# The evolutionary origin of "black" kokanee (*Oncorhynchus nerka*) and their genetic and phenotypic diversity in the Anderson and Seton lakes system

by

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B.Sc., The University of British Columbia, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2014

## Abstract

In order to conserve intraspecific biodiversity, it is crucial to identify genetic and phenotypic variation among populations and to understand the evolutionary processes that drive the evolution and persistence of such variation. *Oncorhynchus nerka* exists as two basic life history forms: the anadromous form, sockeye salmon and the non-anadromous form, kokanee. Unique populations of "black" kokanee are found in Lake Saiko, Japan, and in Anderson Lake and Seton Lake in the southwestern interior of British Columbia. They are distinct from other populations of *O. nerka* in that "black" kokanee spawn between 20 to 70 meters below the surface of each lake and spawn in the winter or early spring, whereas *O. nerka* typically spawns in the autumn in streams or shallow lake beaches. Using mitochondrial DNA and nine microsatellite loci, I investigated the evolutionary origin of eastern and western North Pacific populations of "black" kokanee. Both data sets support the hypothesis that "black" kokanee in Anderson Lake, Seton Lake and Lake Saiko have a polyphyletic origin resulting from repeated episodes of parallel evolution. Further, using variation at nine microsatellite loci, I demonstrated that "black" kokanee in the Anderson and Seton lakes system are genetically distinct from sympatric populations of sockeye salmon in Gates and Portage creeks, and that Anderson and Seton lake "black" kokanee are modestly distinct from one another. Anderson and Seton lake "black" kokanee differ 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. My results provide an example of parallel evolution in north temperate freshwater fishes as well as evidence of yet another facet of distinctive biodiversity within *O. nerka* that needs to be recognized and accounted for in conservation planning for the taxon.

## Preface

I was responsible for data collection, analysis, and interpretation. This project was conducted under supervision of Eric Taylor who contributed to data collection, analysis, and interpretation. Tissue samples were collected with the help of Gene Tisdale (Tisdale Environmental Consulting Inc.), William Alexander and William Terry (residents of Seton Portage). Tissue samples were also provided by Matthew Casselman (UBC Forest and Conservation Sciences), Steve Latham (Pacific Salmon Commission), David Levy (Levy Research Services Ltd), Shannan May-McNally (UBC Zoology), and Tetsuji Nakabo (Kyoto University). Additional DNA samples were from the Beaty Biodiversity Museum's fish DNA archive provided by Eric Taylor (UBC Zoology). Eric Taylor developed the genetic protocols that I used in the project.

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## Acknowledgements

Firstly, I would like to thank my supervisor, Eric (Rick) Taylor, for taking me as a student/getting me out of being a barista for life and keeping me as a student even when I thought I could not do it. Thank you for taking a chance on me and giving me this amazing opportunity. I would like to thank David Levy for introducing Rick to this project and answering all my questions. You were an invaluable resource. Also, thank you to my committee members, Jordan Rosenfeld and Colin Brauner for your contributions and suggestions. Jessica Mackenzie, I cannot thank you enough for the support and experiences that you have given me. You are an amazing person to aspire to be.

Thank you to my fellow lab mates: Monica Yau, Shannan May-McNally, and Jenn Ruskey for your assistance in the lab. Thank you for all the laughs! I wish you all the best to wherever you go from the Taylor Lab. I would also like to thank Rebecca Piercey for all the help she gave me and for listening to me complain about everything. Thank you for driving in a blizzard with no winter tires to Lillooet.

Many people and organizations helped me collect samples and I am grateful to all of them: Matthew Casselman (UBC Forest and Conservation Sciences), Gene Tisdale and colleagues (Tisdale Environmental Consulting), Steve Latham (Pacific Salmon Commission). I honestly believe that William Alexander and his brother William Terry are the nicest people I have ever met. They helped collect Anderson Lake Gwenish and introduced me to the cultural significance of Gwenish and salmon in general.

My office mates have created an amazing support system. Thank you, Kaylee Byers, for being hilarious and listening to all my "problems." You are literally the funniest person I know.

Thank you, Kim Gilbert, for sending me cat pictures and introducing me to other grad students. Thank you, Seth Rudman, for all the stories of the ridiculous things that have happened to you. I could not have asked for three better people to share a tiny office with. Thank you to honorary office mate, Alison Porter, for always distracting me with the long/off topic conversations we have.

I do not think I would have stayed sane without: Nick Adamson, Karen Cheung, Bob Foerster, Michael Hulland, Pearly Li, Jeremy Orsted and Danielle Scott. Thank you all for keeping me laughing. Also, I am grateful to the staff at Bean Around the World for providing me with free coffee and being a refuge from grad school. And thank you to the customers for all your words of encouragement. I am so eternally grateful for Brittney Soobotin and Krista Soobotin. You two are truly amazing. There are no words to describe how supportive you have been.

I do not think I could have asked for a more supportive and helpful family. Thank you Mom and Dad for everything you have done to support me and for taking us every year to see spawning kokanee. Thank you to my siblings: Carmen, Bryce, and Darrin. This was a group thesis and I do not think I would have finished it without the help that each of your provided. I would also like to thank the most important members of the Moreira family: Sampson, Merry, Pippin and the late Salem.

## Dedication

For Carmen, Bryce, and Darrin. Without you I would have no grammar, no computer and no Gwenish. Thank you for the unconditional support.

# Chapter 1: Introduction

My thesis explores the evolutionary origin of "black" kokanee and evaluates the genetic and phenotypic variation that they represent within the Anderson and Seton lakes system. This introductory chapter reviews the importance of conserving biodiversity specifically focusing on fishes, reviews the diversity found within *Oncorhynchus nerka*, and introduces unique populations of *O. nerka*. This chapter ends with my specific research questions, hypotheses and predictions.

## 1.1 Biodiversity and conservation

A major goal of conservation biology is to preserve phenotypic and genetic diversity, as well as the evolutionary processes and adaptive landscapes that produce such variability, and this goal has increasingly been applied to the conservation of intraspecific diversity (Meffe and Carroll 1994; Moritz 2002; Allendorf and Luikhart 2007). Conservation of intraspecific diversity contributes to the persistence of species over a range of habitats and through changing environments (Luck et al. 2003; Duffy 2009; Bolnick et al. 2011). Intraspecific diversity, however, is often overlooked by the general public and by managers because they are focussed on ecosystem and species conservation (Hughes et al. 1997).

Defining intraspecific units of conservation can be conceptually and practically difficult (Moritz 1994; Crandall et al. 2000; Wood and Gross 2008), but there have been various approaches proposed (e.g., Allendorf et al. 1997; Taylor et al. 2011, 2013). Widely distributed species that possess a wealth of diversity are especially problematic because first one needs to gain a better understanding of their current ecological roles and evolutionary diversity before

focusing on the potential biological consequences of their loss (e.g., Taylor et al. 2013). This is especially concerning if such populations are unknowingly declining. Therefore, to conserve intraspecific diversity effectively, we must first document it and secondly understand the processes that have produced and maintained that diversity in a changing environment (Moritz 2002; Pressey et al. 2007). With that said, fishes are a model group to study all levels of biodiversity and conservation.

### 1.2 Diversity of fishes

Fishes make up more than half of all extant vertebrate species and exhibit considerable morphological, ecological, and behavioural diversity at all taxonomic levels (Volff 2005; Helfman et al. 2009). At the intraspecific level, extensive and taxonomically unrecognized diversity occurs in temperate freshwater species (reviewed by Behnke 1972; Taylor 1999). Evidence of such diversity can be seen, for example, in the threespine stickleback (*Gasterosteus aculeatus*, McPhail 1984; Schluter 1996), European whitefish (*Coregonus lavaretus*, Sendek 2004), pygmy whitefish (*Prosopium coulterii*, Gowell et al. 2012), lake whitefish (*Coregonus clupeaformis*, Bernatchez and Dodson 1990), rainbow/steelhead trout (*Oncorhynchus mykiss*, Keeley et al. 2005, 2007), brook char (*Salvelinus fontinalis*, Dynes et al. 1999), brown trout (*Salmo trutta*, Ferguson 1989) and sockeye salmon/kokanee (*Oncorhynchus nerka*, Taylor 1999).

The majority of intraspecific diversity is maintained by some degree of spatial or temporal reproductive isolation that is further reinforced by selection for additional ecological differentiation (e.g. growth rates, age of maturity, habitat and/or food preference) (Behnke 1972). Differences in trophic niches that promote differentiation in feeding ecology (threespine stickleback, lake whitefish, brown trout, references above) are common features promoting

intraspecific divergence (Schulter 1996; Taylor 1999). Salmonids (salmon, trout, char and their relatives), however, exhibit extensive variability in reproductive biology that is expressed as intraspecific diversity in size and time of maturation (e.g., seasonal "races" such as spring and fall Chinook salmon (*Oncorhynchus tschawytsha*, "winter" and "summer" steelhead trout, reviewed in Groot and Margolis 1991; Quinn 2005). Another striking example of divergence in reproductive ecology is the presence of alternative life-history types of *O. nerka*: sockeye salmon and kokanee, as described below.

### *1.3 Oncorhynchus nerka*

Intraspecific diversity, particularly in reproductive ecology and life history, is exceptionally notable in *Oncorhynchus nerka*, which includes two main forms: sockeye salmon, the anadromous (or sea-going) form, and kokanee, the nonanadromous (or freshwater-resident) form (Nelson 1968; Burgner 1991). Both forms are indigenous to tributaries in the North Pacific, ranging from southern Kamchatka and Japan in the western Pacific to the Columbia River in the eastern Pacific (Burgner 1991, Fig. 1.1).

It is generally accepted that contemporary kokanee populations evolved from sockeye salmon multiple times independently in different locations throughout its range in the last 15,000 years (Ricker 1940; Nelson 1968; Foote et al. 1989). This is supported by the observations that: (i) the geographic distribution of kokanee is almost wholly contained within that of sockeye salmon, (ii) sockeye salmon can produce kokanee when introduced to new systems (and *vice versa*), and (iii) kokanee are polyphyletic as inferred from phylogeographic analysis (Ricker 1940, 1959; Nelson 1968; Taylor et al. 1996).

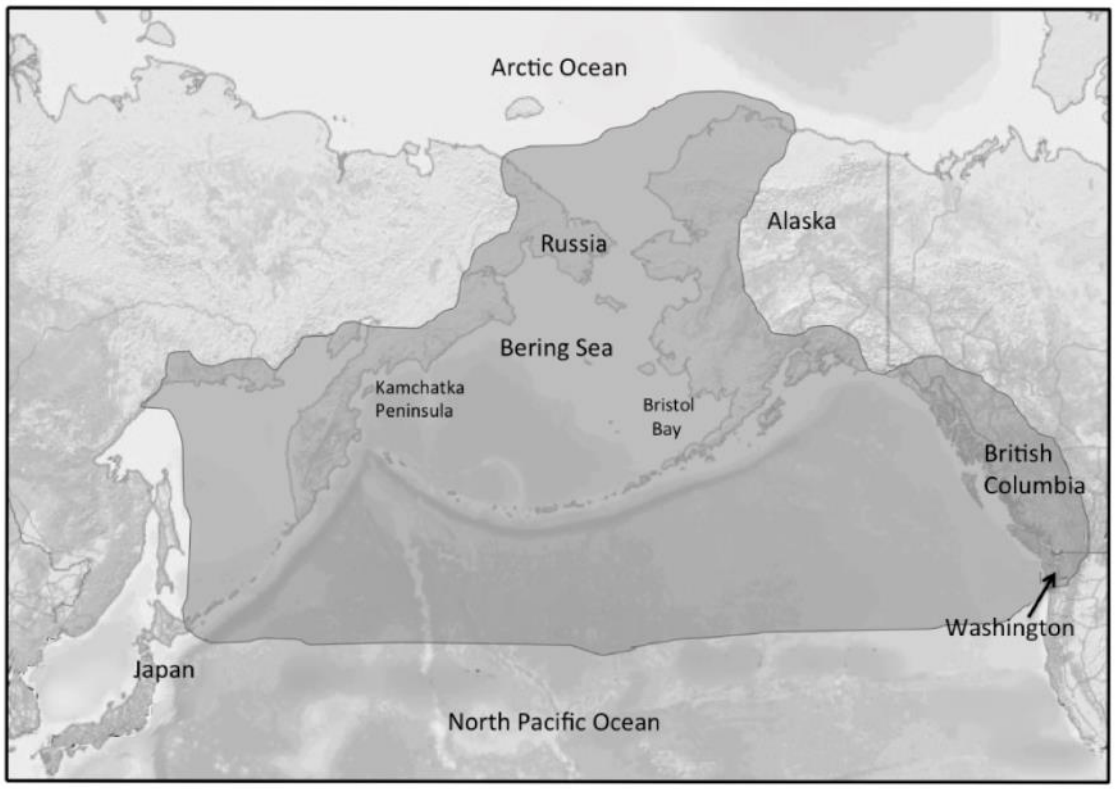


Figure 1.1. Map highlighting the broad natural distribution of *Oncorhynchus nerka* (sockeye salmon and kokanee) ranging from northern Japan to Washington State (map modified from Steinback and Fuller 2005).

Sockeye salmon and kokanee have considerably different life histories. Both sockeye salmon and kokanee adults spawn in streams tributary to lakes or on submerged lakeshore beaches and the young of both forms migrate to a nursery lake. Sockeye salmon then spend one to three years feeding in the lake before migrating to the ocean where they spend another one to three years growing and maturing (Burgner 1991; Wood 1995). From the ocean, they return to freshwater to spawn and die. In contrast, kokanee mature and spawn over two to four years entirely in freshwater (Ricker 1940).

Migratory life history differences between sockeye salmon and kokanee are accompanied by divergence in numerous traits (Nelson 1968). For instance, the two forms differ genetically in

growth and developmental rates (Wood and Foote 1990; Foote et al. 1994), salt-water adaptability (Foote et al. 1992), morphological and meristic traits (Wood and Foote 1996; Foote et al. 1999), and sustained swimming performance (Taylor and Foote 1991). Most of these traits may be a result of selection stemming from the differential physiological demands of anadromy and differences in trophic and predation ecology in the sea and in lakes (e.g., Taylor and Foote 1991). Owing to differences in marine versus freshwater productivity and the demands of migration, sockeye salmon are typically much larger at maturation than kokanee (approximately 40-70 cm versus 20-30 cm, respectively). Assortative mating by size between the forms promotes further divergence and reproductive isolation when the forms are sympatric (Foote and Larkin 1988; Taylor 1999). Sockeye salmon and kokanee do, however, typically exhibit similar colouration: both turn from a countershaded silver on the sides and black/grey on the back to bright red with an olive green head and blackish-red fins when they reach maturity (Craig and Foote 2001; Fig. 1.2).

Molecular analyses based on allozyme allele frequency variation and minisatellite and mitochondrial DNA variation as well as in quantitative traits have shown that sockeye salmon and kokanee are genetically distinct in allopatry as well as when they are sympatric (Foote et al. 1989; Wood and Foote 1990; Taylor et al. 1996; Wood and Foote 1996). Specifically, sockeye salmon and kokanee form distinct gene pools in sympatry (spatially and temporally) in many British Columbia lakes (e.g., Takla, Shuswap and Babine lakes) with the divergent forms found in the same lake often more closely related to each other than they are to the comparable form in different lakes (Taylor et al. 1996; Wood and Foote 1996). Fully viable and fertile hybrids between sockeye salmon and kokanee can, however, be formed experimentally as well as in nature (Wood and Foote 1996). Despite such gene flow in nature, the forms remain genetically

distinct likely as a result of selection against hybrids associated with their very different life histories (Wood and Foote 1990; Wood and Foote 1996).

Figure 1.2. Mature sockeye salmon (top two fish) and kokanee (bottom four fish) sampled from a tributary of Takla Lake, British Columbia. In both the sockeye salmon and kokanee, females are at the top and males are at the bottom (C. Foote, Vancouver Island University). The white scale marker is approximately 1 m in length.

### 1.4 "Black" kokanee: Gwenish

In addition to the fascinating divergence within *O. nerka* represented by sockeye salmon and kokanee, there is also considerable variation within both of these forms. One particularly remarkable and understudied example of such diversity is found within Anderson Lake and Seton Lake in the Bridge/Seton River system of the Fraser River drainage in the southwestern interior of British Columbia (Fig. 1.3). A unique form of kokanee, locally called "Gwenish," is found in these lakes. Gwenish are black at maturity (Fig. 1.4, 1.5) and have spawning behaviour that is distinct within *Oncorhynchus*. Gwenish spawn late in the season, typically starting in

November in Seton Lake and in January in Anderson Lake (Levy 2012). In Anderson Lake, Gwenish are on average three to four years old at maturity, whereas those in Seton Lake are two to three years old at maturity (Morris and Caverly 2004). Additionally, Anderson Lake Gwenish are larger than Seton Lake Gwenish even when fish of the same age class are compared. In both lakes Gwenish spawn between 20 to 70 meters below the surface of the lake (typically greater than 50 meters) (Morris and Caverly 2004; Levy 2012). After spawning, Gwenish float to the lake's surface due to their distended swim bladders (Morris et al. 2003; Levy 2012). Their distended swim bladders give them a deep body profile that is distinct from "regular" kokanee populations (Levy 2012). It is hypothesized that Gwenish seek out deep water sites for spawning owing to the presence of upwelling of relatively warm, oxygenated water at these areas (Morris and Caverly 2004).

In the Anderson and Seton lakes system there are also spawning populations of sockeye salmon in Gates Creek (spawning in July to September) and Portage Creek (spawning in September to November) (Komori 1997). Sockeye salmon have also been observed (in smaller numbers) in the Seton River, which connects Seton Lake to the Fraser River, but they are believed to be occasional strays that enter the river during periods of adverse migratory conditions (Levy 2012). While sockeye salmon and Gwenish are broadly sympatric in both lakes, the sockeye salmon juveniles feed almost exclusively in Seton Lake where the two forms likely compete for trophic resources (Shortreed et al. 2001).

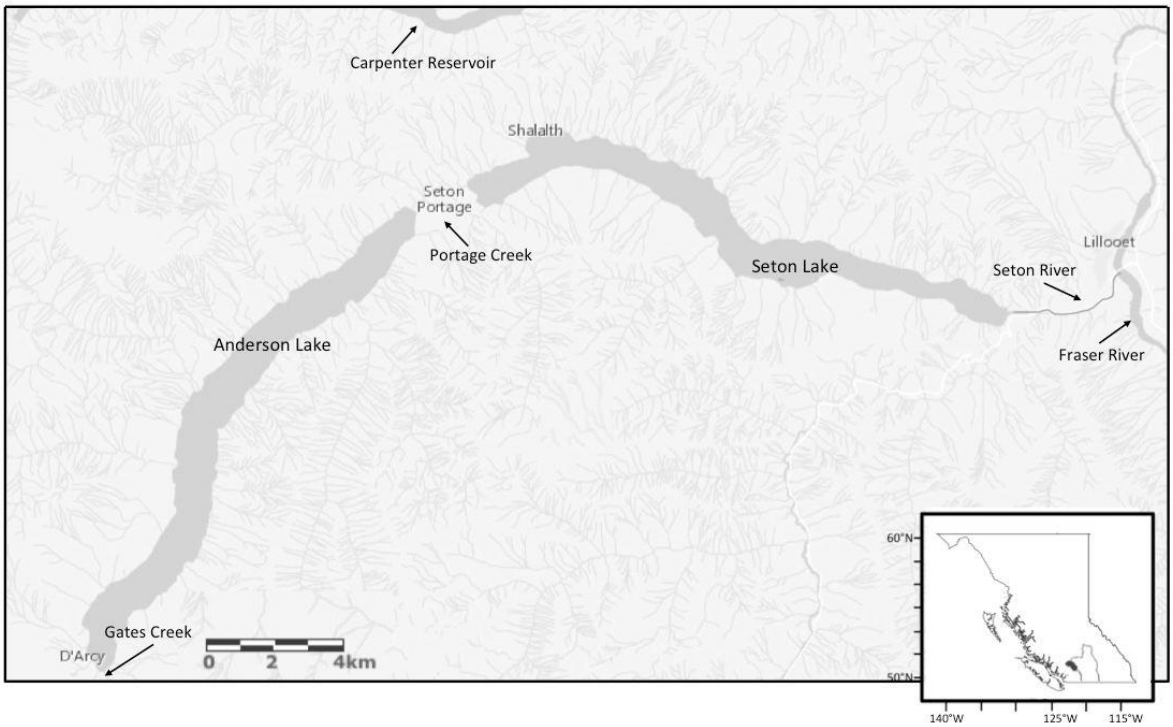


Figure 1.3. Anderson Lake (50.63°N, 122.38°W) and Seton Lake (50.70°N, 122.15°W) in the Bridge/Seton system of BC's southwestern interior. Seton Lake is 24.3 $km^2$ with an average depth of 85 meters. Anderson Lake is 28.3 $km^2$ with an average depth of 140 meters.

Figure 1.4. Mature Gwenish (*Oncorhynchus nerka*) collected from Seton Lake (male above, female below) Lillooet, British Columbia in November 2013 during peak spawning season. Photo by: Amanda Moreira.

Figure 1.5. Mature Gwenish (*Oncorhynchus nerka*) collected from Anderson Lake (female above, male below) Lillooet, British Columbia in January 2014 during peak spawning season. Photo by: Amanda Moreira.

## 1.5 "Black" kokanee: Kunimasu

Remarkably, 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 (near Tokyo) (Fig. 1.6). "Black" kokanee, or "kunimasu", in Lake Saiko, were transplanted from their native Lake Tazawa, in northern Japan, after hydroelectrical development in 1935 increased Lake Tazawa's acidity (Nakabo et al. 2011). It was believed that the introduced kunimasu had gone extinct in Lake Saiko until their rediscovery in 2010 (Nakabo et al. 2011). Eyed eggs were also transplanted into several other lakes, but there is no evidence that these transplants were successful. In Japan, native "regular" kokanee from Lake Akan, on the island of Hokkaido, were

introduced into both Lake Saiko and Lake Tazawa. Consistent with most North American kokanee, these populations spawn in streams in the fall (Nakabo et al. 2011).

