AMOVA and using "form" as an a priori grouping factor (black kokanee or sockeye salmon) in the Anderson–Seton lakes system, the majority of variation was found within populations (95.6%, $P < 0.001$). The among-population – within-form component of the total variation accounted for 4.1% of the total variation ($P < 0.001$). The between-form component of variation representing differences between black kokanee and sockeye salmon accounted for only 0.32% of the total variation, but was statistically significant ($P < 0.001$).

Morphological differentiation

On average, males were longer (standard length) than females in both lakes (all $P < 0.05$; Table 3). In Anderson Lake (both years combined), maximum body depth was positively correlated with standard length in females ($r = 0.826$, $P < 0.0001$) and males ($r = 0.571$, $P = 0.001$). In Seton Lake, maximum body depth was positively correlated with standard length in females ($r = 0.654$, $P = 0.0005$), but not in males ($r = 0.256$, $P = 0.625$). Results from the ANCOVA demonstrated that the slopes of maximum body depth versus length (using $\log_{10}$ values) were not significantly different between the lakes for males ($P = 0.748$) or females ($P = 0.387$). Therefore, the maximum body depth of each sample was standardized to a common body length of 242.3 mm for males and 191.3 mm for females. The adjustment coefficient was calculated using the pooled slope of the $\log_{10}$ maximum body depth versus

Fig. 4. STRUCTURE analysis for seven populations (*x* axis) of *Oncorhynchus nerka* (sockeye salmon and kokanee) assayed at nine microsatellite DNA loci: Chedakuz Creek kokanee (upper Fraser River, British Columbia), Davidson Creek kokanee (upper Fraser River, British Columbia), Meadow Creek kokanee (upper Columbia River, British Columbia), Anderson Lake black kokanee (middle Fraser River, British Columbia), Seton Lake black kokanee (middle Fraser River, British Columbia), Gates Creek sockeye salmon (middle Fraser River, British Columbia), and Portage Creek sockeye salmon (middle Fraser River, British Columbia). Each fish is represented by a thin vertical line, which represents the proportional composition (*Q*) of each fish's genome across six genetic groups (*K*), each represented by different colours. Populations below the thick horizontal line above the graph are all within the Anderson Lake – Seton Lake system.



$\log_{10}$ length regression line using all samples, but separated for males ($b = 1.200$) and females ($b = 1.084$) in tests for sexual dimorphism. Tests for differences between the sexes within each population (Seton Lake, Anderson Lake 2013, Anderson Lake 2014) in size-adjusted maximum body depth were all significant ($P < 0.05$); mean gill raker counts, however, did not differ between the sexes in either lake (both $P > 0.05$; Table 3).

Standard length was significantly different between sampling years for males and females in Anderson Lake (by about 1 cm, $P = 0.0004$ and 0.010, respectively), while size-adjusted maximum body depth was not significantly different between years for males ($P = 0.129$) or females ($P = 0.839$). Despite some differences in standard length between years within Anderson Lake, they were minor compared with differences between lakes, so the temporal samples were combined. Standard length was significantly different between Anderson Lake (combined years) and Seton Lake both for males and females ($P < 0.0001$), while size-adjusted maximum body depth was not ($P > 0.9$; Table 3).

Mean gill raker number was significantly different ($P < 0.006$) between the temporal samples from Anderson Lake. There was, however, no significant difference in mean gill raker number between Anderson Lake and Seton Lake before, or after, combining the two Anderson Lake sampling years ($P > 0.05$). The number of gill rakers ranged 35–42 in Seton Lake (mean = 38), 35–42 in Anderson Lake 2013 (mean = 37), and 37–42 in Anderson Lake 2014 (mean = 39; Table 3). The degree of colouration also varied between the two lakes. Anderson Lake fish exhibited a dramatic, uniform black colour at spawning, while Seton Lake fish were lighter black to olive in colour (e.g., Fig. 1; Moreira 2014).

Table 3. Summary of morphological variation between black kokanee (*Oncorhynchus nerka*) sampled from Anderson Lake in 2013 and 2014 and Seton Lake in 2013, separated by sex.