In addition to being black at maturity, like Gwenish, kunimasu spawn at average depths of 30-40 meters in lakes from March to April (Nakabo et al. 2011). In Lake Tazawa, kunimasu were reported to spawn at depths of 15 to 300 meters in February, and they were also observed spawning year round. It is hypothesized that kunimasu are adapted to the cool water temperatures at these depths (between 3.9 to 5 °C; Nakabo et al. 2011). There have been no reports of floating or distended swim bladders in post-spawning kunimasu, a trait that is observed in Gwenish.

In Japan, "black" kokanee are recognized as a separate species, *Oncorhynchus kawamurae*, based primarily on differences from sympatric "regular" kokanee in gill rakers (36-45 versus 31-35 in "regular" kokanee), pyloric caeca (46-81 versus 55-82), anal fin rays (14-16 versus 13-15), and pectoral fin rays (15-17 versus 15-18) (Nakabo et al. 2014). Genetic divergence from sympatric "regular" kokanee at five microsatellite loci was also reported (Nakabo et al. 2011; Muto et al. 2012). Hybridization between the two types of kokanee was found to be rare, but there is evidence of past introgression although it is not known if such introgression occurred before or after both were introduced into Lake Saiko (Muto et al. 2012).

The similarities between Gwenish and kunimasu, the diversity they represent, and their relatively unknown biology raise several intriguing questions relevant to the evolutionary origin and the potential genetic and morphological distinctiveness within and between these forms of *Oncorhynchus nerka*.


Figure 1.6. Male kunimasu (*Oncorhynchus kawamurae*) collected off Nishin-okoshi, Lake Saiko, Yamanashi, Japan at 40 meters depth (photo courtesy of T. Nakabo).

## 1.6 Research questions

My thesis encompasses two areas of investigation to test hypothesis about the distinctiveness (patterns) and evolutionary origin (process) of "black" kokanee and their relevance to the conservation of this form of *O. nerka*. Concordance among a number of traits, including genotypic and phenotypic variation, can provide strong evidence for distinctiveness within a species (e.g., Rising and Avise 1993; O'Donnell et al. 2004; Taylor et al. 2011). Therefore, I used mitochondrial DNA, nuclear DNA and morphological data sets to assess the distinctiveness and origin of "black" kokanee. Specifically, my research questions were:

1. What is the degree of phylogeographic structure and evolutionary origin of "black" kokanee?

2. Are "black" kokanee in the Anderson and Seton lakes system genetically distinct from each other and from sympatric sockeye salmon within the *Oncorhynchus nerka* complex, and are these two populations of "black" kokanee morphologically divergent from each other?

### 1.7 Hypothesis and predictions

For question 1, I tested the hypothesis that "black" kokanee evolved postglacially at least twice, once in the eastern North Pacific and once in the western North Pacific. Multiple, independent divergences between forms within single taxa are common in freshwater fishes (Ryman and Stahl 1981; McPhail 1984; Hindar et al. 1991; Taylor 1999) including *O. nerka* (Foote el al. 1989; Taylor et al. 1996; Frazer and Russello 2013).

This hypothesis led me to predict that "black" kokanee would form at least two distinct phylogenetic groups: one native to the western Pacific and one native to the eastern Pacific. The "black" kokanee in Anderson Lake and Seton Lake most likely diverged from sockeye salmon populations spawning in Gates Creek or Portage Creek in the Anderson/Seton watershed. Within-lake divergences have been reported in other sockeye salmon/kokanee systems (e.g., Wood and Foote 1996; Taylor et al. 1996; Frazer and Russello 2013).

For question 2, I tested the hypothesis that "black" kokanee are distinct within *O. nerka* not only in colouration and reproductive ecology, but also genetically and morphologically. Across their geographic range, sockeye salmon demonstrate considerable genetic variation and population structure (e.g., Beacham et al. 2006). Kokanee also exhibit substantial genetic differentiation across their range (Wood and Foote 1996) even within lake systems (Taylor et al. 1997; Taylor 2013; Frazer and Russello 2013). Based on previous *O. nerka* population genetic

studies and the phenotypic differences between forms, I predicted that "black" kokanee would be genetically distinct from other populations of kokanee and from sympatric sockeye salmon populations. Additionally, due to differences in spawning timing and location as well as sizes at maturity between "black" kokanee in Anderson and Seton lakes, I predicted that there would be genetic heterogeneity within and between the lakes that will be observable as local population structure.

Morphological variability (standard length, depth and gill raker counts) was also used as a measure of distinctiveness between the "black" kokanee within Anderson Lake and Seton Lake. Morphological variation may reflect local adaptation arising from divergent selection associated with differences in spawning environments, from phenotypic plasticity, or some combination thereof. Previous studies based on morphology in kokanee have shown that meristic traits such as gill rakers and vertebral counts are variable within and among lakes (e.g., Vernon 1957; Nelson 1968).

The description and identification of genetic population structure, biodiversity, and taxonomic relationships in a constantly changing environment are necessary pieces of information to better understand "black" kokanee. Considering only sockeye salmon and kokanee there is already significant diversification within *O. nerka*, and "black" kokanee represent an additional, but understudied, aspect of such biodiversity.

# Chapter 2: The evolutionary origin of "black" kokanee (*Oncorhynchus nerka*)

## 2.1 Introduction

Understanding the patterns and origins of intraspecific diversity is important for conserving as many genetic "building blocks" of individual species as possible (Waples 1995). For populations with distinct phenotypes, a key first step to better understand such diversity is to elucidate its evolutionary origin, especially if similar traits are found in more than one population.

The multiple, independent divergence of similar phenotypic traits is widely recognized as a general phenomenon within species of north temperate freshwater fishes (Waples et al. 2004). For example: various life-history types, ecotypes, and "morphs" have arisen independently in different geographic areas in brown trout (*Salmo trutta*, Hindar et al. 1991), rainbow smelt (*Osmerus mordax,* Taylor and Bentzen 1993), lake whitefish (*Coregonus clupeaformis,* Pigeon et al. 1997), threespine stickleback (*Gasterosteus aculeatus*, McPhail 1984; Schluter 1996), Chinook salmon (*Oncorhynchus tshawytscha,* Waples et al. 2004) and sockeye salmon/kokanee (*Oncorhynchus nerka,* Taylor et al. 1996). Parallel divergences within species are most likely the result of repeated opportunities for geographic isolation from Pleistocene glaciations, widespread dispersal opportunities, and filling of vacant niches in postglacial environments (Rice 1984; Taylor et al. 1996; Schluter 2000).

The taxon *Oncorhynchus nerka* (sockeye salmon and kokanee) represents a fascinating system to study intraspecific diversity, in particular life history and reproductive system variation, and the evolutionary processes that have shaped this diversity in nature. It is generally accepted that extant populations of kokanee are the result of multiple, independent, post-glacial divergences from sockeye salmon throughout the range of *O. nerka* (Ricker 1940; Taylor et al.

1996). Evidence to support this scenario is supported by patterns of genetic similarity and allele sharing between the two forms (Foote el al. 1989; Taylor et al. 1996). The polyphyletic nature of kokanee is in agreement with the observation that the distribution of kokanee is generally contained within that of sockeye salmon (Ricker 1940). Further, kokanee and sockeye salmon populations have been found in lakes previously devoid of *O. nerka* after the introduction of either form suggesting that the forms can give rise to one another (Ricker 1940; Scott 1984; Kaeriyama et al. 1992). Island populations of kokanee (Japan, Vancouver Island and Alaskan islands) have likely resulted from divergence from sockeye salmon colonization after deglaciation (Taylor et al. 1996).

Evidence of parallel evolution of life history characteristics is not restricted to kokanee/sockeye salmon divergences within the *O. nerka* complex. For example, Wood et al. (2008) hypothesized that "lake-type" sockeye salmon (i.e., those where juveniles reside and feed in lakes for at least one year before seaward migration) have evolved multiple, independent times from geographically proximate "sea/river-type" sockeye salmon (i.e., juveniles reside in spawning streams or migrate to sea as age 0 fish) an idea that the authors supported with mitochondrial DNA data. In addition, "beach" and "stream" spawning ecotypes of kokanee appear to have evolved from independent colonization of beach habitats by stream spawning kokanee in five British Columbia lakes (Duncan, Kootenay, Okanagan, Wood, and Tchesinkut lakes) in less than 10,000 years (Taylor et al. 2000; Frazer and Russello 2013). An understudied aspect of diversity within *O. nerka* is the existence of so-called "black" kokanee. "Black" kokanee are found in Lake Saiko, Japan and in Anderson Lake and Seton Lake in the southwestern interior of British Columbia, Canada. The populations on either side of the Pacific Ocean share similar unique spawning behaviour and colouration: deep lake spawning, black

colouration, and late spawning time from November to February in BC (Levy 2012) and March to April in Japan (Nakabo et al. 2011). These unusual features set them apart from sockeye salmon and kokanee which typically spawn in shallow water areas of streams or lake beaches, possess red body colouration and green heads at spawning, and spawn in the late summer to autumn (Burgner 1991).

In this chapter, I tested the hypothesis that "black" kokanee evolved at least twice: once in the eastern North Pacific and once in the western North Pacific, post-glacially. In doing so, I tested alternative hypotheses, single versus multiple origins, concerning the unknown origin of these unique "black" kokanee populations on either side of the Pacific Ocean. If the multiple origins hypothesis is supported then this suggests that selection has played a prominent role in the evolution of "black" kokanee, as it is unlikely that random genetic drift has caused the evolution of similar traits in two geographically distant locations especially given the evidence for parallel evolution in other traits in *O. nerka* and north temperate fishes more generally (Schluter 1996, 2000; Taylor 1999).

To test this hypothesis, I used mitochondrial DNA sequence variation and microsatellite DNA allele frequency variation. Mitochondrial DNA has a maternally, non-recombining mode of inheritance and typically exhibits extensive intraspecific polymorphism (Avise et al. 1987; Thomas et al. 1986). Mitochondrial DNA has been informative in understanding the evolutionary and phylogeographic relationships within species in a variety of taxa (Avise et al. 1987; Bernatchez and Wilson 1998; Avise 2000). In comparison, microsatellites are highly polymorphic, typically neutral, tandem repeat loci and are biparently inherited (Jarne and Lagoda 1996). These features of microsatellites and their high mutation rate have proven useful to understand the genetic relationships below the species level as well as to infer evolutionary

origins among populations (Lehmann et al. 1996; Abdul-Muneer 2014). The use of two independent data sets provides a powerful test to study the evolutionary processes and divergence patterns among "black" kokanee, and *O. nerka* in general.

The independent origin of "black" kokanee populations would reinforce the importance of parallel evolution as a central process of evolutionary change in the history of north temperate fishes while highlighting further diversity found within *Oncorhynchus nerka*.

## 2.2 Methods

### *2.2.1 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 British Columbia (BC, see Fig. 1.3). Anderson Lake has a total surface area of 28.3 $km^2$ and average depth of 140 m. It is fed by Gates Creek and empties into Seton Lake, which is also fed from water diverted from the Bridge River into Carpenter Reservoir. Seton Lake has a surface area of 24.3 $km^2$ and an average depth of 85 m and it empties into the Fraser River at the town of Lillooet. There are two BC Hydro facilities in operation within the watershed: Seton River power dam and Shalath power generation facility. Rail tracks run along the length of both lakes on the north side (Levy 2012).

Samples of *O. nerka* were obtained from a variety of sources across the North Pacific (Tables 2.1, 2.2). "Black" kokanee (post-spawners) samples were collected from the surface of Seton Lake using dipnets on November 20, 2013 with the help of Gene Tisdale (Tisdale Environmental Consulting Inc.). Adipose or pelvic fin samples were taken at the University of British Columbia (UBC) and stored in 95% ethanol for DNA analysis. Anderson Lake samples were collected in January 2013 by David Levy (Levy Research Services Ltd.) and I collected

samples on January 16, 2014. Samples were collected by surveying the beach for post spawning adults and adipose fin samples were taken on site at Anderson Lake and stored in 95% ethanol for DNA analysis.

Japanese "black" kokanee muscle tissue was graciously provided by Tetsuji Nakabo (Kyoto University). Portage Creek sockeye salmon fin tissue samples were provided by Matthew Casselman (UBC Forest and Conservation Sciences). Gates Creek sockeye salmon fin tissue samples were provided by Steve Latham (Pacific Salmon Commission, Vancouver). Hansen Creek, Lake Aleknagik, Alaska, sockeye salmon fin tissue samples were provided by Shannan May-McNally (UBC Zoology). Additional DNA samples from across *O. nerka*'s range were obtained from the Beaty Biodiversity Museum's fish DNA archive (Eric Taylor, pers. comm.) for the analysis of phylogeographic structure (see Table 2.1).

### *2.2.2 Mitochondrial DNA*

Qiagen spin columns were used to extract DNA using the manufacturer's protocol. The final elution was in 100 μL of buffer "AE" supplied by the manufacturer. I used the polymerase chain reaction (PCR) to amplify an approximately 950 base pair (bp) long fragment of the mitochondrial DNA (mtDNA) NADH dehydrogenase-1 (ND1) subunit gene using the primers ND1F (5'-ACCTCGATGTTGGATCAG-3') and ND1R (5'-TATTCGGCCAGGAAAAACAG-3') and the ND2 (approximately 900 bp long fragment) subunit gene using the primers ND2F (5'-AGCACTACCAACGCCTGAC-3') and ND2R (5'-AACAAGGGCTGGGAGATTTT-3') designed by Eric Taylor 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 10x New England Biolabs (NEB) ThermoPol Buffer, 4 μL dNTP (5

mM), 1 µL of each primer (10 µM), 0.3 µL NEB Taq, 37.2 µL distilled autoclaved water, and 1.5 µL of template DNA (between 50 – 100 ng/µL). The PCR conditions were as follows: initial denaturation 95 °C for 3 minutes, followed by 4 cycles of 95 °C denaturation (30 seconds), 55 °C annealing (30 seconds), 72 °C extension (90 seconds), 32 cycles of 92 °C denaturation (30 seconds), 54 °C annealing (30 seconds), 72 °C extension (90 seconds) and a final extension step at 72 °C for ten minutes. The PCR products were checked for quality and quantity on 1.5% agarose gel stained with SYBR Safe DNA gel stain and viewed under ultraviolet light. The PCR products were purified using Qiagen PCR purification columns and eluted in 20 µL distilled autoclaved water. Purified DNA samples were then sequenced in the reverse direction using ND1R and ND2R primers.

### *2.2.3 Mitochondrial DNA data analysis*

Sequences were aligned using the multiple sequence alignment ClustalW in BIOEDIT version 7.2.5 (Hall 1999). I combined the ND1 and ND2 sequences to increase the resolution in detecting distinct haplotypes. I used the software package MEGA version 6 (Tamura et al. 2013) for phylogenetic analysis. The best model for inferring phylogenetic relatedness was estimated using Bayesian Information Criterion (BIC). 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 – see Results) and bootstrapped with 2,000 replicates. *Oncorhynchus tshawytscha* (Chinook salmon) and *Oncorhynchus kisutch* (coho salmon) were used as outgroups (accession numbers AF392054.1 and EF126369.1, respectively).

### *2.2.4 Microsatellite DNA*

For a subset of populations (Table 2.2) 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. Nine microsatellite DNA loci were selected to assay based on clarity of resolution and degree of polymorphism: *Omy*77 (Morris et al. 1996), *Ots*100, 103, and 108 (Nelson and Beacham 1999), *Oki*10 and 29 (Smith et al. 1998), and *One*103, 108, and 110 (Olsen et al. 2000). The PCRs were carried out using fluorescently dye-labeled forward primers in 20 μL volumes using the Qiagen PCR Multiplex Kit following the manufacturer's protocol. Three PCR multiplex groups and one single reaction were performed to increase scoring clarity: 1) *Omy*77, O*ts*103 and *One*103 (annealing temperature: 55 °C), 2) *Ots*100, *Ots*108 and *Oki*29 (57 °C), 3) *One*108, *One*110 and *Oki*10 (57 °C) and 4) *Oki*29 (58 °C). The PCR products were visualized and evaluated on a Beckman-Coulter CEQ 8000 automated genotyper.

### *2.2.5 Microsatellite DNA data analysis*

I used the program MICRO-CHECKER version 2.2.3 (van Oosterhout et al. 2004) to check for evidence of scoring errors and the presence of non-amplifying alleles. Tests for deviations from Hardy-Weinberg Equilibrium for each locus-population combination using an exact test with P values were estimated using a Markov chain method performed in GENEPOP version 4.2 (Raymond and Rousset 1995). Genotypic linkage disequilibrium between all pairs of loci within a population was also tested in GENEPOP using a Markov chain method.

I used the microsatellite allele frequency data to construct a phylogenetic tree using the neighbor-joining method (NJ, Saitou and Nei 1987) and Cavalli-Sforza and Edwards' (1967)

chord distance ($D_C$) as a measure of genetic distance between populations performed in POPULATIONS version 1.2.31 (Langella 2000) and visualized in TREEVIEW version 1.6.6 (Page 1996). I used $D_C$ because it does not make assumptions based on mutation rates or models, only genetic drift (Cavalli-Sforza and Edwards 1967). The NJ tree was bootstrapped with 5,000 replicates.

I also used the model-based Bayesian clustering program STRUCTURE version 2.3.4 (Pritchard et al. 2000) to visually represent and identify distinct genetic populations to demonstrate population independence on either side of the Pacific Ocean. The STRUCTURE analysis used correlated allele frequencies and employed the admixture model with a burn-in period of 100,000 iterations followed by an additional 200,000 iterations. This was replicated five times with the hypothesized number of genetic groups (*K*) ranging from one to seven. This range takes into account each population sampled and any potential additional substructure. Preliminary analyses using a *K* value larger than seven resulted in population structure with very low likelihoods that also were difficult to interpret biologically. The location prior option was used to assist clustering and the basic results did not differ from previous results obtained without using the location prior (Hubisz et al. 2009). Most *Oncorhynchus* species are thought to have survived the Pleistocene glaciations in one or both of a northern refugium in Beringia (the unglaciated portions of the Yukon River Valley and adjacent areas) and a southern refugium in unglaciated portions of the Columbia River and adjacent areas (Lindsey and McPhail 1986; McPhail and Lindsey 1986). Consequently, the STRUCTURE analysis was performed on a subset of the microsatellite data using Meadow Creek as a "southern" refugium outgroup and Hansen Creek as a "northern" refugium outgroup. The program STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used to evaluate the most likely number of populations (Pritchard

et al. 2000) and incorporated the $\Delta K$ method of Evanno et al. (2005) to infer which model of population structure (i.e., value of $K$) is associated with the greatest second-order rate of change of the log probability of the data.

## 2.3 Results

### *2.3.1 Mitochondrial DNA*

After editing the unclear portions of each sequence, I analyzed an 856 base pair (bp) fragment of the ND1 subunit gene from 105 individuals (see Fig. A.1). The ND1 sequences resolved 12 haplotypes that were then sequenced at ND2 in order to increase the power to detect distinct haplotypes and resolve their inter-relationships. The ND2 subunit gene fragment (823 bp after editing) resolved 18 haplotypes in 82 individuals (see Fig. A.2). After combining the aligned fragments, 24 unique haplotypes were resolved in a 1,679 bp fragment from 82 individuals from across *O. nerka*'s geographic range. The 24 combined ND1/ND2 sequences (Fig. 2.1, Table 2.1) 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. The best substitution model under BIC to build a maximum likelihood tree was the Tamura-Nei (1993) model including a Gamma distribution of substitution rates (TN93 + G). The Tamura-Nei (1993) model of substitution accounts for differences in substitution rates between nucleotides and the inequality of nucleotide frequencies (Tamura et al. 2013). Using a discrete Gamma distribution (+G) accounts for the substitution rate varying from site to site in a sequence (Tamura et al. 2013).

The combined ND1/ND2 tree depicts the phylogenetic relationships among haplotypes from sockeye salmon, kokanee and "black" kokanee (Fig. 2.1). 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 and with 58% bootstrap support (Fig. 2.1). There were no phylogenetic trees (separated ND1 or ND2, see Fig. A.1, A.2) that grouped "black" kokanee from the eastern and western Pacific all together. No haplotypes were shared amongst the "black" kokanee populations from the eastern and western Pacific and their haplotypes differed from each by an average of 0.34% sequence divergence (Table 2.1, Fig. 2.1). In contrast, Anderson Lake and Seton Lake "black" kokanee haplotypes clustered with haplotypes from other populations of *O. nerka* from across its range in North America and differed only slightly from other *O. nerka* haplotypes (0.21% sequence divergence, Fig. 2.1). 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 (Table 2.1, Fig. 2.1). Anderson Lake "black" kokanee sequences included two unique haplotypes (10 and 19, Table 2.1).

Table 2.1. Summary of 24 mitochondrial DNA (mtDNA) haplotypes present by location, watershed and sample size in 82 *Oncorhynchus nerka* (sockeye salmon, kokanee, "black" kokanee) from the combined ND1 and ND2 subunit sequences sequenced. Populations of "black" kokanee are represented with an "*". BC = British Columbia, AK = Alaska, Y = Yukon.

| Location | Watershed | Sample size | Haplotypes Present |
|---|---|---|---|
| Klukshu Lake | Alsek River, Y | 7 | 14, 17 |
| Katherine Lake | Alsek River, Y | 3 | 2 |
| Tazimina River | Bristol Bay, AK | 3 | 7, 13 |
| Woodey Island | Bristol Bay, AK | 5 | 2, 13, 17 |
| Hansen Creek | Bristol Bay, AK | 10 | 2, 12, 13, 18, 23, 24 |
| Okanagan River | Columbia River, BC | 1 | 17 |
| Kootenay River | Columbia River, BC | 2 | 8, 17 |
| Cowichan Lake | Cowichan River, BC | 3 | 11, 16 |
| Anderson Lake* | Fraser River, BC | 5 | 2, 10, 17, 19 |
| Seton Lake* | Fraser River, BC | 2 | 2 |
| Sinmax Creek | Fraser River, BC | 1 | 2 |
| Eagle River | Fraser River, BC | 2 | 2 |
| Horsefly River | Fraser River, BC | 2 | 11, 17 |
| Birkenhead Lake | Fraser River, BC | 2 | 2, 22 |
| Adams River | Fraser River, BC | 1 | 11 |
| Little Horsefly River | Fraser River, BC | 2 | 17, 21 |
| Quesnel River | Fraser River, BC | 2 | 11, 20 |
| Gates Creek | Fraser River, BC | 2 | 11 |
| Portage Creek | Fraser River, BC | 2 | 2 |
| Lake Saiko* | Japan | 10 | 1, 4, 9 |
| Kronotsky Lake | Kamchatka, Russia | 10 | 5, 6, 13, 15, 17 |
| Shale Creek | Skeena River, BC | 1 | 3 |
| Dust Creek | Skeena River, BC | 1 | 2 |
| Takla Lake | Skeena River, BC | 1 | 3 |
| Babine Lake | Skeena River, BC | 2 | 11 |

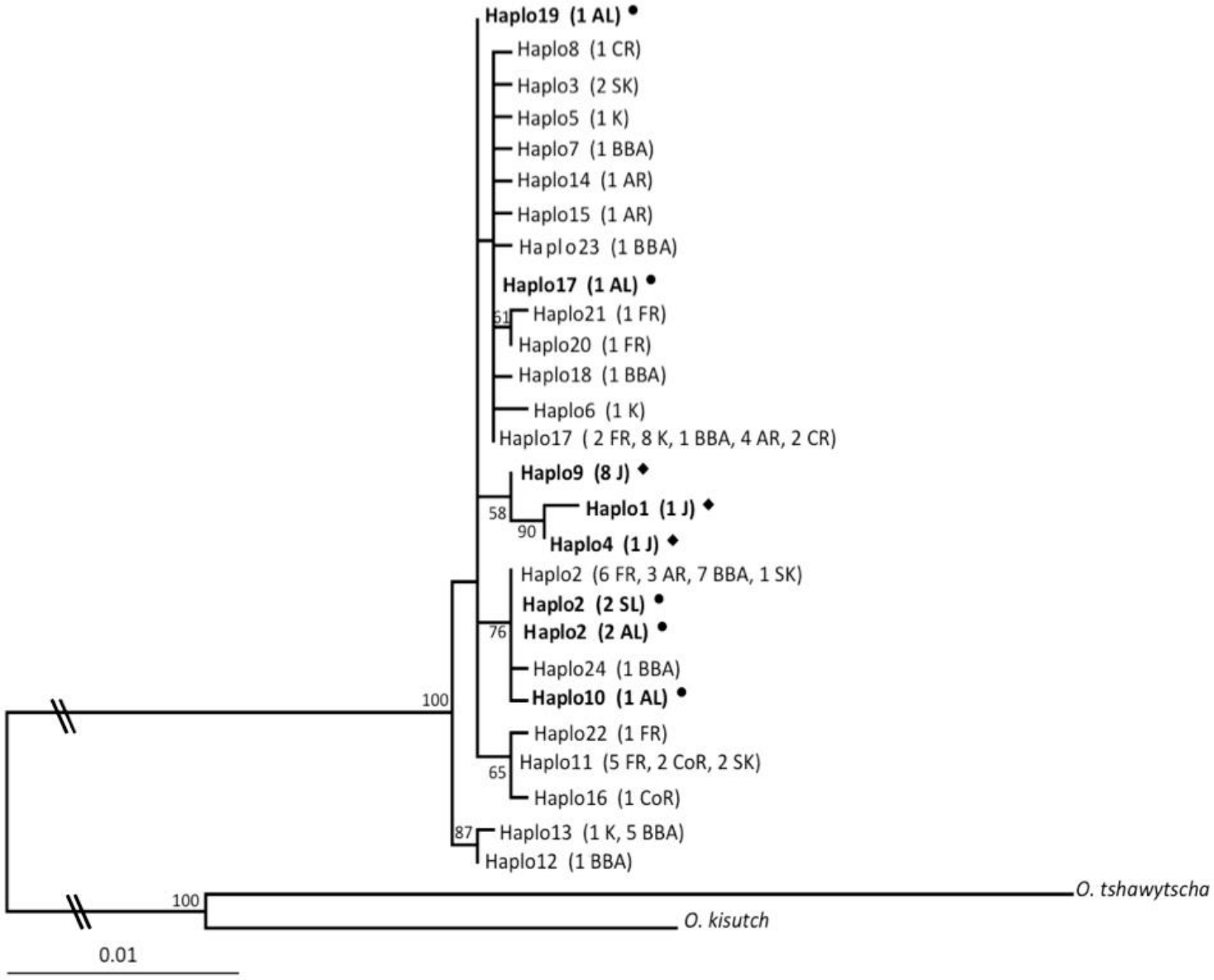


Figure 2.1. Maximum likelihood tree using the TN93 + G model of evolution demonstrating the phylogenetic relationships among 24 haplotypes resolved from combing ND1 and ND2 mitochondrial DNA sequences of *Oncorhynchus nerka* (N=82) from the Columbia River (CR), Cowichan River (CoR), Alsek River (AR), Fraser River (FR), Skeena River (SK), Bristol Bay Alaska (BBA), 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 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 2,000 bootstrap replicates. Dash lines represent a 33% reduction in branch length to enhance visualization among ingroup haplotypes.

### *2.3.2 Microsatellite DNA*

Most locus-population combinations were in Hardy-Weinberg Equilibrium after correcting for multiple comparisons using nine populations (20 out of 81 tests were not) ($P < 0.018$; Narum 2006). Of these 20 tests, and in all but two populations (Chedukuz Creek and Lake Saiko), seven deviations occurred at the *Ots*108 locus, showing evidence of heterozygote deficits that are most likely from null alleles detected by the MICRO-CHECKER analysis. Subsequent analyses were conducted with and without this locus. Other departures from Hardy-Weinberg Equilibrium were not concentrated to any specific loci or population. Interestingly, the *Ots*108 locus was difficult to amplify and genotype in all of the North American populations, but it consistently amplified in the Japanese samples. No genotypic linkage disequilibrium was detected between loci (five out of 324 tests showed significant linkage disequilibrium). Twenty-eight of the 324 tests provided "no information", an intractable situation where all rows or all columns marginal sums are 1 (Rousset 2013). Of these 28 tests, 26 were found in the Japanese populations and this is most likely due to a small sample (see Table A.3 and Chapter 3 for more details).

The NJ tree infers the genetic inter-relationships between the "black" kokanee on either side of the Pacific Ocean along with *O. nerka* populations from Alaska, the Fraser River and the Columbia River (Fig. 2.2). This tree provides further support to my independent evolution hypothesis as Anderson Lake and Seton Lake "black" kokanee clustered separately from Lake Saiko "black" kokanee compared to sockeye salmon populations within the same watershed as well as other Fraser River sockeye salmon and kokanee populations. The same tree was obtained with and without the *Ots*108 locus.

To visually represent and give additional support to my independent origin hypothesis, I used the program STRUCTURE. Using five populations (Hansen Creek and Meadow Creek

populations were used as outgroups) and all nine loci, STRUCTURE returned four as the most likely number of populations ($K = 4$, Fig. 2.3). This was supported by the log-likelihood method (Table 2.3). The $\Delta K$ method supported $K = 2$ as the most likely number of populations grouping Meadow Creek, Hansen Creek, and Lake Saiko together in each independent replicate. The $\Delta$K method supported $K = 4$ as the next most likely model. "Black" kokanee in Anderson Lake and Seton Lake grouped together while all other populations grouped separately from the Anderson/Seton cluster (Fig. 2.3). The same STRUCTURE results were produced without using the *Ots*108 locus with the $K = 4$ model being supported both by the log-likelihood method and the $\Delta K$ method (Table 2.3). There was no result produced by STRUCTURE that grouped the eastern and western "black" kokanee populations together. Further, the STRUCTURE analysis was run using the admixture model, which reflects the proportion of each individual's genome from each hypothesized genetic group ($K$). The limited amount of admixture between "black" kokanee from the eastern and western Pacific demonstrates that they constitute distinct assemblages of alleles (Fig. 2.2, 2.3). For instance, unique alleles 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 2.4). These results are consistent with my mtDNA analysis and support my hypothesis that "black" kokanee on either side of the Pacific Ocean have had independent origins.

Table 2.2. Summary of location, drainage, final sample size (after missing data were removed) and 'form' of *Oncorhynchus nerka* (kokanee, sockeye salmon, "black" kokanee) used for microsatellite analysis. "*" represents populations used in the STRUCTURE analysis. BC = British Columbia, AK = Alaska.

| Location | Drainage | Sample size | Form |
|---|---|---|---|
| Chedakuz Creek | Fraser River, BC | 29 | kokanee |
| Davidson Creek | Fraser River, BC | 31 | kokanee |
| Meadow Creek* | Kootenay River, BC | 30 | kokanee |
| Anderson Lake* | Fraser River, BC | 80 | "black" kokanee |
| Seton Lake* | Fraser River, BC | 47 | "black" kokanee |
| Gates Creek | Fraser River, BC | 41 | sockeye salmon |
| Portage Creek | Fraser River, BC | 36 | sockeye salmon |
| Hansen Creek* | Bristol Bay, AK | 20 | sockeye salmon |
| Lake Saiko* | Japan | 12 | "black" kokanee |

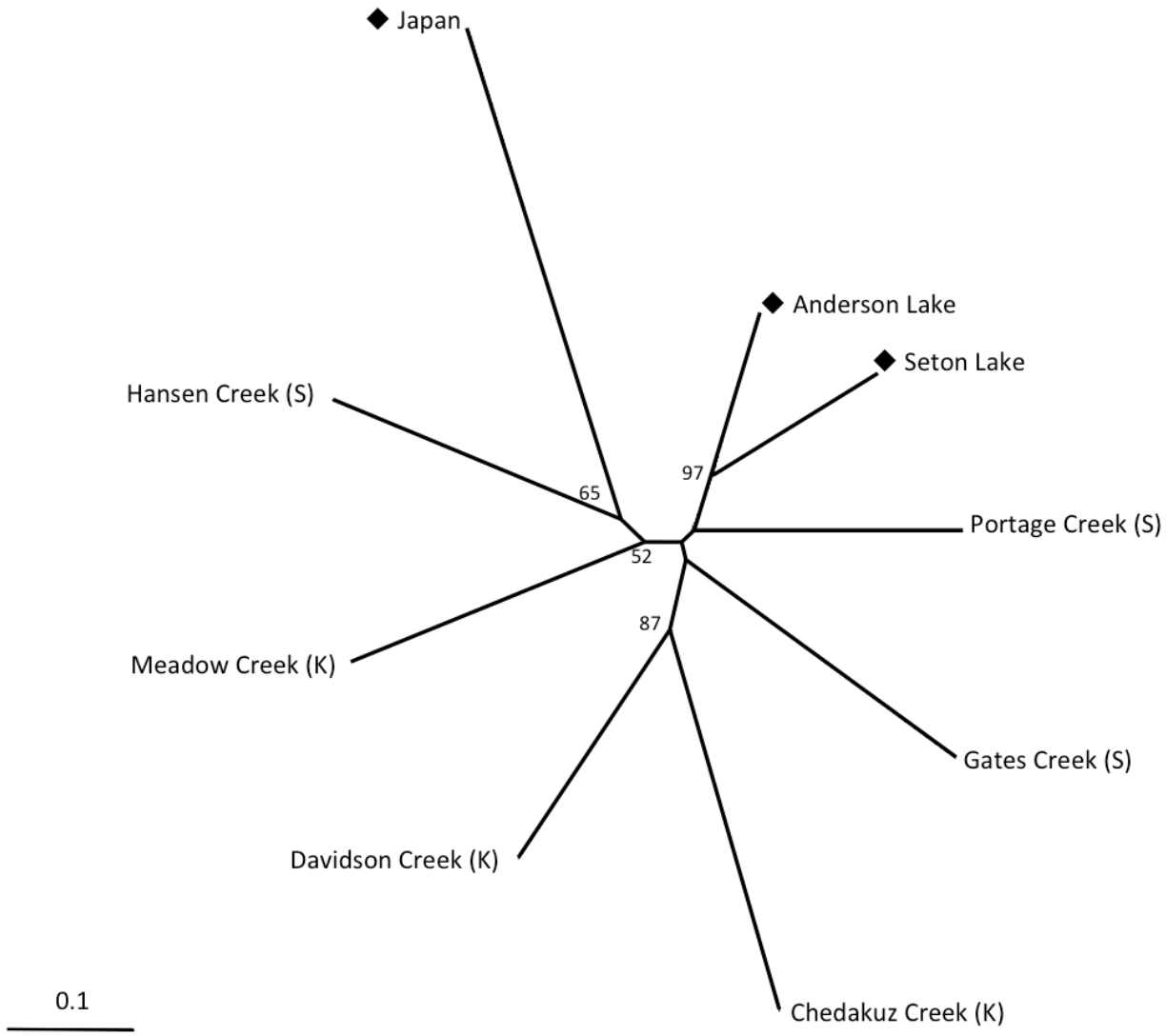


Figure 2.2. Neighbor-joining tree constructed using Cavalli-Sforza and Edwards (1967) chord distance ($D_C$) among nine microsatellites loci from nine populations of *Oncorhynchus nerka*, sockeye salmon (S) and kokanee (K) from the Fraser River drainage (Gates Creek, Portage Creek, Anderson Lake, Seton Lake, Chedakuz Creek, Davidson Creek), Columbia River drainage (Meadow Creek), Bristol Bay Alaska (Hansen Creek) and Lake Saiko (Japan). "◆" represents "black" kokanee. Numbers represent percentage of 5,000 bootstrap replicates (percentages less than 50% have been removed).

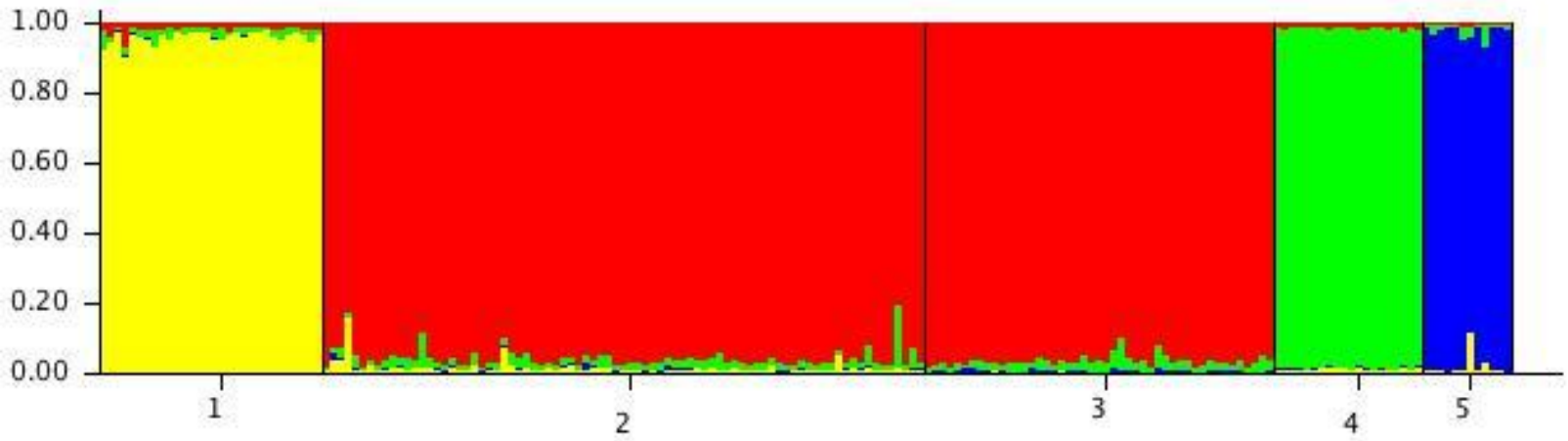


Figure 2.3. The STRUCTURE bar plot of *Oncorhynchus nerka* (sockeye salmon and kokanee) sampled from five populations (x axis) assayed at nine microsatellite loci from: Meadow Creek (1), Anderson Lake (2), Seton Lake (3), Hansen Creek (4), and Lake Saiko (5). The most likely *K* value (genetic groups = colours) is four, grouping Anderson and Seton lake "black" kokanee into one (largely "red") population. Each vertical line represents an individual fish and the different colours represent the genetic contribution (*Q*, y axis) of the four genetic groups.

Table 2.3. Results of five independent runs using nine microsatellite loci in STRUCTURE testing for the most likely number of genetic groups (*K*) using the posterior probability (mean LnP(*K*)) and the Evanno et al. (2005) method (Δ*K*). NA = not applicable. "*" represents independent runs without using the *Ots*108 locus.

| K | Mean LnP(*K*) | Δ *K* | Mean LnP(K)* | Δ*K** |
|---|---|---|---|---|
| 1 | -9601.52 | NA | -8644.64 | NA |
| 2 | -9305.4 | 73.623252 | -8400.6 | 33.079668 |
| 3 | -9203.86 | 0.605418 | -8259.4 | 2.35082 |
| 4 | -9051.02 | 2.914966 | -8149.4 | 73.069696 |
| 5 | -9259.96 | 0.905084 | -8212.7 | 0.612666 |
| 6 | -9049.58 | 20.16409 | -8240.42 | 0.74373 |
| 7 | -9273.42 | NA | -8326.94 | NA |

Table 2.4. Overall frequency of unique alleles per locus in "black" kokanee found in both Anderson Lake and Seton Lake (Gwenish, *Oncorhynchus nerka*), in Lake Saiko (kunimasu, *Oncorhynchus kawamurae*) and alleles found both in Gwenish and kunimasu, but not in the other *O. nerka* populations sampled. Frequencies calculated from the total number of alleles/locus/population.

| | Frequency of unique alleles | | |
|---|---|---|---|
| | Gwenish | Kunimasu | Both |
| *Omy*77 | 0.006 | 0.000 | 0.019 |
| *Ots*103 | 0.011 | 0.000 | 0.083 |
| *Oki*29 | 0.015 | 0.000 | 0.018 |
| *Ots*108 | 0.003 | 0.000 | 0.009 |
| *Ots*100 | 0.013 | 0.054 | 0.000 |
| *Oki*10 | 0.026 | 0.003 | 0.017 |
| *One*108 | 0.022 | 0.009 | 0.003 |
| *One*110 | 0.010 | 0.000 | 0.000 |
| *One*103 | 0.042 | 0.000 | 0.024 |

## 2.4 Discussion

### *2.4.1 Polyphyletic origin of "black" kokanee*

My results suggest a polyphyletic origin of "black" kokanee from at least two independent episodes of divergence on either side of the Pacific Ocean. This inference is supported by two independent data sets: the relatively high bootstrap support for distinct clusters of "black" kokanee in the mitochondrial DNA (mtDNA) tree, the lack of clustering by phenotype in the microsatellite tree and the general lack of alleles shared between the eastern and western North Pacific "black" kokanee populations. "Black" kokanee in Japan and in the Anderson/Seton lakes system do not share any mtDNA haplotypes and they did not form a monophyletic grouping. Further, both data sets showed that "black" kokanee from Anderson Lake and Seton Lake were genetically more similar to each other and to other Fraser River sockeye salmon and kokanee populations than they are to Japanese "black" kokanee. The STRUCTURE results and neighbor-joining tree also demonstrate the genetic similarity between Anderson Lake and Seton Lake "black" kokanee and their joint similarity to other Fraser River populations. This suggests that "black" kokanee from the Anderson/Seton lake system are more similar to kokanee and sockeye salmon within the same geographic area than to "black" kokanee from Japan. Additionally, very few unique alleles were shared amongst Japanese and Anderson/Seton "black" kokanee populations.

A post-glacial origin for "black" kokanee is supported by the lack of deep phylogenetic structure, low variation and large proportion of low frequency haplotypes (19 out of 24 haplotypes resolved). The overwhelming evidence of post-glacial polyphyletic origin of "regular" kokanee following multiple independent divergences between the forms of *O. nerka* further supports my findings (Foote et al. 1989; Taylor et al. 1996; Wood and Foote 1996; Taylor

et al. 1997; Frazer and Russello 2013). Furthermore, my findings are consistent with post-glacial parallel origins in other species complexes of north temperate freshwater fishes: *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 been much more widespread across *O. nerka*'s geographic range, but, due to historical events, only two populations persisted. The lack of deep genetic distance, the existence of only two distantly located populations, and the lack of any known geographically intermediate populations despite the wide occurrence of kokanee, makes this alternative unlikely.

### *2.4.2 Phenotypic divergence*

"Black" kokanee show parallel patterns of phenotypic (colour and reproductive ecology) divergence and my results provide evidence of genotypic divergence with both nuclear and mitochondrial DNA. Demonstrating parallel evolution of similar phenotypes by genetic divergence can implicate the role of natural selection. Inferences can be made that "black" kokanee populations on either side of the Pacific Ocean experience similar ecological conditions that have promoted parallel patterns of phenotypic and genetic divergence (Bell and Foster 1994; Schluter and Nagel 1995). The reproductive habitats and behaviour of "black" kokanee may result in unique selective regimes that promote parallel evolution of phenotypes as potential adaptations to their distinct reproductive ecology. Therefore, deep water and late spawning behaviour may be accompanied by selection for particular, but as yet unknown traits that have resulted in differentiation from "regular" kokanee and sockeye salmon. Evidence of unique

colouration and/or behaviour of adaptive significance can be seen in other salmonids: lake whitefish (*Coregonus clupeaformis*, Pigeon et al. 1997), beach/stream spawning kokanee (*O. nerka*, Taylor et al. 2000; Frazer and Russello 2013), Atlantic salmon (*Salmo salar*, Garcia de Leaniz et al. 2007), masu salmon (*O. masou*, Kano et al. 2010) and was reviewed in Fraser et al. (2011).

In Lake Saiko, sympatric stream spawning kokanee are known to be reproductively isolated from "black" kokanee and the two rarely hybridize (Nakabo et al. 2011; Muto et al. 2012). Comparatively, juvenile sockeye salmon reside and feed both in Anderson Lake and Seton Lake prior to migrating to the ocean, but there have been no observations of stream or beach spawning "black" kokanee in these lakes. The unique reproductive strategy of "black" kokanee could be the result of competition for stream spawning sites and/or food in both Lake Saiko and the Anderson/Seton lakes system. For instance, the deep water spawning habitats of "black" kokanee may be associated with distinct thermal regimes leading to different times at first feeding for sockeye salmon and "black" kokanee in the same lake (Morris and Caverly 2004). In addition, there may be more deep water spawning habitat in relation to stream spawning habitat that could facilitate deep water spawning. Morris and Caverly (2004) suggested that deep spawning would also reduce the risk of offshore alevin (larvae) and fry transport from spring winds that would normally occur in the much shallower stream or beach spawning sites.