	Anderson Lake		Seton Lake
Trait	2013	2014	2013
Males			
N	15	15	6
SL	255.3 (1.95)*	265.4 (1.62)*	152.2 (1.15)†
AD	87.9 (1.41)	85.5 (0.63)	86.5 (1.67)
Females			
N	15	4	24
SL	243.8 (2.75)*	252.2 (1.00)*	148.4 (0.73)†
AD	62.4 (0.45)	62.7 (1.12)	62.5 (0.48)
GR	37 (0.32)*	39 (0.39)*	38 (0.51)

Note: All measurements are in millimetres (mean, SE). Sexually dimorphic traits include standard length (SL) and size-adjusted maximum body depth (AD, standardized to a mean standard length of 242.2 and 191.3 mm for males and females, respectively. Gill rakers (GR) were not sexually dimorphic and means are for males and females combined. The asterisk (*) represents a significant difference between sampling years ($P < 0.001$), and the "†" symbol represents significant a difference between the lakes ($P < 0.001$).

Discussion

O. nerka phylogeography

Genetic studies typically resolve two major lineages of *O. nerka* that are hypothesized to have resulted from differentiation and divergence in two glacial refugia, Beringia in the north and Cascadia in the south, after the end of the last glacial period (Varnavskaya et al. 1994; Taylor et al. 1996; Allendorf and Seeb 2000; Winans and Urawa 2000; Beacham et al. 2006). Further, within-refugium divergences are often reflected in the current patterns of diversity across the range of a species (Waples et al. 2008). Although interrelationships among some groups of mtDNA haplotypes were not fully resolved, our results provide some support for a modest phylogeographic break in *O. nerka* associated with the hypothesized divergence from at least two refugia during the most recent (Wisconsinan) glaciation: a northern (Beringian) refuge and a southern (Cascadian) refuge. This division was observed in the mtDNA tree (at 87% bootstrap) support for a small group consisting of Alaskan haplotypes that were distinct from other haplotypes in more southern localities in North America. Similar phylogeographic distinctions resulting from past historical events are found in other species of Pacific salmonids (e.g., *Oncorhynchus mykiss*: Taylor 1995; *Oncorhynchus gorbuscha*: Churikov and Gharrett 2002; *O. tshawytscha*: Waples et al. 2004) and a variety of other fishes (e.g., Bernatchez and Dodson 1990; Bernatchez and Wilson 1998) and are consistent with our general understanding of phylogeography of the Pacific Basin (e.g., McPhail and Lindsey 1970; Jacobs et al. 2004).

Diphyletic origin of black kokanee

The mitochondrial and microsatellite DNA data sets both suggest at least a diphyletic origin of black kokanee from two independent episodes of divergence on either side of the Pacific Ocean. This inference is supported by the observations that black kokanee in Japan and in the Anderson–Seton lakes system did not share any mtDNA haplotypes and they did not form a monophyletic grouping in either the mtDNA or microsatellite-based trees. Each black kokanee assemblage also possessed different sets of apparently unique microsatellite alleles, although more samples of Japanese black kokanee (i.e., a sample size at least equal to North American black kokanee) and perhaps samples of regular kokanee from Japan would make this latter inference stronger. A multiple origin model for black kokanee is consistent with the multiple lines of evidence of postglacial, polyphyletic origin of regular kokanee from sockeye salmon (Foote et al. 1989; Taylor et al. 1996, 1997; Wood and Foote 1996; Frazer and Russello 2013)

and of the role of parallel evolution in the origin of postglacial reproductively isolated forms within other species complexes in north temperate freshwater fishes (e.g., *Salmo trutta*: Hindar et al. 1991; *Osmerus mordax*: Taylor and Bentzen 1993; *Coregonus clupeaformis*: Pigeon et al. 1997; *Gasterosteus aculeatus*: McPhail 1984; Schluter 1996; and *Oncorhynchus tshawytscha*: Waples et al. 2004).