The deep water spawning sites being used by "black" kokanee may be associated with geothermal and/or groundwater upwelling that provides favorable temperature and oxygen content for spawning and incubation (Morris et al. 2003). Alternatively, it is hypothesized that "black" kokanee could be locally adapted to spawn at cool water temperatures (Nakabo et al. 2011). In Lake Saiko, the water temperature below 30 meters is estimated to be less than 5 °C

during spawning and temperatures at putative spawning sites in Anderson Lake and Seton Lake are estimated to be around 4 °C (Morris et al. 2003; Nakabo et al. 2011). Comparatively, "regular" kokanee and sockeye salmon typically spawn in approximately 9-13 °C water (Burgner 1991; Nakabo et al. 2011).

Decreased light intensity at depth may be causally related to the unusual colour of these "black" kokanee populations. Light clarity decreases with increasing depth (Moss 1998) and, as a result of spawning at such great depths; the characteristic nuptial red colouration of *O. nerka* could potentially be selected against because red-colour attenuates rapidly with depth (Reimchen 1989). 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 is associated with low water clarity In addition, decreased visibility to predators would also result from colour loss (Moodie 1972). Given that carotenoids are energetically costly (Martinkappi et al. 2009), and that "black" kokanee flesh does not appear to contain an excess amount of carotenoids, any energy associated with carotenoid deposition could be allocated elsewhere. The eggs are a pale orange colour and appear to contain carotenoids (personal observation). Further research is needed to test these hypotheses in "black" kokanee.

Recognizing that these "black" kokanee populations are relatively understudied and that the habitats in which "black" kokanee occur are probably not unique to Anderson Lake, Seton Lake or Lake Saiko, "black" kokanee could potentially be in other north temperate lakes. Dunn Lake north of Kamloops, for example, may also produce "black" kokanee, but there is no information regarding their spawning behaviour beyond that the kokanee are dark green to almost black in colour when spawning (Gene Tisdale, pers. comm.).

Even if natural selection favours the same phenotype in populations of "black" kokanee, mutations in different genes or different pathways could have also produced similar phenotypes (Conte et al. 2012; Lemay 2013). Alternatively, random genetic drift could have caused the divergence of "black" kokanee, although this is unlikely to account for similar phenotypes repeatedly and independently, especially considering the evolutionary history of *O. nerka* across its geographic range.

### *2.4.3 Low genetic diversity*

The low levels of mtDNA sequence variation that I resolved are consistent with previous studies showing low biochemical and molecular variation within *O. nerka* in comparison to other species of Pacific salmon. For example, Allendorf and Utter (1979) examined allozyme allele frequency variation and found that *O. nerka* has the lowest levels of heterozygosity compared to other Pacific salmon and trout except coho salmon (*O. kisutch*). Taylor et al. (1997) amplified a highly variable portion of mtDNA control region in *O. nerka* and only resolved three haplotypes from across its geographic range. While Neilson et al. (1994) amplified the same portion in rainbow trout (*O. mykiss*), coho and Chinook salmon (*O. tshawytscha*) from 22 streams in northern California and resolved nine, five and six haplotypes, respectively. In another study, Taylor et al. (1996) surveyed allele variation at two minisatellite loci from across *O. nerka*'s range and found that two alleles at each of the two loci accounted for approximately 95% of the total variation. Churikov et al. (2001) sequenced seven regions of the mitochondrial genome (accounting for 97% of the genome) of three Pacific salmon species, pink (*O. gorbuscha*) sockeye, and chum (*O. keta*) salmon and two thirds of the haplotypes resolved in sockeye salmon were restricted to the ND1/ND2 segment. Also, mtDNA nucleotide diversity of sockeye salmon

was comparable to that of pink salmon and chum salmon, but haplotype diversity was substantially lower (Churikov et al. 2001).

Japanese kokanee and sockeye salmon populations have substantially lower levels of genetic variability at allozyme (Winans and Urawa 2000) and microsatellite loci (Young et al. 2004, Beachman et al. 2006) compared to North American and Russian populations. Additionally, most Japanese populations sequenced at the cytochrome *b* segment were monomorphic for a single haplotype (Yamamoto et al. 2011). Although my sample sizes were modest for Japanese kokanee, my results are consistent with low molecular variability in Japanese *O. nerka*. All 13 ND1 sequences were monomorphic (Fig. A.1). After combining the ND1 sequences with ND2 sequences only two additional haplotypes were resolved. The low genetic variation observed in Japanese *O. nerka* can probably be explained by the transplantation history (e.g., Tokui 1964; Masaoka et al. 1997) and that Japan is on the southwestern periphery of native *O. nerka'*s range (Yamamoto et al. 2011).

### *2.4.4 Phylogeographic structure*

There are 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 (Varnakskaya et al.1994; Taylor et al. 1996; Allendorf and Seeb 2000; Winans and Urawa 2000; Beacham et al. 2006). Within refugium divergences are often reflected in the current patterns of diversity across the range of a species (Waples et al. 2008). My findings provide some support for a minor 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) and the microsatellite DNA NJ tree (at 65% bootstrap support for a grouping linking Alaskan *O. nerka* and Japanese *O. nerka*). Similar phylogeographic distinctions resulting from past historical events are found in other species of Pacific salmon (e.g., *O. mykiss*: Taylor 1995, *O. gorbuscha*: Churikov and Gharrett 2002, *O. tshawytscha*: Waples et al. 2004), 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).

This phylogeographic distinction would become clearer with a larger sample size and inclusion of more populations from across *O. nerka*'s geographic range but that is beyond the scope of this chapter. I would predict that Japanese "black" kokanee would group with a more northern group (such as suggested by the microsatellite tree) and Anderson/Seton "black" kokanee would group with a southern group (Beacham et al. 2006). My results hint at multiple divergences within these phylogeographic lineages, which would also become clearer with a broader survey.

### *2.4.5 Conclusions*

It is important to understand how intraspecific diversity originates especially when natural selection is potentially driving the evolution of genetic variation. Similar ecological specializations observed in "black" kokanee in Lake Saiko and in the Anderson/Seton lakes system resulted from repeated evolutionary events rather than from a single divergence from the rest of *O. nerka*. In conclusion, my findings suggest that "black" kokanee have evolved at least twice across the North Pacific Ocean. This chapter has provided evidence from concordance of

two independent data sets of the role of parallel evolution in the origin of north temperate fish biodiversity particularly within *O*. *nerka,* and has revolved yet another nuance in the evolutionary history of Pacific salmon.

# Chapter 3: Genetic and morphological differentiation of "black" kokanee (*Oncorhynchus nerka*) within the Anderson and Seton lakes system

## 3.1 Introduction

### *3.1.1 Biocomplexity*

The greatest challenge and perhaps the most important component in conservation biology is identifying and preserving intraspecific variation (Behnke 1972; Moritz 2002; Wood and Gross 2008). In particular, phenotypic and genetic variation among populations is a critical aspect of conservation (Allendorf and Luikhart 2007). Phenotypic specialization and genetic structure provide information regarding local adaptation and demographic independence of populations, which can influence recovery probability of declining populations (Taylor et al. 2000; Hoffmann and Willi 2009; Suk and Neff 2009). Intraspecific variation can buffer ecosystems from future changes in environmental conditions and lead to long-term sustainability of ecosystem services and functions (Bengtsson et al. 1997; Hilborn et al. 2003; Duffy 2009; Schindler et al. 2010). This "biocomplexity" is known to increase stability in populations and ecosystems. For example, Bristol Bay, Alaska, sockeye salmon (*Oncorhynchus nerka*) exist as numerous distinct and locally adapted populations, and conserving this "biocomplexity" has played an important role in maintaining stability in overall abundance of the population complex in the face of a fluctuating environment as well as providing the basis for a sustainable fishery (Hilborn et al. 2003).

### *3.1.2 Diversity within* Oncorhynchus nerka*: sockeye salmon and kokanee*

*Oncorhynchus nerka* provides an outstanding example of the broad range of diversity that can be found within a single species. First, *O. nerka* exists as two common life-history forms: the

sockeye salmon or anadromous (sea-going) form and the kokanee or non-anadromous (permanently freshwater-resident) form (Burgner 1991; Quinn 2005). Further, both within sockeye salmon and kokanee, extensive variation exists at multiple spatial and temporal scales, most of which is heritable. For example, variation in growth and maturation (Wood and Foote 1990, 1996), biochemical/molecular traits (Foote et al. 1989; Taylor et al. 1996; Wood and Foote 1996), colouration (Craig and Foote 2001; Craig et al. 2005), swimming ability (Taylor and Foote 1991) and morphology (Burgner 1991; Taylor and Foote 1991; Foote et al. 1999) have been well documented.

A most intriguing aspect of *O. nerka* is that of its variation in life history and reproductive behaviour that has been well documented (e.g., Wood 1995; Wood et al. 2008). Briefly, sockeye salmon exist as two main ecotypes: the sea/river type and the lake type. Sea/river type sockeye salmon spawn in tributaries with no access to lakes and juveniles feed and grow in side channels for several months to two years before migrating to the ocean where they grow further and initiate maturation before returning to freshwater streams and lake beaches to spawn (Wood 1995). The lake type of sockeye salmon typically spends one to two years in a lake before migrating to the ocean (Burgner 1991; Wood 1995). Lake type sockeye salmon can be further divided into beach-spawners and stream-spawners (Burgner 1991).

Considerable variation exists in kokanee some of which remains relatively understudied. Kokanee can also be divided into beach- and stream-spawning ecotypes (Burgner 1991; Taylor et al. 1997). Stream spawners are similar to sockeye salmon in both morphology and life history. They ascend tributary streams to spawn in gravel substrate and typically females defend spawning territories (Taylor et al. 1997; Shepherd 2000). In contrast, beach spawning kokanee spawn on submerged lakeshore beaches between 15 to 100 cm in depth and later than stream

spawners (Shepherd 2000). Beach spawners typically have less pronounced colouration and no mate or territory defense has been observed (Shepherd 2000; Frazer and Russello 2013).

### *3.1.3 "Black" kokanee*

A relatively newly documented and understudied aspect of the variation within kokanee is the phenomenon known as the so-called "black" kokanee, which are distinguished from "regular" kokanee by the intense black nuptial colouration compared to the typical red-body/green head colouration. Curiously, to date "black" kokanee have only been documented in a single lake in Japan and in two lakes in the Fraser River drainage of BC: Anderson Lake and Seton Lake (see chapter 2 and Fig. 1.3).

Along with their distinct colour, some of the spawning behaviour of "black" kokanee in Anderson Lake and Seton Lake appears to be unique not only within *O. nerka* but within *Oncorhynchus* generally. They spawn at depths of 20 to 70 meters (typically greater than 50 meters) on small gravel substrate with peak spawning November (Seton Lake) and in January (Anderson Lake) (Morris and Caverly 2004). Substrate size at putative spawning sites varies with depth and typically consists of small gravel but large gravel and finer substrates have also been observed (Morris and Caverly 2004). Large cobble and boulder substrates observed at putative spawning locations in Anderson Lake suggest that some "black" kokanee may be broadcast spawning (Morris and Caverly 2004). In addition, "milling" or schooling behaviour has been observed in spawning "black" kokanee Anderson Lake, but remains unknown in Seton Lake. Deep water spawning is thought to cause swim bladder distension due to rising to the surface too quickly resulting in their unusual post-spawning floating (Morris and Caverly 2004; Stable 2004). Interestingly, some "floaters" are still alive (personal observations).

Differences between the populations in both lakes are also observed. The majority of Anderson Lake spawners are three years of age, with a small number being four or five. In contrast, Seton Lake spawners are predominantly two years of age with few being three years (Morris and Caverly 2004). Differences in mean fork length between fish from the lakes correspond to the age differences with Anderson Lake males and female kokanee being larger than Seton Lake males and females (Morris and Caverly 2004). Black colouration in Anderson Lake fish is more striking in comparison to the duller Seton Lake spawners (personal observation, see Figs. 1.4, 1.5).

"Black" kokanee play a significant ecological role in maintaining the biodiversity in the Anderson/Seton lakes system as well as adding to the productivity and complexity of the lakes' food webs. They provide food for piscivorous fishes such as bull trout (*Salvelinus confluentus*) and rainbow trout (*O. mykiss*) and for terrestrial predators such as bald eagle (*Haliaeetus leucocephalus*) and cougar (*Puma concolor*) (Morris and Caverly 2004). "Black" kokanee also likely play a role in nutrient cycling (Richey et al. 1975). Further, "black" kokanee are important socially and culturally to the St'át'imc First Nation, which form the majority of the population in the surrounding communities in the Bridge/Seton system. "Black" kokanee are used as a crop fertilizer, a winter food source, and a way to transfer traditional St'át'imc knowledge (William Alexander, pers. comm.).

In order to better understand the management needs of "black" kokanee in Anderson Lake and Seton Lake, we need to understand if the two lakes house distinct populations of "black" kokanee. If the two lakes' populations are genetically distinct, for instance, then this suggests that they might also be demographically distinct and require independent management in terms of habitat protection and harvest levels (see Palsbøll et al. 2007; Lowe and Allendorf

2010). Indeed, the differences in size and age at maturity between Anderson and Seton lakes' "black" kokanee strongly suggest that the two populations are not the same demographically.

In this chapter, I used microsatellite allele variation at nine loci to test the hypothesis that "black" kokanee are genetically distinct from each other and from broadly sympatric sockeye salmon within the Anderson/Seton lakes system. Such information is important to better understand the hierarchical nature of diversity within *O. nerka* both conceptually, but also in the context of 'designatable units' (DUs) and Canada's *Species at Risk Act*. A DU is an intraspecific unit of diversity that is both "discrete" (spatially or genetically) from other such units and where such discreteness is "significant" to the evolutionary legacy of the species (COSEWIC 2013; Taylor et al. 2013). This could be especially important because sockeye salmon are scheduled for assessment by the Committee on the Status of Endangered Wildlife in Canada in the near future (E. Taylor, pers. comm.). Specifically, I used microsatellite allele frequency variation to estimate the levels of divergence within and among populations. Genetic variation at neutral loci can provide a measure of population independence and are an ideal tool to investigate genetic diversity due to their high mutation rates and high levels of polymorphism (Suk and Neff 2009). This is the first study to quantify the level of genetic variation within the Anderson/Seton lakes system.

Furthermore, I conducted a morphological analysis (standard length, maximum body depth and gill raker counts) to test the extent of phenotypic variation within and between Anderson Lake and Seton Lake "black" kokanee. My morphological analysis provides an additional measure of diversity within a single lake system, variation that potentially reflects local adaptation from differences in spawning environments, or phenotypic plasticity (Nelson 1968; Taylor et al. 1997). Identifying population structure based on molecular and morphological

data will help to better understand the nature of *O. nerka* biocomplexity that exists in Anderson Lake and Seton Lake.

## 3.2 Methods

### *3.2.1 Microsatellite DNA*

Samples used in my microsatellite assay consisted of kokanee/sockeye salmon fin tissue from three lake systems: Tatelkuz Lake, Anderson/Seton lakes (both in the Fraser River drainage system) and Kootenay Lake (Columbia River drainage system) (Fig. 3.1). Tatelkuz Lake (upper Chedakuz Creek N = 30, Davidson Creek N = 31) and Kootenay Lake (Meadow Creek N = 30) kokanee samples were collected in 2012 and supplied by Eric Taylor. Tatelkuz Lake and Kootenay Lake kokanee were used as "outgroups" from the Fraser River and Columbia River, respectively, to place the scale of molecular variation between "black" kokanee and sockeye salmon from the Anderson/Seton lakes system within the context of variation between watersheds in the Fraser River and between the Fraser and Columbia rivers. Anderson Lake "black" kokanee samples were collected in January 2013 by David Levy (Levy Research Services Ltd.) (N = 40) and I collected samples on January 16, 2014 (N = 44). Samples were collected by surveying the beach for post spawning adults and adipose fin samples were taken on site. I collected "black" kokanee (post-spawners) from the surface of Seton Lake on November 20, 2013 (N = 48) using dipnets and with the help of Gene Tisdale (Tisdale Environmental Consulting Inc.). Portage Creek sockeye salmon (N = 40) fin tissue was collected in 2013 and supplied by Matthew Casselman (UBC Forest and Conservation Sciences). Gates Creek sockeye salmon (N = 41) fin tissue were collected in 2013 and supplied by Steve Latham (Pacific Salmon Commission) (summarized in Table 3.1). Both Gates Creek and Portage Creek sockeye salmon

populations are native to the Anderson/Seton system and both have a history of receiving transplants from other sockeye populations, but only the transplantations into Portage Creek (from the lower Adams River also in the Fraser River drainage) were thought to be at least partially successful (Withler 1982) (see Fig. 1.3). Fin tissue was stored in 95% ethanol for later genetic analysis. The procedures and materials used for microsatellite amplification at the nine loci are outlined in Chapter 2.

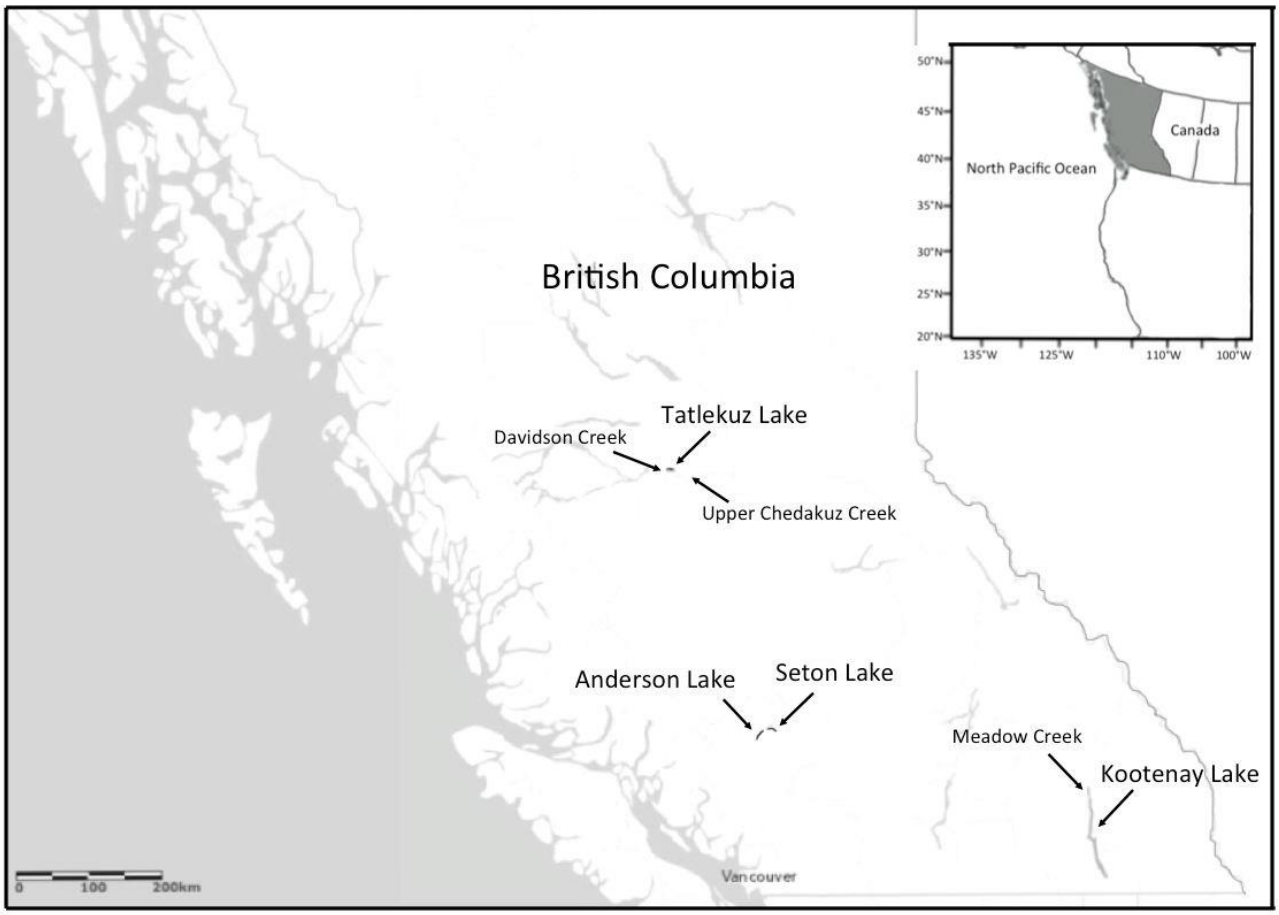


Figure 3.1. Map of British Columbia showing the three lake systems: Tatlekuz Lake, Anderson/Seton lakes system in the Fraser River drainage and Kootenay Lake in the Columbia River drainage, used in the microsatellite analysis of sockeye salmon and kokanee (*Oncorhynchus nerka*).

Table 3.1. Summary of location, drainage, final sample size (after missing data was removed) and 'form' of *Oncorhynchus nerka* (kokanee, sockeye salmon, "black" kokanee) used for microsatellite analysis.

| Location | Drainage | Sample size | Form |
|---|---|---|---|
| Chedakuz Creek | Fraser River, BC | 29 | kokanee |
| Davidson Creek | Fraser River, BC | 31 | kokanee |
| Meadow Creek | Kootenay River, BC | 30 | kokanee |
| Anderson Lake 2013 | Fraser River, BC | 37 | "black" kokanee |
| Anderson Lake 2014 | Fraser River, BC | 43 | "black" kokanee |
| Seton Lake | Fraser River, BC | 47 | "black" kokanee |
| Gates Creek | Fraser River, BC | 41 | sockeye salmon |
| Portage Creek | Fraser River, BC | 36 | sockeye salmon |

### *3.2.2 Microsatellite DNA data analysis*

I used the program MICRO-CHECKER version 2.2.3 (van Oosterhout et al. 2004) to check for evidence of scoring errors and the presence of non-amplifying alleles (null alleles). Tests for deviations from Hardy-Weinberg Equilibrium for each locus-population combination using an exact test with P values were estimated using a Markov chain method performed 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, I performed basic population genetic summary statistics (number of alleles per locus, allelic richness, observed heterozygosity and expected heterozygosity) in FSTAT version 2.9.3.2 (Goudet 1995) and GENEPOP.

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 were corrected for multiple simultaneous tests as outlined in Narum

(2006). Although in most instances, temporal variation is usually minor in comparison to spatial variation in salmonid fishes (e.g., Beacham et al. 2004; Taylor and Yau 2013), I tested for differentiation between temporal samples from Anderson Lake (2013, 2014) before proceeding with subsequent analyses.

Microsatellite variation was partitioned hierarchically by within population, among population and among "black" kokanee and sockeye salmon populations within the Anderson/Seton lakes system by using an analysis of molecular variance (AMOVA) performed in GenAlEx version 6.5 (Peakall and Smouse 2012).

I used the model based Bayesian clustering method in STRUCTURE version 2.3.4 (Pritchard et al. 2000) to assess the hypothesized differences between populations of kokanee, sockeye salmon and "black" kokanee. The STRUCTURE analysis used correlated allele frequencies and employed the admixture model 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 population sampled and any potentially additional substructure. Preliminary analyses using a *K* value larger than eight resulted in population structure with very low likelihoods complicating biological interpretation. I used ten independent runs for each *K* to ensure results were consistent. The Anderson Lake and Seton Lake "black" kokanee populations did not give consistent results using five iterations so I used ten iterations to provide more consistent results. This is potentially caused by the weak differentiation between the two "black" kokanee populations (see below). The analysis was run with and without the location prior option that is used to assist clustering (Hubisz et al. 2009). The STRUCTURE analysis was performed on the entire microsatellite data set as well as with

the four populations from the Anderson and Seton lakes system with the same parameter set and *K* ranging from one to seven. The program STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used to evaluate the most likely number of populations and incorporated the Δ*K* method of Evanno et al. (2005) to infer which model of population structure (i.e., value of *K*) is associated with the greatest second-order rate of change of the log probability of the data, and therefore most likely.

Discriminant analysis of principal components (DAPC) was used to visualize the variation among populations within the Anderson and Seton lakes system using kokanee from Meadow Creek, upper Chedakuz Creek and Davidson Creek as outgroups. 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). This method makes no assumptions about the underlying population genetic model, Hardy-Weinberg Equilibrium or linkage disequilibrium and it uses an analysis of variance model to quantify between group differentiation (Jombart et al. 2010). The DAPC analysis was conducted in R (R Development Core team 2012) using the ADEGENET package (Jombart et al. 2010).

### *3.2.3 Morphological analysis*

"Black" kokanee from Anderson Lake (sampled in 2013 N = 30 and 2014 N = 19) and Seton Lake (sampled in 2013 N = 30) were used for morphological analysis. Carcasses from both lakes were kept on ice until arrival at UBC where they were frozen for subsequent morphological analysis. Samples were thawed overnight and then preserved in 10% formalin for a minimum of four days (Seton Lake samples) to a maximum of one week (Anderson Lake samples owing to their larger size). Swim bladders were deflated to assist in preservation. Samples were then

rinsed and soaked in water for 24 hours before being measured and then stored in 45% isopropyl alcohol. Basic morphological measurements included standard length and the distance from the insertion of the pectoral fin to the insertion of the dorsal fin (used as a measure of maximum body depth). Measurements were taken on the left side of the fish and the sex of each fish was recorded.

The first gill arch on the left side was removed and stained in 1% potassium hydroxide with Alizarin red for two hours. Gill arches were rinsed and then soaked in distilled water for approximately 12 hours before gill rakers were counted using a dissecting microscope. Samples were then preserved in 45% isopropyl alcohol.

### *3.2.4 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 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, I 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. 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 significant 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. I compared the means of each measurement between Anderson Lake and Seton Lake as well as between sampling years using *t*-tests for independent samples.

## 3.3 Results

### *3.3.1 Within population microsatellite variation*

#### 3.3.1.1 Hardy-Weinberg Equilibrium and linkage disequilibrium

Only one locus (*Ots*108) consistently showed evidence of one or more null alleles after analysis with MICRO-CHECKER. This locus also had lower than expected heterozygosity in seven out of the eight populations analyzed. Subsequent analyses were performed with and without *Ots*108 for comparison. Other loci showed minor evidence for null alleles, but no other locus showed consistent evidence across populations. There was no evidence of large allele dropout or scoring error due to stuttering.

Out of 72 tests for Hardy-Weinberg Equilibrium for each locus population combination, 17 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 17 tests, seven were at *Ots*108 therefore the deficiencies of heterozygotes are most likely caused by non-amplifying alleles. The remaining ten tests were not concentrated at one specific locus or population. Tests for linkage disequilibrium between loci

resulted in statistically significant departures in 11 out of 288 tests but departures were not concentrated on specific locus pairs and were less frequent than expected by chance alone therefore each locus represents an independent measure of genetic variation.

#### 3.3.1.2 Temporal variation

Anderson Lake was sampled in 2013 and in 2014. Allele frequencies in 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 an increase 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, I combined the temporal Anderson Lake samples for the subsequent analysis.

#### 3.3.1.3 Allelic richness and genetic diversity

The mean number of alleles per locus corrected for different sample sizes (allelic richness) ranged from 7.50 (*Omy*77) to 21.03 (*One*103) (Table 3.2). Averaged across all nine loci and populations, allelic richness was 13.10 per locus per population. Genetic diversity (expected heterozygosity) averaged across loci ranged from 0.58 (Chedakuz Creek) to 0.89 (Seton Lake), and ranged from 0.67 (*Omy*77) to 0.93 (*Oki*10) when averaged across populations. Genetic diversity averaged across all seven populations and loci was 0.81. Chedakuz Creek and Gates Creek consistently exhibited the lowest levels of within population diversity in my analysis (Table 3.2). 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 average 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 3.2).

Table 3.2. Summary statistics at nine microsatellite loci in sockeye salmon (S), kokanee (K), and "black" kokanee (B) (*Oncorhynchus nerka*) from Chedakuz Creek K (CC), Davidson Creek K (DC), Meadow Creek K (MC), Anderson Lake B (2013 and 2014 combined) (AL), Seton Lake B (SL), Gates Creek S (GC), and Portage Creek S (PC). Number of alleles/locus (A), expected heterozygosity ($H_E$), observed heterozygosity ($H_O$), allelic richness ($A_R$) and sample size (N) for each loci and population. Significant departures from Hardy-Weinberg Equilibrium after correcting for multiple comparisons are represented by * ($P < 0.018$).

| Population | Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Omy*77 | *Ots*103 | *Oki*29 | *Ots*108 | *Ots*100 | *Oki*10 | *One*108 | *One*110 | *One*103 | Average |
| **CC** | | | | | | | | | | |
| A | 5 | 9 | 5 | 5 | 4 | 11 | 7 | 6 | 7 | 6.56 |
| $H_E$ | 0.465 | 0.829 | 0.651 | 0.388 | 0.333 | 0.842* | 0.755 | 0.683* | 0.729 | 0.58 |
| $H_O$ | 0.448 | 0.655 | 0.759 | 0.276 | 0.345 | 0.862 | 0.714 | 0.448 | 0.586 | 0.57 |
| $A_R$ | 4.897 | 8.93 | 5 | 4.965 | 3.966 | 10.93 | 7 | 5.999 | 6.931 | 6.51 |
| N | 29 | 29 | 29 | 29 | 29 | 29 | 28 | 29 | 29 | 28.89 |
| **DV** | | | | | | | | | | |
| A | 5 | 14 | 10 | 5 | 3 | 21 | 11 | 10 | 18 | 10.78 |
| $H_E$ | 0.781 | 0.905 | 0.841 | 0.719* | 0.351* | 0.951 | 0.832* | 0.883 | 0.933 | 0.8 |
| $H_O$ | 0.742 | 0.71 | 0.839 | 0.161 | 0.226 | 0.935 | 0.774 | 0.742 | 0.839 | 0.66 |
| $A_R$ | 5 | 13.677 | 9.613 | 4.992 | 2.992 | 20.385 | 10.701 | 9.887 | 17.653 | 10.54 |
| N | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31 |
| **MC** | | | | | | | | | | |
| A | 10 | 18 | 11 | 14 | 17 | 25 | 16 | 16 | 22 | 16.56 |
| $H_E$ | 0.657 | 0.951* | 0.702 | 0.862* | 0.917 | 0.94 | 0.933 | 0.916* | 0.956 | 0.87 |
| $H_O$ | 0.633 | 0.767 | 0.767 | 0.414 | 0.8 | 0.9 | 0.8 | 0.9 | 0.933 | 0.77 |
| $A_R$ | 9.793 | 17.853 | 10.6 | 13.86 | 16.716 | 24.056 | 15.79 | 15.53 | 21.516 | 16.19 |
| N | 30 | 30 | 30 | 29 | 30 | 30 | 30 | 30 | 30 | 29.89 |

| Population | Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Omy*77 | *Ots*103 | *Oki*29 | *Ots*108 | *Ots*100 | *Oki*10 | *One*108 | *One*110 | *One*103 | Average |
| **SL** | | | | | | | | | | |
| A | 9 | 19 | 16 | 16 | 16 | 32 | 14 | 15 | 36 | 19.22 |
| $H_E$ | 0.759 | 0.926 | 0.847 | 0.903* | 0.867 | 0.963 | 0.825 | 0.9 | 0.971* | 0.89 |
| $H_O$ | 0.674 | 0.907 | 0.872 | 0.333 | 0.787 | 0.935 | 0.778 | 0.851 | 0.915 | 0.78 |
| $A_R$ | 7.675 | 16.849 | 14.37 | 14.792 | 14.417 | 26.285 | 11.777 | 13.478 | 29.605 | 16.58 |
| N | 46 | 43 | 47 | 42 | 47 | 46 | 45 | 47 | 47 | 45.56 |
| **AL** | | | | | | | | | | |
| A | 12 | 24 | 23 | 17 | 20 | 38 | 16 | 17 | 54 | 24.56 |
| $H_E$ | 0.843 | 0.933 | 0.7 | 0.810* | 0.897 | 0.966* | 0.874 | 0.891 | 0.968 | 0.88 |
| $H_O$ | 0.759 | 0.872 | 0.725 | 0.416 | 0.835 | 0.825 | 0.825 | 0.887 | 0.975 | 0.79 |
| $A_R$ | 9.178 | 17.616 | 15.345 | 12.784 | 15.247 | 27.493 | 12.162 | 12.771 | 32.047 | 17.18 |
| N | 79 | 78 | 80 | 77 | 79 | 80 | 80 | 80 | 80 | 79.22 |
| **GC** | | | | | | | | | | |
| A | 7 | 13 | 12 | 8 | 12 | 16 | 6 | 9 | 23 | 11.78 |
| $H_E$ | 0.747 | 0.702 | 0.701* | 0.732* | 0.843 | 0.884 | 0.776 | 0.741 | 0.872 | 0.78 |
| $H_O$ | 0.8 | 0.683 | 0.585 | 0.067 | 0.725 | 0.829 | 0.675 | 0.61 | 0.775 | 0.64 |
| $A_R$ | 6.669 | 10.891 | 9.97 | 7.926 | 10.113 | 13.706 | 5.7 | 7.604 | 18.513 | 10.12 |
| N | 40 | 41 | 41 | 30 | 40 | 41 | 40 | 41 | 40 | 39.33 |
| **PC** | | | | | | | | | | |
| A | 10 | 13 | 17 | 12 | 12 | 25 | 14 | 18 | 22 | 15.89 |
| $H_E$ | 0.852 | 0.898 | 0.708 | 0.787* | 0.781 | 0.951* | 0.87 | 0.928 | 0.951 | 0.86 |
| $H_O$ | 0.806 | 0.889 | 0.714 | 0.241 | 0.694 | 0.833 | 0.778 | 0.972 | 1 | 0.77 |
| $A_R$ | 9.286 | 12.311 | 15.078 | 11.894 | 10.861 | 23.029 | 13.163 | 16.795 | 20.929 | 14.82 |
| N | 36 | 36 | 35 | 29 | 36 | 36 | 36 | 36 | 34 | 34.89 |

#### 3.3.1.4 Unique alleles in "black" kokanee

Most unique alleles were found at low frequency and most occurred in Anderson Lake and not in Seton Lake (Table 3.3). The total frequency of unique alleles found in Anderson Lake was 0.0086 and in Seton Lake was 0.0050. A majority (68.5%) of unique alleles found in the "black" kokanee populations were found at *Oki*10 (frequency = 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 "black" kokanee were typically also shared with Portage Creek sockeye salmon rather than Gates Creek sockeye salmon.

Table 3.3. Overall frequency of unique alleles per locus in "black" kokanee from Anderson Lake (AL), only Seton Lake (SL) or unique to both populations of "black" kokanee (Both). Frequencies calculated from the total number of alleles/locus/population.

| | Frequency of unique alleles | | |
|---|---|---|---|
| | AL | SL | Both |
| *Omy*77 | 0.004 | 0.002 | 0.005 |
| *Ots*103 | 0.008 | 0.002 | 0.000 |
| *Oki*29 | 0.011 | 0.011 | 0.017 |
| *Ots*108 | 0.008 | 0.002 | 0.000 |
| *Ots*100 | 0.002 | 0.000 | 0.000 |
| *Oki*10 | 0.007 | 0.008 | 0.017 |
| *One*108 | 0.007 | 0.008 | 0.002 |
| *One*110 | 0.002 | 0.005 | 0.003 |
| *One*103 | 0.037 | 0.012 | 0.011 |

*3.3.2 Among population microsatellite variation:*

### 3.3.2.1 $F_{ST}$ comparisons

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 when all seven populations were considered together.

The mean level of pairwise divergence (estimated using θ) among all seven populations and all nine loci was 0.080 (95% confidence intervals 0.051 – 0.112) (Table 3.4). Chedakuz Creek and Gate Creek consistently had the highest level of mean pairwise divergence from the other populations, 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 $F_{ST}$ amongst populations from this system was 0.041 (95% confidence intervals 0.029 – 0.055). Gates Creek showed the greatest divergence from all other populations, $F_{ST}$ = 0.071 (range = 0.059 – 0.083). All pairwise comparisons between populations, including the outgroup populations ($P < 0.014$) and without them ($P < 0.020$), were significant after adjusting for multiple tests (Narum 2006) (Table 3.4). Anderson and Seton lake "black" kokanee and Portage Creek sockeye salmon consistently showed the lowest mean level of divergence from each other ($F_{ST}$ = 0.019 and 0.035, respectively, all $P < 0.020$).

When I removed the *Ots*108 locus, the mean level of divergence among all populations was $F_{ST}$ = 0.069 (95% confidence intervals 0.046 – 0.096) and all pairwise comparisons were significant after adjusting for multiple comparisons ($P < 0.014$). When only the Anderson and Seton lakes system populations were compared, the mean level of divergence across the eight loci was $F_{ST}$ = 0.036 (95% confidence intervals 0.027 – 0.045) and all pairwise comparisons were significant after adjusting for multiple comparisons ($P < 0.020$). The program FreeNA

(Chapuis and Estoup 2007) was used to estimate $F_{ST}$ taking into account null alleles. These results did not change the significance levels of my results using all nine loci or after removing *Ots*108.

Considering the low level of differentiation between the two "black" kokanee populations, I used a *G*-test, which evaluates the distribution of alleles among populations to test for genetic differentiation and which is typically more powerful than $F_{ST}$-based analyses (Rousset 2013). Three of the nine loci were found to be significantly different between the two populations (*Omy*77, *Oki*29, and *One*108, $P < 0.05$). Using all nine loci, the "black" kokanee populations in Anderson and Seton lakes were significantly different from each other ($P < 0.05$). The results did not change after removing *Ots*108.

Table 3.4. Pairwise $F_{ST}$ comparisons between Anderson/Seton lakes (Fraser River) "black" kokanee (*) and sockeye salmon, Tatelkuz Lake (Fraser River) kokanee and Kootenay Lake (Columbia River) kokanee (*Oncorhynchus nerka*) using nine microsatellite loci (including *Ots*108). 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 ($P < 0.0137$).

| | **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 |
| **PC** | | | | | | |

#### 3.3.2.2 Bayesian analysis of population structure

The STRUCTURE analysis using all seven populations and nine loci suggested that $K = 6$ was the most likely number of populations and it grouped Anderson Lake and Seton Lake "black" kokanee into one population (supported by the log-likelihood method Table 3.5, Fig. 3.2). The $\Delta K$ method supported $K = 2$ as the most likely number of populations and $K = 6$ as the second most likely. A $K = 2$ highlights the high level differentiation of Gates Creek sockeye salmon and Chedakuz Creek kokanee (Fig. 3.2). Portage Creek sockeye salmon (and to a lesser extent Gates Creek) showed signs of admixture with the "black" kokanee populations, but were suggested to be separate genetic populations. This is consistent with my $F_{ST}$ analysis and DAPC results (see below). The Chedakuz Creek and Davidson Creek kokanee populations (Tatlekuz Lake system) grouped separately but showed a small amount of admixture with one another. As a Columbia River outgroup, Meadow Creek kokanee also formed their own genetic population. Removing the *Ots*108 locus returned the same results.

When the Anderson and Seton lakes system populations were analyzed alone using all nine loci, a $K = 3$ was supported by the log-likelihood method (grouping "black" kokanee together, Fig. 3.3). The $\Delta K$ method supported a model of $K = 2$ (Table 3.5) grouping Portage Creek sockeye salmon and the two "black" kokanee populations together. This is consistent with the low levels of differentiation between these populations, the clustering observed in the DAPC analysis (see below) and the Gates Creek population being quite distinct. The results were the same with and without *Ots*108. Neither the log-likelihood method nor the $\Delta K$ method supported a model of $K = 4$ associated with separation of the two "black" kokanee populations although slight distinction is observed in their patterns of admixture (Fig. 3.4).

Table 3.5. Results of ten independent runs in STRUCTURE testing for the most likely number of genetic groups (*K*) using the posterior probability (mean LnP(*K*)) and the Evanno et al. method (*ΔK*). NA = not applicable. "*" represents independent runs using all nine loci and all seven populations and "◆" represents independent runs using all nine loci and four populations from the Anderson and Seton lakes system with *K* ranging from one to seven

| K | Mean LnP(K)* | *ΔK* * | Mean LnP(K)◆ | *ΔK*◆ |
|---|---|---|---|---|
| 1 | -14327.43 | NA | -9859.51 | NA |
| 2 | -13638.24 | 31.410751 | -9511.12 | 19.500255 |
| 3 | -13230.04 | 5.99219 | -9466.71 | 1.228284 |
| 4 | -12950.46 | 16.084129 | -9482.21 | 0.929703 |
| 5 | -12853.32 | 0.581451 | -9553.79 | 1.351255 |
| 6 | -12627.09 | 18.011541 | -9538.12 | 1.036939 |
| 7 | -12819.49 | 1.05612 | -9742.43 | NA |
| 8 | -12788.13 | NA | NA | NA |

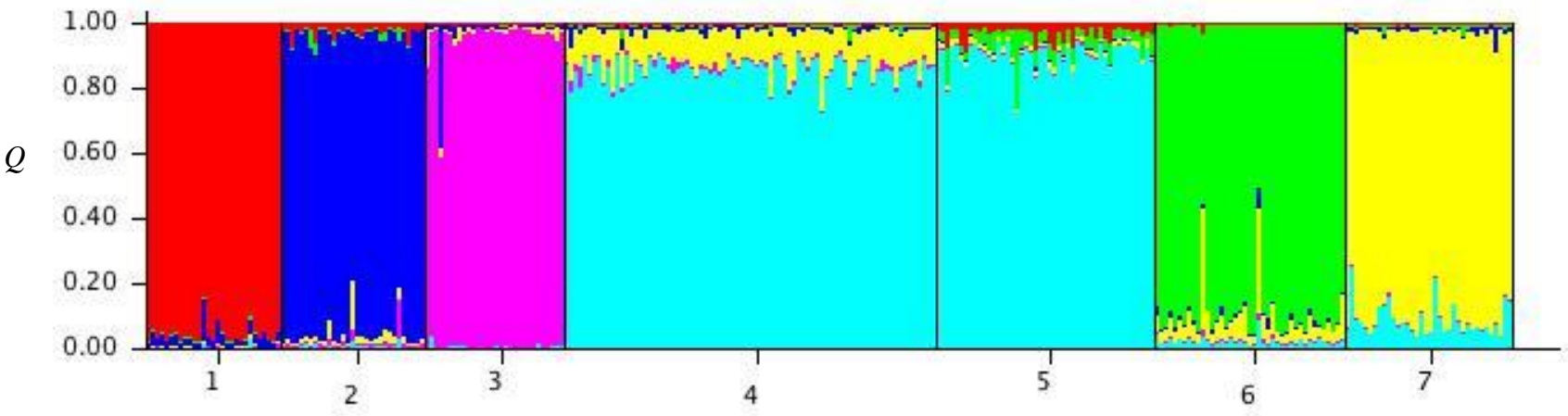


Figure 3.2. The STRUCTURE bar plot for seven populations (x axis) of *Oncorhynchus nerka* (sockeye salmon and kokanee) sampled from Chedakuz Creek (1), Davidson Creek (2), Meadow Creek (3), Anderson Lake (4), Seton Lake (5), Gates Creek (6), and Portage Creek (7) and assayed at nine microsatellite DNA loci. The most likely model was $K = 6$ (supported by log-likelihood), which grouped "black" kokanee into one genetic group (light blue). Each vertical line represents an individual fish and the different colours represent the genetic contribution ($Q$, y axis) of the six genetic groups.