Alternatively, black kokanee populations could have had a single origin and once existed in many areas and dispersed to their present locations on different sides of the Pacific Ocean, with local extinctions explaining their current highly disjunct distributions. Indeed, our mtDNA data included one tree representing black kokanee monophly that we could not exclude. Yet, the shallow genetic divergence (e.g., mean 0.25% sequence divergence) between *O. nerka* haplotypes from the northwestern and northeastern Pacific Ocean (this study) suggests that the divergence is relatively recent (postglacial) and, therefore, cannot be explained by dispersal across Asian and North American sub-basins in fresh water. Further, the lack of any known geographically intermediate populations of black kokanee, despite the wide occurrence of kokanee in general, along with the apparent rarity of the black phenotype makes the single origin hypothesis very unlikely.

The black kokanee from Japan and from Anderson and Seton lakes exhibit similar phenotypic differentiation from regular kokanee in terms of colour and reproductive ecology. The distinctive reproductive habitats of black kokanee probably resulted from divergent selective regimes that promote parallel evolution of phenotypes as potential adaptations to their distinct reproductive ecology (e.g., Blair et al. 1993; Lemay and Russello 2015). Certainly, considerable evidence exists in other systems of potentially adaptive differentiation in reproductive behaviour in salmonids and other fishes (Pigeon et al. 1997; Taylor et al. 2000; Frazer and Russello 2013; Garcia de Leaniz et al. 2007; Kano et al. 2010; reviewed in Fraser et al. 2011). For instance, the deepwater and the winter–spring spawning behaviour of black kokanee both in Japan and North America may have promoted the evolution of their distinctive nuptial colouration. Light penetration decreases and red colour attenuates with increasing depth (Moss 1998), especially during the winter spawning period of black kokanee. Consequently, as a result of spawning at such great depths, the characteristic nuptial red colouration of *O. nerka* could be selected against. For example, Reimchen (1989) found that the loss of red nuptial colouration in male threespine sticklebacks (*Gasterosteus aculeatus*) and the resulting black colouration in several localities in western North America was associated with low water clarity in lakes with high tannin content. In addition, decreased visibility to predators could also result from colour loss at depth (Moodie 1972). Given that the mobilization of carotenoids from salmon flesh to the skin and eggs is energetically costly (Craig and Foote 2001; Martinkappi et al. 2009), selection may favour reallocation of energy associated with carotenoid deposition to other metabolic processes if red colouration, especially in the skin, is not advantageous.

Within-watershed divergences between black kokanee

The level of genetic differentiation between black kokanee populations in Anderson and Seton lakes was modest, but statistically significant, and comparable to the levels of differentiation found using neutral loci between beach and stream spawning kokanee in other systems (Taylor et al. 2000; Frazer and Russello 2013). These modest levels of neutral differentiation may result from a recent, postglacial time frame for divergence, recent and (or) historical gene flow between black kokanee populations, and (or) high effective population sizes. For instance, *O. nerka* colonized the Anderson and Seton lakes system only about 11 000 years ago, and adult population sizes were recently estimated at 5000 in Seton Lake and 150 000 in Anderson Lake (Wood 1995; A.R. Morris, BC Conservation Foundation, and A. Caverly, BC Ministry of Environment, Kamloops, BC, unpublished data).