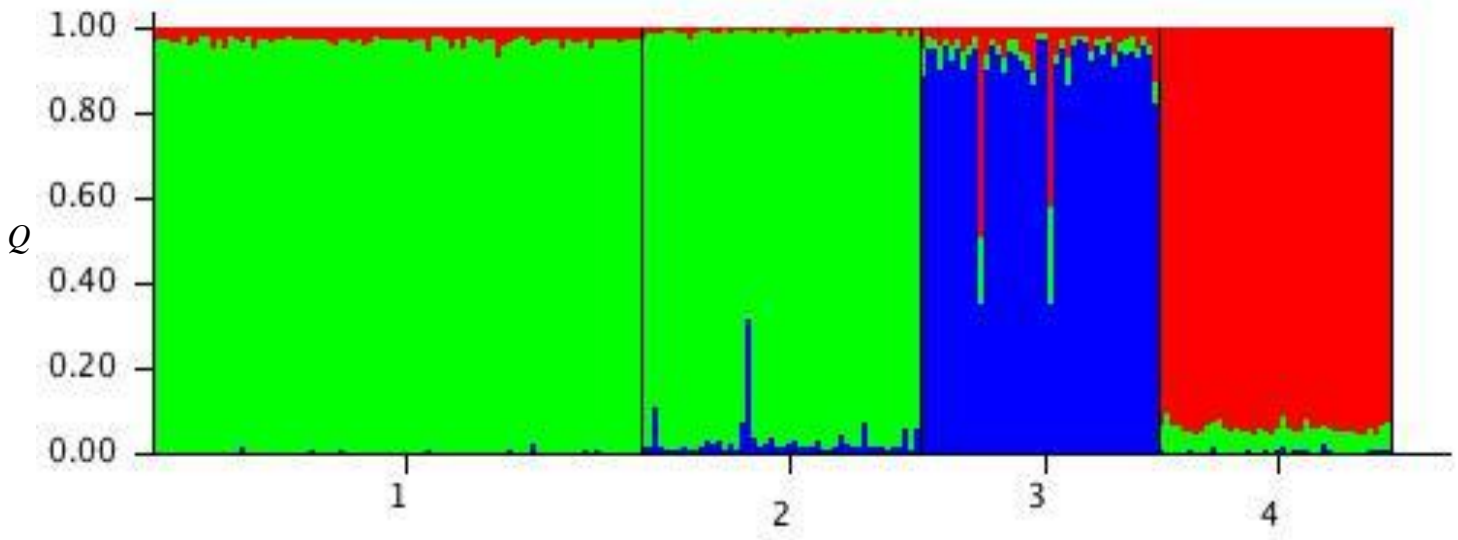


Figure 3.3. The STRUCTURE bar plot for "black" kokanee and sockeye salmon (*Oncorhynchus nerka*) sampled from the Anderson/Seton lakes watershed and assayed at nine microsatellite DNA loci. "black" kokanee from Anderson Lake (1), Seton Lake (2), and sockeye salmon from Gates Creek (3), Portage Creek (4). The most likely model was $K = 3$ (supported by log-likelihood), which grouped "black" kokanee into one genetic group (largely green). Relatively low admixture of allele frequencies with the other genetic groups (red and blue) demonstrates the uniqueness of "black" kokanee within the Anderson/Seton system, Fraser River drainage. Each vertical line represents an individual fish and the different colours represent the genetic contribution ($Q$, y axis) of the three genetic groups.

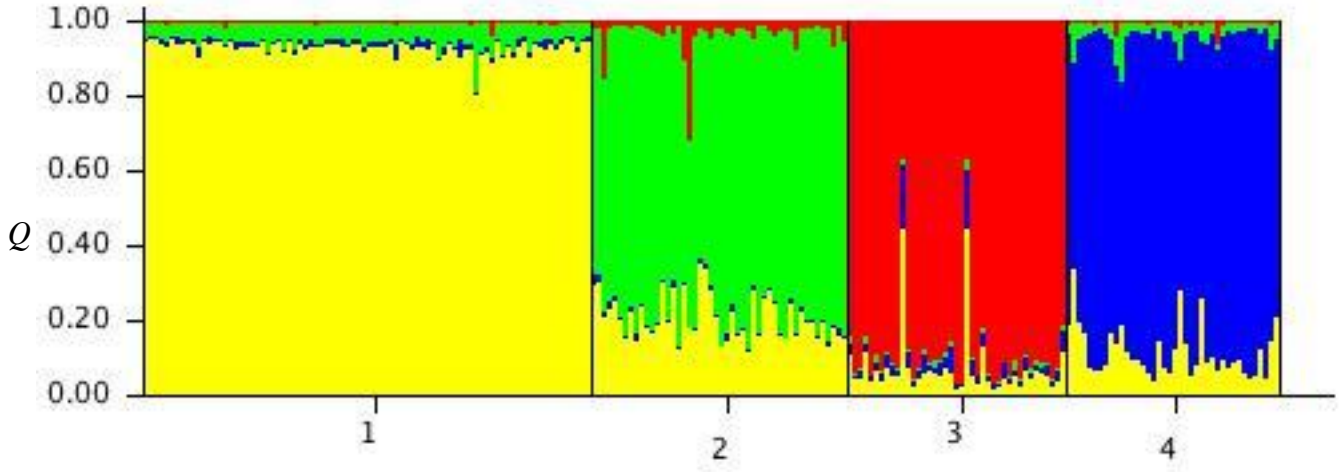


Figure 3.4. The STRUCTURE bar plot for "black" kokanee and sockeye salmon (*Oncorhynchus nerka*) sampled from the Anderson/Seton lakes watershed and assayed at nine microsatellite DNA loci. "Black" kokanee from Anderson Lake (1), Seton Lake (2), and sockeye salmon from Gates Creek (3), Portage Creek (4). In this case, $K = 4$ was modeled which reveals some distinction between the two "black" kokanee populations (1, 2).

#### 3.3.2.3 DAPC analysis

The DAPC analysis using all nine loci highlighted the differences and similarities among the seven populations retaining 95 principal components (PCs) representing 90% of the total variation in the data set across six discriminant functions (Fig. 3.5). The DAPC plots illustrated the distinctiveness of the Chedakuz Creek and Gates Creek populations, and, similarly to the STRUCTURE results, "black" kokanee clustered closely with Portage Creek sockeye salmon. 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 other *O. nerka* (Fig. 3.6). The same results were observed after removing the *Ots*108 locus. 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 both DAPC plots indicates that they are genetically similar to one another.

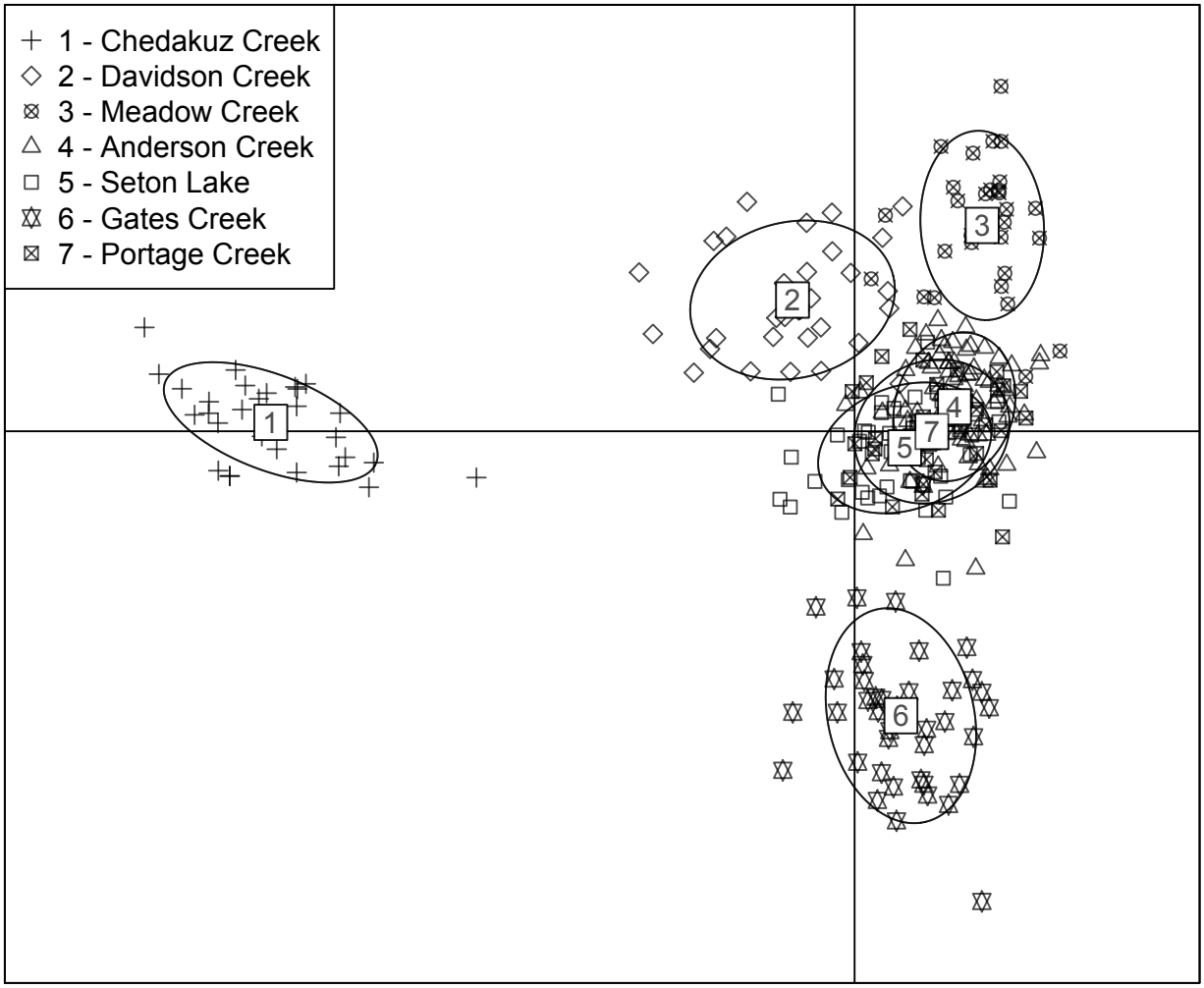


Figure 3.5. Discriminant analysis of principal components (DAPC) highlighting the genetic variability among populations and minimizing variation within populations among seven *Oncorhynchus nerka* populations assayed at nine microsatellite loci. Ninety percent of the variation was retained as by 95 principal components. Takelkuz Lake watershed (Fraser River): Chedakuz Creek kokanee (1), Davidson Creek kokanee (2), Kootenay Lake watershed (Columbia River): Meadow Creek kokanee, and Anderson/Seton watershed (Fraser River): (4) Anderson Lake "black" kokanee, (5) Seton Lake "black" kokanee, (6) Gates Creek sockeye salmon and (7) Portage Creek sockeye salmon.

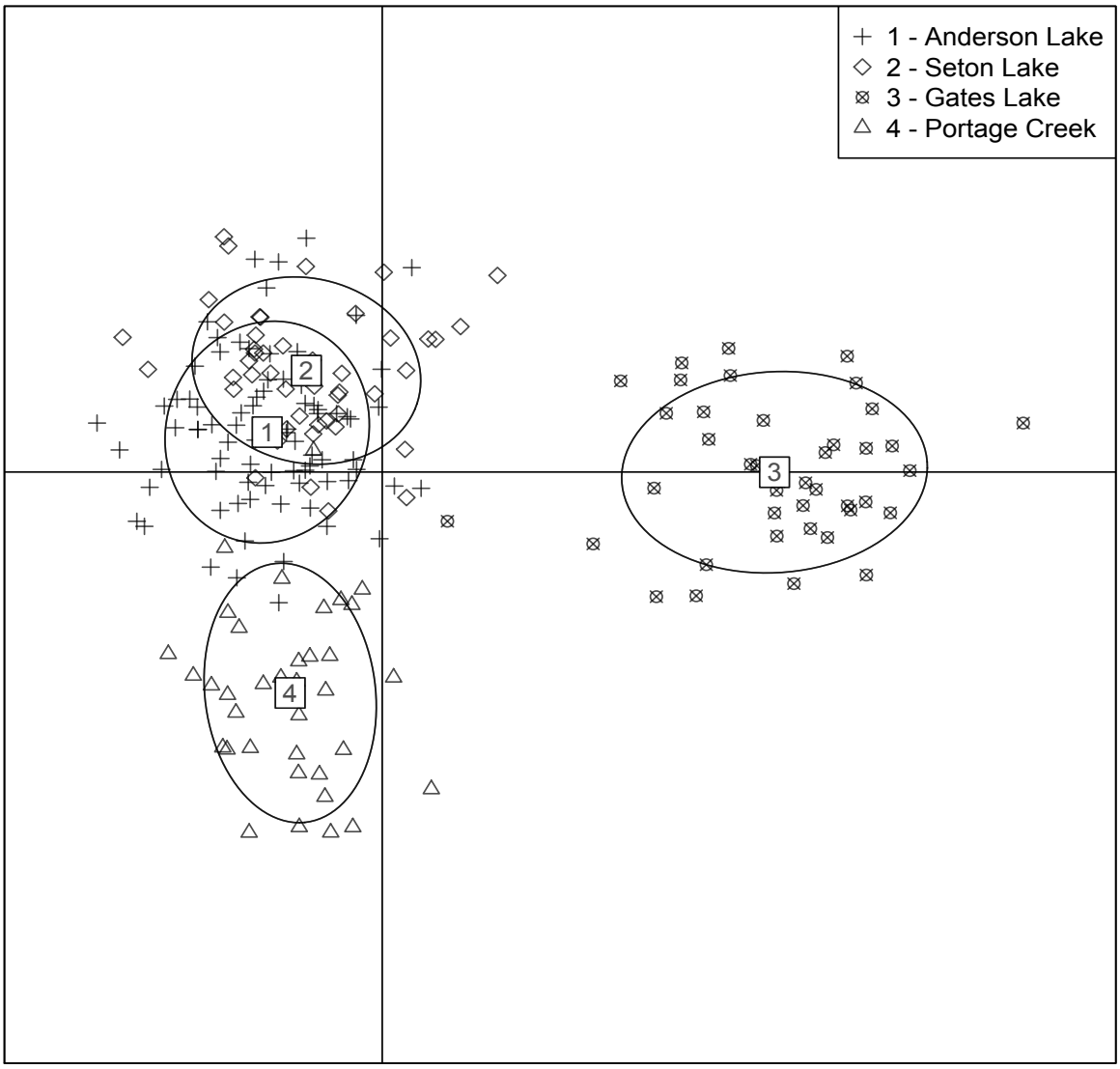


Figure 3.6. Discriminant analysis of principal components (DAPC) highlighting the genetic distinctiveness of "black" kokanee populations from: (1) Anderson Lake and (2) Seton Lake from that of sockeye salmon from: (3) Gates Creek and (4) Portage Creek sockeye salmon (Fraser River drainage), (*Oncorhynchus nerka*) assayed at nine microsatellite loci. Eighty-five percent of the variation was retained as 68 principal components.

#### 3.3.2.4 Hierarchical analysis of genetic structure

When the microsatellite variation 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%, $P < 0.001$) (Table 3.6). Among populations-within form variation accounted for 4.1% of the total variation ($P < 0.001$). Between "black" kokanee and sockeye salmon variation accounted for only 0.32% of the total variation, but was significant ($P < 0.001$). The magnitude or significance of the results did not change when I removed *Ots*108.

Table 3.6. Microsatellite variation using nine loci partitioned hierarchically using analysis of molecular variance in Anderson and Seton Lake "black" kokanee and sockeye salmon (*Oncorhynchus nerka*). Among form variation represents the difference between "black" kokanee and sockeye salmon.

| Source of Variation | % variation | F-value | P-value |
|---|---|---|---|
| Within population | 95.57 | 0.044 | < 0.001 |
| Among all populations | 4.12 | 0.041 | < 0.001 |
| Between forms | 0.32 | 0.003 | < 0.001 |

### *3.3.3 Morphological differentiation:*

#### 3.3.3.1 Within population variation

On average, males were larger (standard length and adjusted maximum body depth) than females in both lakes for (Table 3.7). Tests for differences between the sexes within each population (Seton Lake, Anderson Lake 2013, Anderson Lake 2014) for standard length and size-adjusted maximum body were all significant ($P < 0.05$). Mean gill raker counts did not differ between the sexes in either lake (both $P > 0.05$).

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).

Table 3.7. 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. All measurements are in mm (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 mm and 191.3 mm for males and females, respectively). Gill rakers (GR) were not sexually dimorphic. "*" represents significant difference between sampling years (P < 0.001). "◆" represents significant difference between the lakes (P < 0.001).

| Trait | Anderson Lake 2013 | Anderson Lake 2014 | Seton Lake 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) |

#### 3.3.3.2 Between population variation

Results from the analysis of covariance (ANCOVA) demonstrated that the slopes of maximum body depth versus length (using $\log_{10}$ values) were not significantly different 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 $\log_{10}$ length regression line using all samples but separated for males (b = 1.200) and females (b = 1.084). Adjusting maximum body depth to a common standard length separately for males and females removed any positive correlation between depth and length in either lake ($r < 0.100$, $P > 0.5$) therefore effectively controlling for any effects of body size.

Standard length was significantly different between sampling years for males (P = 0.0004) and females (P = 0.010) in Anderson Lake 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 between years within Anderson Lake, they were minor compared to differences between lakes so I combined the temporal samples to highlight the striking difference in size of maturity between the lakes. Standard length was significantly different between Anderson Lake (combined years) and Seton Lake both for males and females ($P < 0.0001$, Fig. 3.7, 3.8) while size-adjusted maximum body depth was not ($P > 0.9$, Table 3.7).

Mean gill raker number was found to be 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 sampling years in Anderson Lake ($P > 0.05$). The number of gill rakers ranged from 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 3.7).

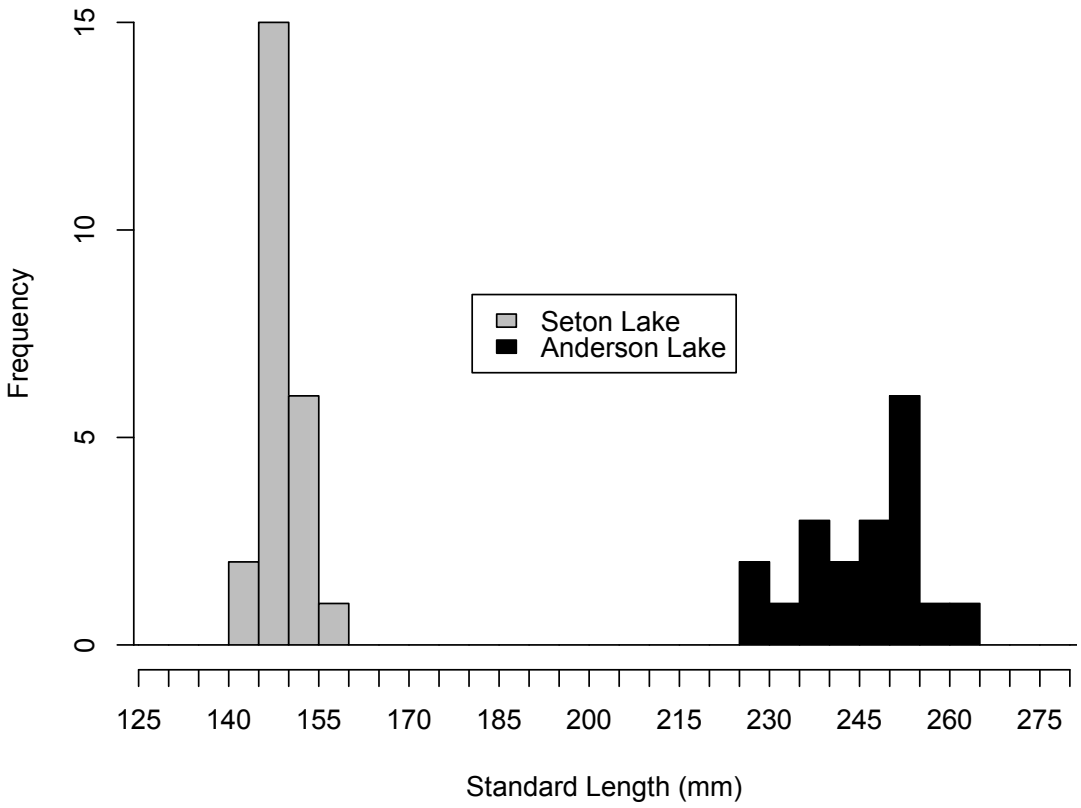


Figure 3.7. Frequency distribution of standard lengths highlighting the differences in standard length (mm) between female kokanee (*Oncorhynchus nerka*) sampled from Anderson Lake (combined 2013 and 2014 data sets), mean = 245.45 mm (95% confidence intervals ± 4.86 mm) and Seton Lake, 148.40 mm (± 1.51 mm) ($P < 0.0001$).

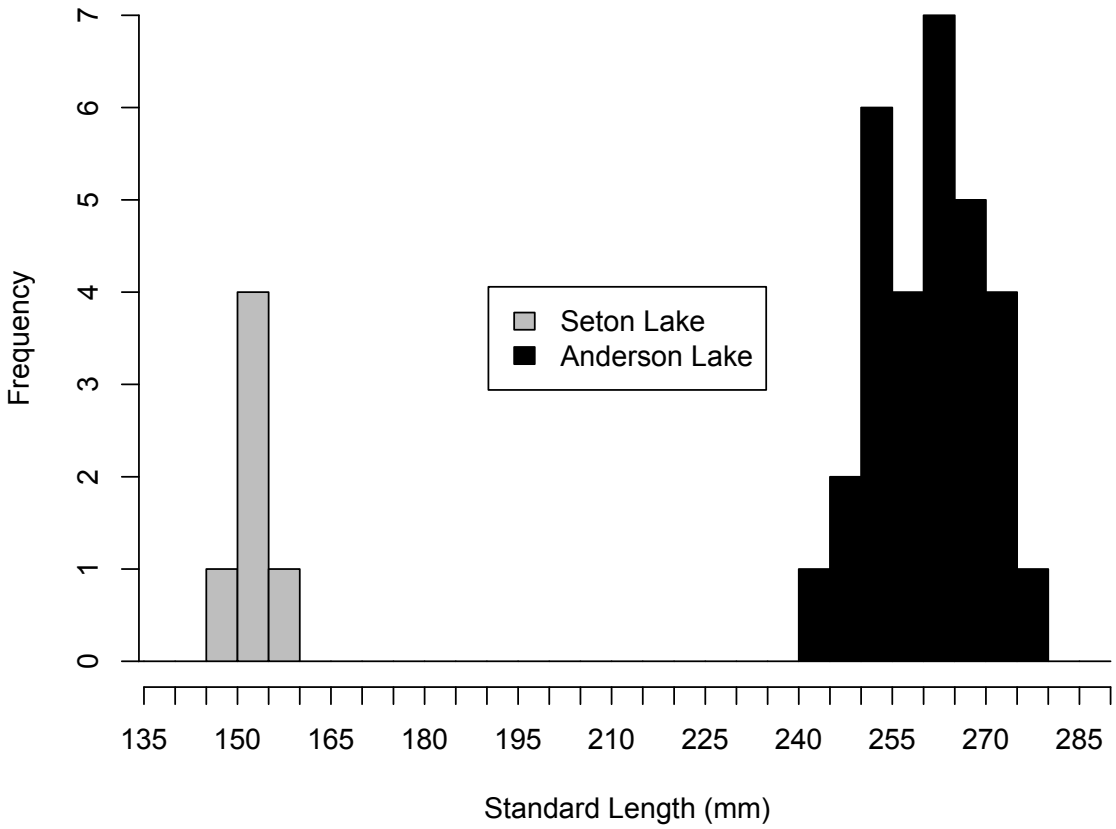


Figure 3.8. Frequency distribution of standard length highlighting the statistically significant differences in standard length (mm) between male kokanee (*Oncorhynchus nerka*) sampled from Anderson Lake (combined 2013 and 2014 data sets), 260.36 mm (± 3.19 mm) and Seton Lake, 152.24 mm (± 2.95 mm) ($P < 0.0001$).

## 3.4 Discussion

### *3.4.1 Within population genetic variation*

Levels of within population variation in the Anderson and Seton lakes system were comparable, on average, to other population genetic studies of kokanee and sockeye salmon although comparisons should be used with some caution as different loci were used and each study is unique in terms of sample sizes and geographic scale of study. For example, Taylor et al. (2000) surveyed Okanagan Lake beach and stream spawning kokanee using eight microsatellite loci

(three of which were used in my analysis) and reported five to 23 alleles per locus and expected heterozygosities ranging from 0.49 to 0.91 (averaged across the six populations studied). In comparison to Anderson Lake and Seton Lake populations, the number of alleles per locus corrected for sample size ranged from 10 to 17 and expected heterozygosities ranged from 0.78 to 0.89. Comparing individual populations in the Okanagan Lake study of similar sample sizes, Anderson Lake and Seton Lake *O. nerka* had, on average, more alleles per locus. Furthermore, Young et al. (2004) studied 11 populations of *O. nerka* (sockeye salmon and kokanee) in the Lake Sammamish/Lake Washington basin using nine microsatellite loci; three of the same loci were used in my analysis. They reported relatively high levels of diversity with the mean number of alleles per locus ranging from 5 to 49 and heterozygosities averaged across populations ranging from 0.74 to 0.96 (Young et al. 2004). These comparisons suggest that within population variation between Anderson and Seton lakes *O. nerka* and that of spawning ecotypes of kokanee (Okanagan Lake system) and sympatric kokanee and sockeye salmon (Lake Sammamish/Lake Washington basin) are comparable across broad geographic areas.

### *3.4.2 Among population genetic variation*

My results demonstrate 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 discriminant analysis of principal components (DAPC). Comparing levels of population differentiation between studies should be interpreted with some caution because of the different markers used, the number of populations surveyed and the geographic scale of the study. Nevertheless, sympatric populations of kokanee and sockeye salmon are known to be genetically distinct even when they spawn at the same time

and in the same stream. For example, Foote et al. (1989) found allele frequency differences between sympatric kokanee and sockeye salmon in Takla, Babine and Shuswap lakes in British Columbia. Similar results were reported by Taylor et al. (1996) in the same three lakes as well as in other sympatric populations ranging from Kamchatka to the Columbia River using minisatellite loci. 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 to 0.5% variation within forms between tributaries.

Young et al. (2004) found significant genetic divergence (0.071, measured as coancestry distances, i.e., $d = -\ln(1-\theta)$; Weir 1996) using nine microsatellite loci between sympatric Issaquah Creek (Lake Sammamish basin, Washington State) kokanee and sockeye salmon. Differentiation may be the result of different spawning times between kokanee (early August) and sockeye salmon (late October) (Young et al. 2004). Interestingly, this level of differentiation is comparable to the level of differentiation between Anderson and Seton lakes "black" kokanee and Gates Creek sockeye salmon when measured as coancestry distances (0.072 and 0.061, respectively).

Levels of differentiation between sympatric forms of *O. nerka* may represent a widespread phenomenon within the species that have only recently become apparent with current advances in genetic analysis. Frazer and Russello (2013) analyzed differentiation between sympatric beach and stream spawning kokanee in five British Columbia lakes (Wood, Kootenay, Okanagan, Duncan, and Tcheskinut lakes) using 15 neutral loci and 15 expressed sequence tag (EST) linked loci that exhibit signatures of directional selection. Using only neutral loci, significant levels of differentiation were found in Okanagan, Kootenay, and Wood lakes ($F_{ST}$ = 0.005, 0.008, and 0.032, respectively) (Frazer and Russello 2013). In contrast, divergence

between beach and stream spawning kokanee in two other lakes was found to be virtually non-existent ($F_{ST}$ = -0.001, and 0.000 in Duncan and Tcheskinut lakes, respectively, Frazer and Russello 2013). Using lake-specific EST linked loci, levels of divergence were found to be much greater between beach and stream-spawning kokanee in each lake: Duncan $F_{ST}$ = 0.017, Kootenay $F_{ST}$ = 0.071, Okanagan $F_{ST}$ = 0.081, Wood $F_{ST}$ = 0.126, and Tcheskinut $F_{ST}$ = 0.074 (Fraser and Russello 2013). Using loci that may be influenced by natural selection can be effective for management when neutral loci demonstrate modest divergence as a result of recent divergence or weak population structure (Russello et al. 2011). Levels of neutral genetic differentiation between sympatric "black" kokanee and sockeye salmon in the Anderson/Seton lakes system are generally comparable (and higher than in some cases) to differences between sympatric beach and stream spawning kokanee (cf. Frazer and Russello 2013). In addition, Taylor et al.'s (2000) microsatellite analysis of beach and stream spawning kokanee in Okanagan Lake reported an $F_{ST}$ = 0.018 as the level of population differentiation which is slightly lower than I observed between sympatric sockeye salmon and "black" kokanee in the Anderson/Seton lakes system ($F_{ST}$ = 0.023).

Two of the three outgroups used in my analysis were chosen based on their relative distinctiveness within their respective watersheds; providing an appropriate comparison to evaluate the distinctiveness of "black" kokanee. Kokanee from upper Chedakuz Creek were found to be highly distinct within the Tatelkuz Lake system (Taylor 2013). Upper Chedakuz Creek kokanee had an average level of divergence of $F_{ST}$ = 0.148 when compared to seven other kokanee populations within Tatelkuz Lake system using six of the same loci used in my study compared to an average level of differentiation among the other kokanee populations of from $F_{ST}$ = 0.0517 to 0.0685 (Taylor 2013). Taylor (2013) suggested that the high divergence of the upper

Chedakuz Creek's kokanee was related to greater average distance from the other populations in the watershed and because it was part of a different, but interconnected, lake system (Kuyakuz Lake). Upper Chedakuz Creek kokanee were also larger than the other populations of kokanee, which highlights their distinctiveness within the watershed (Taylor 2013).

Kokanee from Meadow Creek of the West Arm of Kootenay Lake (Columbia River system) are also known to be distinct, with an average level of divergence of $F_{ST} = 0.018$, when compared to four other Kootenay Lake populations, two of which were stocked with Meadow Creek kokanee (Anders et al. 2007). Meadow Creek kokanee's relatively high level of divergence in the Kootenay Lake system may be related to its distant location at the head of the north arm of the lake (Anders et al. 2007). Both Chedakuz Creek and Meadow Creek kokanee are distinct within their respective watershed, some of which can be attributed, at least in part, to their relatively isolated locations. These outgroups provide context to the levels of differentiation found in "black" kokanee.

Within the Anderson/Seton lake system, the average level of divergence of "black" kokanee from Portage Creek and Gates Creek sockeye salmon was 0.022 and 0.062, respectively. Given the level of differentiation between Portage Creek and Gates Creek sockeye salmon and "black" kokanee, temporal and spatial segregation could account for the observed divergence between these ecotypes of *O. nerka*. For instance, spawning timing and location are both known to cause genetic differentiation between sympatric populations of *O. nerka* (Taylor et al. 2000; Fillative et al. 2003; Lin et al. 2008; Muto et al. 2012; Frazer and Russello 2013) and other species within *Oncorhynchus* (McGregor et al. 1998; Hendry et al. 2002). In Anderson and Seton lakes, both sockeye salmon populations are stream spawners with different spawning times both from each other and "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 mixed stock fisheries analysis using 14 microsatellites and four single nucleotide polymorphism loci (Steve Latham, Pacific Salmon Commission, Vancouver, pers. comm.). 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 sympatric sockeye salmon. The "mosaic" population structure of *O. nerka,* whereby neighboring lake populations are not always the most similar genetically to one another (Wood 1994, 1995; Beacham et al. 2004), could explain this pattern (Wood 1995; Withler et al. 2000).

Consistent with my analysis, several studies (e.g., Withler et al. 2000, Beacham et al. 2004, 2006) have found Gates Creek sockeye salmon to be quite distinct not only from other Fraser River populations, but also those throughout its range. Withler et al. (2000) reported that Gates Creek sockeye salmon had fewer alleles per locus than other Fraser River populations and a low proportion of rare alleles. Additionally, most pairwise $F_{ST}$ comparisons between Gates Creek sockeye salmon populations and other Fraser River sockeye salmon populations were greater than 0.100 (Withler et al. 2000). Withler (1982) attributed the low diversity of Gates Creek sockeye salmon and their atypical allele frequencies to founder events and/or random genetic drift as a result of a small population size. Another possible explanation, although unlikely, could be the effect of previous transplantation of over 15 million eggs and juveniles from the Birkenhead River (lower Fraser River) to Gates Creek between 1915-1930, but there is

no evidence to support that this transplantation effort was successful or resulted in introgression (Withler 1982; Withler et al. 2000).

Portage Creek has a more successful history of transplantation. In 1950, 300,000 eyed eggs were transplanted from the lower Adams River (Thompson River drainage) to Portage Creek and 193,000 juveniles were transplanted into Anderson Lake (Aro 1979; Withler et al. 2000). Prior to the transplant, there was a small, native population in Portage Creek. While there is no evidence for substantial introgression, there is a high degree of genetic similarity between Portage Creek sockeye salmon and the lower Adams River reflecting some level of gene flow following these transplantations (Withler et al. 2000).

Interestingly and consistent with both the $F_{ST}$, DAPC and STRUCTURE analyses, "black" kokanee within both Anderson and Seton lakes tended to be more similar to the Portage Creek sockeye salmon rather than the sockeye salmon from Gates Creek (Table 3.4; Figs. 3.3, 3.6). The observed level of admixture between the "black" kokanee populations and Portage Creek sockeye salmon may reflect some level of gene flow between the populations, but it is unknown whether this is reflective of pre-transplantation conditions or some effect of transplantation of sockeye from other systems (see above). Alternatively, the observation that Gates Creek sockeye salmon are so highly differentiated from most Fraser River populations could result in "black" kokanee grouping with Portage Creek sockeye salmon more as a consequence of the highly distinct nature of Gates Creek sockeye salmon than any real affinity with Portage Creek sockeye salmon *per se*. Regardless, "black" kokanee as a group are significantly distinct from sympatric sockeye salmon, as revealed by the hierarchical analysis of genetic structure.

### *3.4.3 Anderson and Seton lakes "black" kokanee*

For most loci, Anderson Lake "black" kokanee had a higher number of unique alleles compared to Seton Lake "black" kokanee (Table 3.3). This is most likely due to the large sample size after combining temporal samples. Both populations did share a relatively high frequency of alleles that were not found in the other *O. nerka* populations sampled, especially at *Oki*10, *Oki*29, and *One*103 (Table 3.3).

The level of differentiation between "black" kokanee populations was modest, but statistically significant using all nine loci and without *Ots*108. This result was consistent across all analyses including the DAPC and STRUCTURE analyses that most clearly demonstrated their genetic similarity to one another (Fig. 3.4, 3.6). Additionally, using genetic diversity as a test statistic (*G*-test) showed that the two "black" kokanee populations are significantly different at three loci (*Omy*77, *Oki*29, and *One*108) and when all loci are considered together. These modest levels of differentiation are comparable (and higher in some instances) to the levels of differentiation found using neutral loci between beach and stream spawning kokanee (see above, Fraser and Russello 2013). These modest levels of differentiation may result from a recent, post-glacial time frame for divergence, recent and/or historical gene flow between "black" kokanee populations or high effective population sizes. For instance, *O. nerka* colonized the Anderson and Seton lakes system about 11,000 years ago and adult population size were recently estimated at 5,000 in Seton Lake and 150,000 in Anderson Lake (Wood 1995; Morris and Caverly 2004).

The relatively weak, but significant differentiation that was observed between the "black" 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. 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)

### *3.4.4 Morphological variation*

Perhaps one of the more fascinating results was the substantial size difference between mature "black" kokanee in the two lakes. A large proportion of Anderson Lake "black" kokanee mature at ages three and four years, which contrasts with that in Seton Lake. In Seton Lake, "black" kokanee mature predominately at age two with a smaller proportion at age three. When the fish are compared at a common age class of three, Anderson Lake fish were still found to be larger (218.22 mm fork length in Anderson Lake compared to 198.11 mm fork length in Seton Lake) (Morris and Caverly 2004). I also found Anderson Lake "black" kokanee were larger than Seton Lake "black" kokanee (254.61 mm and 149.16 mm, respectively), but I did not compare sizes at specific age classes.

A previous survey of Anderson Lake and Seton Lake "black" kokanee (Morris and Caverly 2004) that was conducted in the pre-spawning season (early June) found that Seton Lake fish were larger than Anderson Lake fish (189.00 mm mean fork length and 143.25 mm, respectively) when samples of the same age class were compared (age three). By contrast, Morris and Caverly (2004) later surveyed "black" kokanee after spawning and reported that Anderson Lake fish were larger than Seton Lake fish when the same age class was compared (see above). Potentially, this could suggest that the age-specific growth rates vary between the two lakes. Life history theory predicts that there are fitness related trade-offs between growth, reproduction and survival (Fisher 1930; Hirshfield and Tinkle 1975; Roff 1984). Furthermore, increased adult survival favours reduced reproductive effort and delayed maturation while increased juvenile

growth rate favours increased reproductive effort and early maturation (Hutchings 1993). Therefore, different growth rates between juvenile and adult "black" kokanee in both lakes may account for the observed age-specific size differences and age of maturity. Hutchings (1993) suggested that there might be age-specific variation in growth rate and survival among three populations of brook trout (*Salvelinus frontinalis*). Comparisons between Anderson and Seton lake "black" kokanee populations in terms of age of maturity and growth rates combined with information on age-specific survival and age-specific growth rates would help to elucidate the basis for the differences in age of maturity (Roff 1984; Hutchings 1993).

Differences in standard length (and associated age of maturation) of adult "black" kokanee spawners could potentially reflect adaptations to different spawning environments (Blair et al. 1993; reviewed by Taylor 1991 and Fraser et al. 2011). Seton Lake is turbid from the inflow of glacial water from the Bridge River and Carpenter Reservoir, but Anderson Lake is very clear. Additionally, Anderson Lake has a higher plankton density than Seton Lake (2,622 mg dry mass/$m^2$ and 422 mg dry mass/$m^2$, respectively) (Shortreed et al. 2001). Interestingly, Geen and Andrews (1961) and Shortreed et al. (2001) both found that a variable, but substantial proportion of Gates Creek sockeye salmon fry migrate straight through Anderson Lake to reside and feed in Seton Lake. For example in 2000, densities of juvenile sockeye salmon and kokanee in Anderson Lake were estimated, using hydroacoustic and trawl surveys, at 1,057 fish per hectare and otolith strontium to calcium ratios (used to infer seawater entry) indicated that less than 5% of juvenile *O. nerka* sampled in Anderson Lake were sockeye salmon. In comparison, Seton Lake juvenile densities were estimated at 289 fish per hectare (Shortreed et al. 2001). The lower estimated population density of adult Seton Lake "black" kokanee (only about 5,000 adults versus about 150,000 in Anderson Lake) could explain the lower juvenile densities and result in

lower competition for food in Seton Lake. Additionally, the Carpenter Reservoir and Bridge River diversions introduce sediment that may accumulate on the lake bottom, reducing the spawning habitat quality for "black" kokanee in Seton Lake (Morris et al. 2003).

The increased turbidity in Seton Lake probably decreases the visibility in the water column and thus the feeding efficiency of piscivorous fishes, particularly relative to that of planktivorous fishes like *O. nerka* (De Robertis et al. 2003). In Anderson Lake and Seton Lake, bull trout prey on "black" kokanee and juvenile sockeye salmon; therefore, the increased turbidity in Seton Lake may be beneficial for juvenile sockeye salmon because they will be less visible to predators, but their feeding rate will not be substantially affected (Morris and Caverly 2004; De Robertis et al. 2003). All together, the different biotic and abiotic conditions in Seton Lake could explain why a greater proportion of sockeye salmon juveniles feed in Seton Lake compared to Anderson Lake. Consequently, the younger age and smaller size at maturity in of "black" kokanee in Seton Lake may be attributed to the increased competition from sockeye salmon within the lake especially given the lower plankton biomass. Additional studies are needed to test these hypotheses.

Gill raker counts did not differ significantly between Anderson Lake and Seton Lake "black" kokanee. Gill raker counts are known to occasionally vary among kokanee populations within the same lake (e.g., Vernon 1957; Kurenkov 1977, but see Taylor et al. 1997). The lack of differences observed in my results suggests that the feeding ecology (size and composition of prey items) is similar between the populations of "black" kokanee in the two lakes. 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. These differences in gill raker counts 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 degree of colouration also varied between the two lakes. Anderson Lake fish exhibit a dramatic, uniform black colour at spawning and, unlike the Seton Lake fish, this colour did not fade after spawning. Seton Lake fish were lighter black to olive in colour, which faded to almost white on the ventral side after spawning. These differences in colour observed between the two lakes could result from water colour/transparency. Seton Lake is glacially turbid whereas Anderson Lake is very clear. Glacial turbidity can be associated with "brighter" coloured fish (e.g. Northrup et al. 2010). Further, the jaws and caudal fins of Seton Lake "black" kokanee were worn out or even missing, but Anderson Lake samples rarely had worn out caudal fins suggesting that the spawning substrate may be composed of large gravel or the spawners are removing sediment. The differences in physical damage from spawning could result from differences in spawning substrates and/or that broadcast spawning may occur in Anderson Lake, but not in Seton Lake (Morris and Caverly 2004).

### *3.4.5 Comparisons with the Japanese "black" kokanee (kunimasu)*

In Japan, stream-spawning kokanee population structure is characterized by low genetic differentiation with average levels of $F_{ST}$ ranging from -0.008 to 0.032 (Kogura et al. 2011). This low level of divergence is most likely due to intensive transplantation history and resultant mixing of diverse gene pools and perhaps to ancestral populations founded by a common gene pool because they are on the southern periphery of *O. nerka*'s geographic range in the western Pacific (Kogura et al. 2011). In Japan, kokanee populations are native to Lake Akan and Lake Shikotsu (on Hokkaido), but have been transplanted to more than 60 lakes with populations

being established in 22 lakes, including Lake Saiko (Yamamoto et al. 2011). Native spawning populations of sockeye salmon do not occur in Japan; the Abira River sockeye salmon population was derived from kokanee in Lake Shikotsu (Burgner 1991; Nakabo et al. 2011).

By contrast, striking pairwise differentiation was observed between sympatric "black" kokanee and stream spawning kokanee in Lake Saiko, Japan ($F_{ST}$ = 0.134) and between "black" kokanee and stream spawning kokanee in Lake Akan ($F_{ST}$ = 0.142) (Nakabo et al. 2011). In comparison, stream spawning kokanee in Lake Akan and Lake Saiko are dramatically less differentiated from each other ($F_{ST}$ = -0.0005) (Nakabo et al. 2011). While there is a strong level of divergence between "regular" kokanee and "black" kokanee in Japan, especially considering the low genetic diversity in other Japanese populations, their differentiation is consistent with levels of differentiation among some populations of sympatric and allopatric North American kokanee or sockeye salmon populations (Varnakskaya et al.1994; Beacham et al. 2004; Nakabo et al. 2011). By contrast, my results suggest that "black" kokanee in the Anderson/Seton lake system have weaker differentiation from sympatric sockeye salmon populations than that reported between Japanese "black" kokanee and sympatric "regular" kokanee (Nakabo et al. 2011). Although it is difficult to make direct comparisons owing to the different loci used in each of the studies, the apparently lower differentiation observed in the Anderson/Seton system may be due to differences in effective population sizes within each system resulting in lower genetic diversity or to the fact that some of the Japanese populations involve introduced populations. Additionally, the Anderson Lake and Seton Lake "black" kokanee could represent a more recent divergence compared to Japanese "black" kokanee.

Interestingly, the phenotypes of North American and Japanese "black" kokanee tended to be quite similar. The standard length of Japanese mature "black" kokanee males and females

ranged from 178.0 mm to 268.7 mm and 183.2 mm to 235.6 mm, respectively (Nakabo et al. 2014). These lengths are more comparable to Anderson Lake (males: 241.7 mm to 278.8 mm, females: 226.3 mm to 261.8 mm) than to Seton Lake "black" kokanee (males: 147.5 mm to 155.3 mm, females: 140.7 mm to 155.9 mm). Japanese "black" kokanee gill raker counts ranged from 36 to 45 (mean 39, Nakabo et al. 2014) compared to 34 to 42 gill rakers for Anderson and Seton lakes "black" kokanee. At maturity in both sexes, colouration was described as olive green to black when alive and becoming darker when dead (see Fig. 1.4 to 1.6; Nakabo et al. 2011, 2014). These phenotypic similarities provide further evidence to support the hypothesis that natural selection may be promoting parallel evolution of "black" kokanee phenotypes (see Chapter 2).

### *3.4.6 Conclusions*

In summary, my findings highlight the genetic and morphological distinction of "black" kokanee in Anderson Lake and Seton Lake 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 to the striking differences in age and size at maturity.