The genetic differentiation between kokanee populations in Anderson Lake and Seton Lake could be the product of different spawning locations and times. The peak spawning time in Anderson Lake is the beginning to the middle of January, and in Seton Lake it is the beginning to the middle of November (A.R. Morris, BC Conservation Foundation, and A. Caverly, BC Ministry of Environment, Kamloops, BC, unpublished data). Temporal and spatial isolation are well-documented processes by which salmonid populations can diverge from one another (e.g., Hendry et al. 1995; Hendry and Day 2005). A large proportion of Anderson Lake black kokanee mature at ages 3 and 4 years, which contrasts with that in Seton Lake, which tend to mature at 2 years of age, with some maturing at 3 years of age, but even when fish are compared at a common age class of 3 years, Anderson Lake fish are larger (218.2 mm fork length in Anderson Lake compared with 198.1 mm fork length in Seton Lake; A.R. Morris, BC Conservation Foundation, and A. Caverly, BC Ministry of Environment, Kamloops, BC, unpublished data). Differences in standard length (and associated age of maturation) and colouration of adult black kokanee spawners between Anderson and Seton lakes could reflect adaptations to different spawning environments, phenotypic plasticity owing to differences in growth opportunity between the two lakes, or a combination of both (Blair et al. 1993; Northrup et al. 2010; reviewed by Taylor 1991 and Fraser et al. 2011). Certainly, the two lakes differ in a number of attributes. For instance, Seton Lake is turbid from the inflow of glacial water from the Bridge River and Carpenter Reservoir, but Anderson Lake is very clear (A.R. Morris, BC Conservation Foundation, and A. Caverly, BC Ministry of Environment, Kamloops, BC, unpublished data). Further, Anderson Lake has a higher plankton density than Seton Lake (2622 and 422 mg dry mass·m^{-2}, respectively) (K.S. Shortreed, K.F. Morton, K. Malange, J.M.B. Hume, Fisheries and Oceans Canada, Cultus Lake, BC, unpublished data).

Size-adjusted body depths and gill raker counts, however, did not differ significantly between Anderson Lake and Seton Lake black kokanee. The lack of differences in gill raker counts suggests that the feeding ecology (size and composition of prey items) is similar between the populations of black kokanee in the two lakes (cf. Taylor et al. 1997). By contrast, Kurenkov (1977) reported mean gill raker counts of "few-rakered" kokanee (mean gill rakers = 32) and "many-rakered" kokanee (mean gill rakers = 43) in Lake Kronotsky, Kamchatka, Russia, that were associated with dramatically different diets of the two morphs of kokanee; the "many-rakered" kokanee fed on plankton, while the "few-rakered" kokanee fed on macrobenthos (Kurenkov 1977). The lack of differences in size-adjusted body depth is, perhaps, to have been expected given that Anderson and Seton lakes black kokanee both are reported to spawn at depths of >20 m under what are likely similarly low light regimes. Differences in body depths of mature *O. nerka* are, however, typically associated with contrasting conditions experienced by fish spawning in shallow versus deepwater spawning habitats (e.g., Blair et al. 1993; Hendry and Quinn 1997).

Interestingly, the morphology of North American and Japanese black kokanee tended to be quite similar to one another. The standard length of Japanese mature black kokanee males and females ranged from 178.0 to 268.7 mm and 183.2 to 235.6 mm, respectively (Nakabo et al. 2014). These lengths are more comparable to Anderson Lake (about 245 to 265 mm) than to Seton Lake black kokanee (about 140 to 156 mm). Japanese black kokanee gill raker counts ranged from 36 to 45 (mean 39; Nakabo et al. 2014) compared with 34 to 42 gill rakers for Anderson and Seton lakes black kokanee. At maturity, colouration was described in both sexes as olive green to black when alive and becoming darker when dead (Nakabo et al. 2011, 2014). Despite, therefore, our evidence of their independent origins and great physical distance

from one another, the physical similarities between two groups of black kokanee imply that they share similar ecological attributes perhaps related to similarity in spawning and feeding habitats and behaviour.

Within-watershed divergences between black kokanee and sockeye salmon

We demonstrated significant microsatellite divergence between black kokanee and sockeye salmon from Anderson Lake and Seton Lake. Consistent differences were resolved using F_{ST}, Bayesian analysis of population structure, and in DAPC. Sympatric populations of kokanee and sockeye salmon are known to be genetically distinct from one another even when they spawn at the same time and in the same stream. For instance, Wood and Foote (1996) studied variation at 15 allozyme loci and found that 18% of variation was due to differences between kokanee and sockeye salmon spawning at the same place and time in Takla Lake compared with 0.5% variation within forms between tributaries (see also Foote et al. 1989).