These results have several management and conservation implications. First, my data supports the potential classification of "black" kokanee as a distinct designatable unit (DU) within *O. nerka* under Canada's *Species at Risk Act*: (i) they are genetically distinct from sympatric sockeye salmon; (ii) their unusual reproductive ecology can reasonably be inferred to be the result of local adaptation; and (iii) Anderson Lake and Seton Lake are the only two known lakes in Canada that produce "black" kokanee (see Chapter 4). Furthermore, the modest, yet

significant, genetic differentiation between Anderson Lake and Seton Lake "black" kokanee in addition to their differences in size and age at maturity and spawning time suggests that the two populations are also demographically independent and that they might qualify as separate DUs. Overall, "black" kokanee exemplify novel diversity found within a taxon that is already renowned for spectacular levels of intraspecific diversity of evolutionary and conservation significance (e.g., Ricker 1972; Hendry 2001; Hilborn et al. 2003).

# Chapter 4: General Discussion

## 4.1 Summary of results

My thesis addresses some important questions regarding the unknown evolutionary origin of "black" kokanee and the genetic and phenotypic diversity they represent within the Anderson/Seton lakes system and within *Oncorhynchus nerka*. My thesis has resulted in three general findings:

1) "Black" kokanee in Anderson Lake, Seton Lake and Lake Saiko have a polyphyletic origin caused by repeated episodes of parallel evolution in the eastern and western North Pacific Ocean. This is supported by the polyphyletic relationship of mitochondrial DNA haplotypes and the lack of microsatellite admixture between eastern and western North Pacific "black" kokanee at nine loci. The independent origin of "black" kokanee populations reinforces the phenomenon of parallel evolution in the history of north temperate fishes (e.g., threespine stickleback, sockeye and kokanee, whitefish pairs; see reviews by Schluter 1996 and Taylor 1999).
2) "Black" kokanee in Anderson Lake and Seton Lake are genetically distinct from broadly sympatric populations of sockeye salmon that spawn in Gates Creek and Portage Creek within the Anderson/Seton system, and also from other populations of Fraser River and Columbia River kokanee. The genetic differences within the Anderson/Seton system between "black" kokanee and sockeye salmon populations are most likely the result of spatial and temporal differences in spawning.

3) The striking morphological distinction represented in standard length at maturity between the two populations of "black" kokanee in the Anderson/Seton lakes system contrasts with their modest, but significant, genetic differentiation. Differences in sizes at maturity may be the result of different levels of competition, predation, turbidity and/or plankton levels between the lakes in addition to potential life history trade-offs. There were no differences observed in gill raker numbers, suggesting that the two populations of "black" kokanee have broadly similar feeding ecology. Further, the black colouration is also uniquely different between the two lakes perhaps as a result of local adaptation.

## 4.2 Conservation implications

Anderson Lake and Seton Lake are the only known lakes in North America that produce "black" kokanee (also known locally as "Gwenish"). Historically, Gwenish were extremely numerous; local residents recall seeing Anderson and Seton lake beaches with piles of post-spawning Gwenish (William Alexander, local Tsalalh resident, pers. comm.). This is, unfortunately, no longer the case. Local residents hypothesized that the reduction in abundance of Gwenish has been driven by the effects of increased sedimentation from the Seton Dam project, which is thought to be affecting their spawning habitat (William Alexander, pers. comm.). Effects of overfishing, hydroelectrical development, climate change, and other anthropogenic habitat changes may also have contributed to population declines in the Anderson/Seton lakes system (Levy 2012).

The apparent decline of Anderson/Seton "black" kokanee combined with the results of my thesis may have important implications for species at risk assessment in Canada. For instance, my data could be used to assess whether "black" kokanee in Anderson Lake and Seton

Lake qualify as distinct designatable units (DU) within *O. nerka* under the *Species at Risk Act* and as per The Committee for the Status of Endangered Wildlife in Canada's (COSEWIC) guidelines for recognizing conservation units within taxa (COSEWIC 2013). Designatable units are defined as "species, subspecies, variety, or geographically or genetically distinct population that may be assessed by COSEWIC, where such units are both discrete and evolutionarily significant" (COSEWIC 2013). "Discrete" can be defined as occupying a distinct biogeographic region, being genetically distinct, or having a major range disjunction. In the context of "black" kokanee, my results have provided evidence to support the "discreteness" of these unique populations. "Black" kokanee are genetically differentiated from other populations of sockeye salmon and kokanee across their range and they exhibit unique reproductive ecology and colour, all of which can be used to help define them as "discrete."

Defining a population as "evolutionarily significant" includes evidence for some combination of: deep phylogeographic divisions, occupying a unique habitat such that local adaptations are likely to have arisen, being the only surviving assemblage within the taxon's natural range and/or if the DU went extinct it would cause a gap in the species range (COSEWIC 2013). While I did not find deep phylogeographic lineages, the black colouration and unique deep-water spawning behaviour clearly qualifies as unique local adaptations. In addition, Anderson Lake and Seton Lake are the only known lakes that produce "black" kokanee in Canada (and none have been reported from the United States).

The conservation status of "black" kokanee is also a concern for local First Nations surrounding the Anderson and Seton lakes system. "Black" kokanee were identified by First Nations as a species of special concern in the Bridge/Seton Water Use Plan process (Morris and Caverly 2004; David Levy, Larry Casper, Tsalalh Chief, pers. comm.). The St'át'imc First

Nation forms the majority of the population in the surrounding communities in the Bridge/Seton system. They value protecting the environment for future generations and living in harmony with all living things (Larry Casper, pers. comm.). Gwenish are used as a crop fertilizer, a winter food source, and as a way to transfer traditional St'át'imc knowledge. The cultural connection to fish and the services they provides increases the importance of studying such an understudied, but not undervalued, fish.

### 4.3 Future studies

Understanding how intraspecific diversity originates and quantifying this diversity are important aspects to consider when conducting studies on biodiversity. My research is the first biological study on the evolutionary history and genetic variation of "black" kokanee in the Anderson and Seton lakes system and the first using the three known populations of "black" kokanee across the extensive range of *O. nerka*. Answering questions regarding the evolutionary history of, and quantifying genetic divergences among, these understudied populations of *O. nerka* have hopefully bridged some gaps regarding the generally unknown ecology and evolution of "black" kokanee.

Additionally, my thesis has presented new aspects of their biology to study. A better understanding of the specific spawning locations of "black" kokanee in Japan and the Anderson and Seton lakes system would help elucidate how deep spawning originated and the associated costs and benefits of it. In Anderson Lake and Seton Lake the cumulative effects of hydroelectric dams and rail systems on putative spawning locations and rearing habitat need to be studied further. Also, investigation into what causes the unusual black colour of these kokanee populations could potentially help answer other questions regarding the unknown ecology of

these fish and the genetics of pigmentation. Assaying with outlier loci such as expressed sequence tag (EST) linked loci that exhibit signatures of directional selection (see Russello et al. 2011; Frazer and Russello 2013) would also provide insight into local adaptation in "black" kokanee. Assaying with outlier loci would especially be beneficial considering the low levels of divergence observed with neutral loci between Seton Lake and Anderson Lake "black" kokanee. A more in depth morphological analysis using juvenile and adult "black" kokanee would help understand the life history differences between the Anderson and Seton lake populations. Considering that there are more questions than answers regarding "black" kokanee, the future research possibilities are enticing.

## 4.4 Conclusion

Studies of genetic and phenotypic variation are crucial in order to conserve the maximum evolutionary potential of a species (Fraser and Bernatchez 2001). Additionally, quantifying levels of genetic variation is beneficial because the stability of an ecosystem, and its ability to respond to a fluctuating or changing environment, appears to increase with increasing genetic variation (Hilborn et al. 2003; Pressey et al. 2007; Hughes et al. 2008). "Black" kokanee not only provide another example of parallel evolution found in north temperate freshwater fishes, but these unique populations of kokanee also increase the diversity found within a single species. The striking variation in black colouration, timing and depth of spawning, and morphological differences that exist among the three known populations of "black" kokanee have made this a fascinating system to study.

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# Appendix

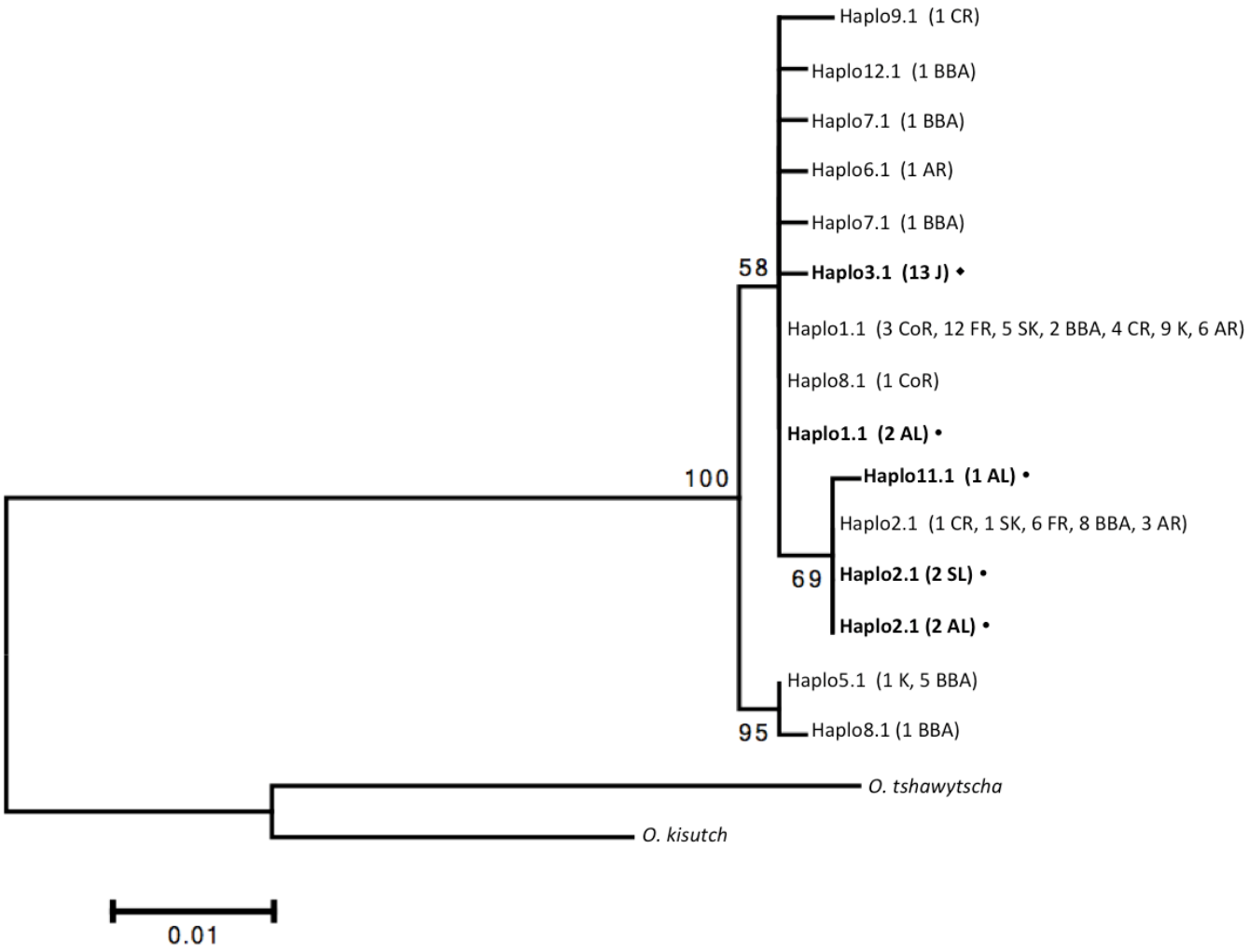


Figure A.1. Maximum likelihood tree using the HKY model of evolution demonstrating the phylogenetic relationship among 12 haplotypes resolved from ND1 mitochondrial DNA sequences of *Oncorhynchus nerka* (N=105) from the Columbia River (CR), Cowichan River (CoR), Alsek River (AR), Fraser River (FR), Skeena River (SK), Bristol Bay Alaska (BBA), 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 in brackets. 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 1000 bootstraps. "◆" represent Japanese "black" kokanee. "•" represent Anderson Lake and Seton Lake "black" kokanee.

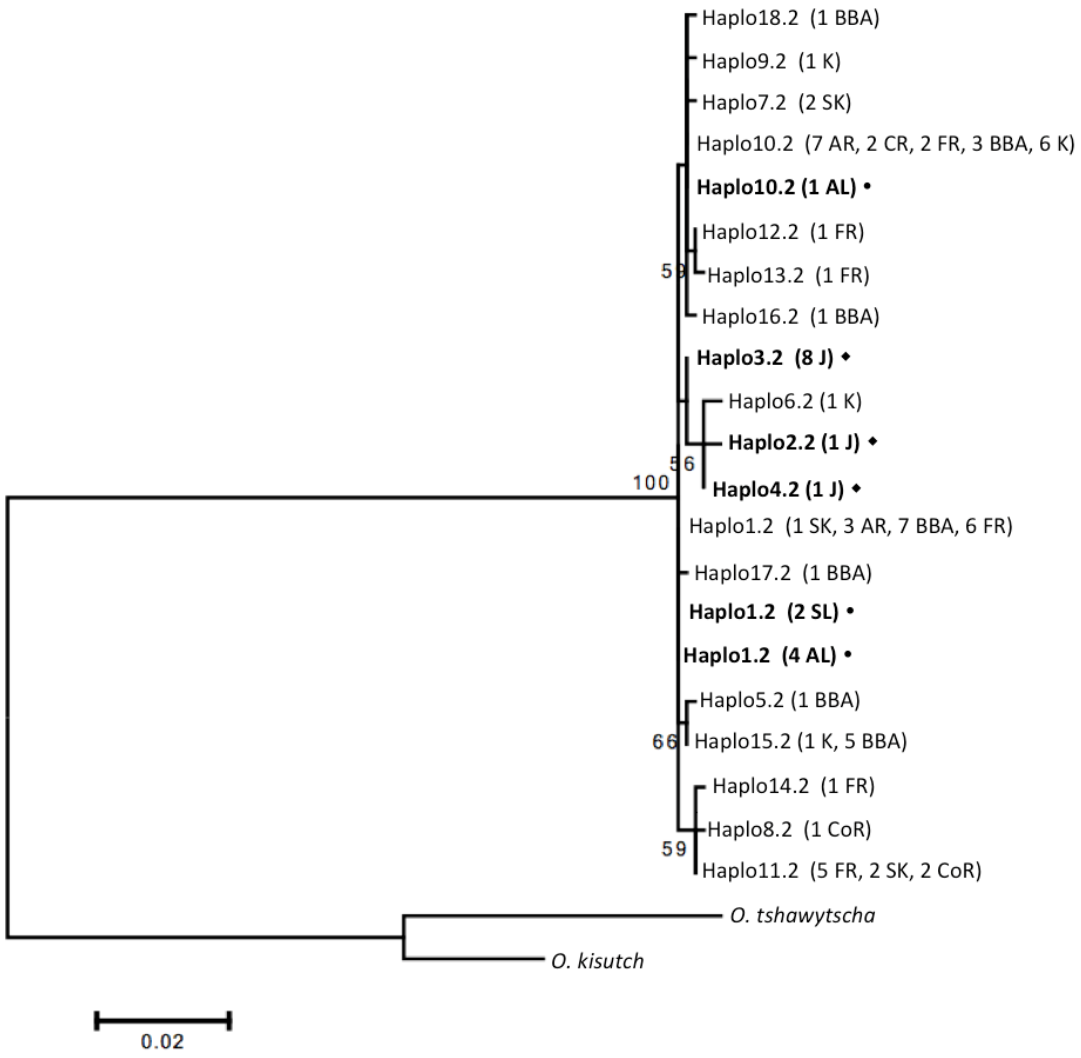


Figure A.2. Maximum likelihood tree using the TN93+G model of evolution demonstrating the phylogenetic relationship among 18 haplotypes resolved from ND2 mitochondrial DNA sequences of *Oncorhynchus nerka* (N=82) from the Columbia River (CR), Cowichan River (CoR), Alsek River (AR), Fraser River (FR), Skeena River (SK), Bristol Bay Alaska (BBA), 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 in brackets. 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 1000 bootstraps. "◆" represent Japanese "black" kokanee. "•" represent Anderson Lake and Seton Lake "black" kokanee.

Table A.3. Summary statistics at nine microsatellite loci in sockeye salmon/kokanee (*Oncorhynchus nerka*) from Chedakuz Creek (CC), Davidson Creek (DV), Meadow Creek (MC), Seton Lake (SL), Anderson Lake (AL), Hansen Creek, Alaska (HCA), Gates Creek (GC), Portage Creek (PC), and Lake Saiko, Japan (J). Number of alleles/locus (A), expected heterozygosity ($H_E$), observed heterozygosity ($H_O$), and sample size (N) for each loci and population. Significant departures from Hardy-Weinberg equilibrium after correcting for multiple comparisons are represented by * ($P < 0.018$).

| Population | Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Omy77* | *Ots103* | *Oki29* | *Ots108* | *Ots100* | *Oki10* | *One108* | *One110* | *One103* | Average |
| **CC** | | | | | | | | | | |
| A | 5 | 9 | 5 | 5 | 4 | 11 | 7 | 6 | 7 | 6.56 |
| He | 0.465 | 0.829 | 0.651 | 0.388 | 0.333 | 0.842 | 0.755 | 0.683* | 0.729 | 0.57 |
| Ho | 0.448 | 0.655 | 0.759 | 0.276 | 0.345 | 0.862 | 0.714 | 0.448 | 0.586 | 0.57 |
| N | 29 | 29 | 29 | 29 | 29 | 29 | 28 | 29 | 29 | 28.89 |
| **DV** | | | | | | | | | | |
| A | 5 | 14 | 10 | 5 | 3 | 21 | 11 | 10 | 18 | 10.78 |
| He | 0.781 | 0.905 | 0.841 | 0.719* | 0.351* | 0.951 | 0.832 | 0.883 | 0.933 | 0.88 |
| Ho | 0.742 | 0.710 | 0.839 | 0.161 | 0.226 | 0.935 | 0.774 | 0.742 | 0.839 | 0.66 |
| N | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31.00 |
| **MC** | | | | | | | | | | |
| A | 10 | 18 | 11 | 14 | 17 | 25 | 16 | 16 | 22 | 16.56 |
| He | 0.657 | 0.951* | 0.702 | 0.862* | 0.917 | 0.940 | 0.933 | 0.916 | 0.956 | 0.86 |
| Ho | 0.633 | 0.767 | 0.767 | 0.414 | 0.800 | 0.900 | 0.800 | 0.900 | 0.933 | 0.77 |
| N | 30 | 30 | 30 | 29 | 30 | 30 | 30 | 30 | 30 | 29.89 |

| Population | Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Omy77* | *Ots103* | *Oki29* | *Ots108* | *Ots100* | *Oki10* | *One108* | *One110* | *One103* | Average |
| **SL** | | | | | | | | | | |
| A | 9 | 19 | 16 | 16 | 16 | 32 | 14 | 15 | 36 | 19.22 |
| He | 0.759 | 0.926 | 0.847 | 0.903* | 0.867 | 0.963 | 0.825 | 0.900 | 0.971* | 0.87 |
| Ho | 0.674 | 0.907 | 0.872 | 0.333 | 0.787 | 0.935 | 0.778 | 0.851 | 0.915 | 0.78 |
| N | 46 | 43 | 47 | 42 | 47 | 46 | 45 | 47 | 47 | 45.56 |
| **AL** | | | | | | | | | | |
| A | 12 | 24 | 23 | 17 | 20 | 38 | 16 | 17 | 54 | 24.56 |
| He | 0.843 | 0.933 | 0.700 | 0.81* | 0.897 | 0.966 | 0.874 | 0.891 | 0.968 | 0.88 |
| Ho | 0.759 | 0.872 | 0.725 | 0.416 | 0.835 | 0.825 | 0.825 | 0.887 | 0.975 | 0.79 |
| N | 79 | 78 | 80 | 77 | 79 | 80 | 80 | 80 | 80 | 79.22 |
| **HCA** | | | | | | | | | | |
| A | 5 | 12 | 9 | 7 | 13 | 13 | 11 | 11 | 21 | 11.33 |
| He | 0.637 | 0.916 | 0.830 | 0.871 | 0.903 | 0.935 | 0.893 | 0.895 | 0.964 | 0.87 |
| Ho | 0.656 | 0.900 | 0.895 | 0.143 | 0.700 | 0.667 | 0.950 | 0.950 | 0.950 | 0.76 |
| N | 20 | 20 | 19 | 14 | 20 | 18 | 20 | 20 | 20 | 19.00 |
| **GC** | | | | | | | | | | |
| A | 7 | 13 | 12 | 8 | 12 | 16 | 6 | 9 | 23 | 11.78 |
| He | 0.747 | 0.702 | 0.701 | 0.732* | 0.843 | 0.884 | 0.776 | 0.741 | 0.872 | 0.78 |
| Ho | 0.800 | 0.683 | 0.585 | 0.067 | 0.725 | 0.829 | 0.675 | 0.610 | 0.775 | 0.64 |
| N | 40 | 41 | 41 | 30 | 40 | 41 | 40 | 41 | 40 | 39.33 |

| Population | Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Omy77* | *Ots103* | *Oki29* | *Ots108* | *Ots100* | *Oki10* | *One108* | *One110* | *One103* | Average |
| **PC** | | | | | | | | | | |
| A | 10 | 13 | 17 | 12 | 12 | 25 | 14 | 18 | 22 | 15.89 |
| He | 0.852 | 0.898 | 0.708 | 0.787* | 0.781 | 0.951* | 0.870 | 0.928 | 0.951 | 0.86 |
| Ho | 0.806 | 0.889 | 0.714 | 0.241 | 0.694 | 0.833 | 0.778 | 0.972 | 1.000 | 0.77 |
| N | 36 | 36 | 35 | 29 | 36 | 36 | 36 | 36 | 34 | 34.89 |
| | | | | | | | | | | |
| **J** | | | | | | | | | | |
| A | 4 | 12 | 8 | 5 | 4 | 9 | 10 | 7 | 12 | 7.89 |
| He | 0.720 | 0.923 | 0.894 | 0.807 | 0.723 | 0.944 | 0.883 | 0.837 | 0.924 | 0.85 |
| Ho | 0.333 | 0.909 | 0.833 | 0.833 | 0.667 | 0.200 | 0.833 | 0.750 | 0.917 | 0.70 |
| N | 12 | 11 | 12 | 12 | 12 | 10 | 12 | 12 | 12 | 11.67 |