The relatively high level of divergence between black kokanee from Anderson and Seton lakes and sockeye salmon from Portage Creek and Gates Creek was much higher than that between the two populations of black kokanee (F_{ST} = 0.012). Temporal and spatial segregation during spawning could account for the observed divergence between Anderson and Seton lakes' black kokanee and sockeye salmon ecotypes. For instance, spawning timing and location are both known to cause genetic differentiation between sympatric populations of *O. nerka* (Taylor et al. 2000; Fillatre et al. 2003; Lin et al. 2008; Frazer and Russello 2013; Muto et al. 2013) and other species within *Oncorhynchus* (McGregor et al. 1998; Hendry et al. 2002). In the Anderson and Seton lakes system, both sockeye salmon populations are stream spawners with different spawning times both from each other and from black kokanee. Portage Creek is a late run population with peak spawning occurring in September to November (Beacham et al. 2004). Gates Creek is an early summer run population, and peak spawning typically occurs in July to September (Beacham et al. 2004).

The high level of distinctiveness of black kokanee from sympatric sockeye salmon in the Anderson–Seton lakes system is consistent with recent genetic mixed stock fisheries analysis in the Fraser River basin (S. Latham, Pacific Salmon Commission, Vancouver, BC, personal communication). Here, the black kokanee samples (which do not occur in the baseline samples from the Fraser River) were more similar to sockeye salmon from **outside** the Anderson–Seton lakes system (e.g., early Stuart River, Harrison River) than to sockeye salmon within the Anderson–Seton system (S. Latham, Pacific Salmon Commission, Vancouver, BC, personal communication). The "mosaic" population structure of *O. nerka*, whereby neighboring lake populations are not always the most similar genetically to one another, could explain this pattern (Wood 1995; Withler et al. 2000; Beacham et al. 2004).

Interestingly and consistent across the F_{ST}, DAPC, and STRUCTURE analyses, black kokanee within both Anderson and Seton lakes tended to be more similar to the sockeye salmon from Portage Creek rather than to the sockeye salmon from Gates Creek. The higher level of admixture between the black kokanee populations and Portage Creek sockeye salmon may reflect some level of past or current gene flow between the populations. Further, differentiation between sympatric black kokanee and stream spawning regular kokanee in Lake Saiko, Japan (F_{ST} = 0.134) and between black kokanee and stream spawning regular kokanee in Lake Akan (F_{ST} = 0.142) was much higher than observed in the Anderson–Seton lakes black kokanee and sockeye salmon (Nakabo et al. 2011).

Implications for conservation

Our evidence that black kokanee in Lake Saiko and in the Anderson–Seton lakes system have evolved at least twice across the North Pacific Ocean suggests the importance of habitat variability and parallel evolution to the origin of biodiversity in *O. nerka* and freshwater fishes more generally. This implies that the maintenance of habitat variability (e.g., provision both of shallow and deepwater spawning habitats) is critical to the origin and maintenance of such biodiversity. Further, black kokanee in Anderson Lake and Seton Lake were found to be genetically distinct from sympatric sockeye salmon, from *O. nerka* in other areas, and, to some extent, from each other. By contrast, the genetic differences observed between the Anderson Lake and Seton Lake black kokanee populations are relatively modest compared with the striking differences in age and size at maturity. Such differences suggest that the two populations of North American black kokanee are, to some extent at least and despite their spatial interconnectedness, demographically independent from sympatric sockeye salmon and from each other. Consequently, to promote the long-term persistence of the current biodiversity of *O. nerka* within the Anderson–Seton system, these distinctions need to be recognized and used to design separate management regulations and protocols in terms of, for instance, harvest levels and habitat protection (cf. Hilborn et al. 2003; Palsbøll et al. 2007). Overall, black kokanee exemplify novel diversity within a taxon that is already renowned for "biocomplexity", variability that has been demonstrated to be important to the persistence of *O. nerka* across diverse and fluctuating environments (e.g., Ricker 1972; Hendry 2001; Hilborn et al. 2003).

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