AN ABSTRACT OF THE THESIS OF

Kendra L. Hoekzema for the degree of Master of Science in Fisheries Science presented on May 14, 2013.

Title:

Phylogenetic and Morphometric Analysis of Speckled Dace (*Rhinichthys osculus*) from Oregon's Great Basin

Abstract approved: _____

Brian Sidlauskas

Speckled dace (*Rhinichthys osculus*) is a small cyprinid that is geographically widespread throughout western North America, and the most frequently occurring fish in Oregon. Because of the genetic and morphological variation in this species across its range, it has been referred to as a "species complex" and no revision to its taxonomy has occurred since 1984. We investigated the phylogenetics, population genetics, and morphometrics of speckled dace from Oregon's Great Basin region to describe the genetic and morphological variation of speckled dace, identify any unrecognized taxonomic diversity, and to test the validity of Foskett Spring speckled dace, an undescribed putative subspecies that occurs in a single spring within Warner Valley in Southeast Oregon. We collected morphometric and genetic data from Foskett Spring and the surrounding basins

(Warner, Goose Lake, Lake Abert, Silver Lake, and Malheur), as well as Stinking Lake Spring (located within Malheur). We created Maximum Likelihood and Bayesian phylogenetic trees from mitochondrial ND2 and nuclear S7 sequence data and genotyped eight microsatellite loci. We identified three genetic clades within our samples area that warrant species-level status: Malheur stream dace, Stinking Lake Spring dace, and dace from the other four basins. Population structure was detected at the basin-level and separated Foskett Spring dace from other dace in Warner Valley. The geometric and linear morphometric data indicate that overall body shape did not differ among fish occupying streams in these basins, except for in Warner Valley dace, which had larger heads. The two populations of spring dace were distinct from stream dace by having more posterior dorsal fins, and were distinct from each other in head size. With this data we recommend species status for Malheur stream dace, Stinking Lake Spring dace, and dace from the other basins. Because of the strong morphological and slight genetic distinction of Foskett Spring speckled dace, ESU status is recommended, and this population will likely continue to be protected under the ESA.

Phylogenetic and Morphometric Analysis of Speckled Dace (*Rhinichthys osculus*) from Oregon's Great Basin

by

Kendra L. Hoekzema

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kendra L. Hoekzema, Author

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Chapter 1 – General Introduction

1.1 Introduction

The ability to identify and designate species reliably is fundamental to the development of effective conservation and management strategies. However, species boundaries are often poorly understood because species, especially fish species (Turner, 1999), are difficult to distinguish using morphological approaches. In areas of complex biogeographic history, identifying species can be more difficult because of unknown modes of speciation, but this can be abetted by a united genetic and morphological approach. Combining multiple tools allows for increased detection of cryptic diversity, which will help revise the confusing taxonomy of potentially well-known species.

Speckled dace (*Rhinichthys osculus*, Figure 1.1) are a well-known species that are geographically widespread throughout western North America, as far south as Sonora, Mexico and north into British Columbia, Canada (Lee, 1980; Page and Burr, 1998). These dace are part of the family Cyprinidae, the carps and minnows, and subfamily Leuciscinae, the true minnows. In Oregon, *Rhinichthys osculus* is found in many isolated subbasins and interior drainages and is the most frequently occurring freshwater fish (Bond et al., 1988). This ubiquity results because speckled dace are capable of living in diverse habitats from small springs

or streams to large rivers and deep lakes, but they prefer habitat that includes clear, well oxygenated water, with movement due to a current or waves (Page and Burr, 1998).

Figure 1.1: Speckled dace (*Rhinichthys osculus*) from Goose Lake Basin in Eastern Oregon

Because of the widespread distribution of speckled dace within most drainages along the Pacific coast including the Great Basin and Colorado River basin, this species has been a frequent target for phylogeographic studies, looking at the genetic patterns among basins (Oakey et al., 2004; Pfrender et al., 2004; Smith and Dowling, 2008). These studies have shown high genetic differentiation among the major drainage basins that speckled dace occupy. Speckled dace also vary geographically in meristics, color, size, and body proportions (Hubbs et al., 1974), and exhibit large differences in morphological traits (Hubbs and Miller, 1948; Woodman, 1992; Scott and Crossman, 1998). There are countless local variants that occupy an almost endless number of habitats, ranging from major river systems to isolated springs (Hubbs et al., 1974). This genetic and morphological variation has led speckled dace to be referred to as a species complex (Page and Burr, 1998),

where unrecognized variation across its range indicates more taxonomic diversity within the current concept of *Rhinichthys osculus*.

The taxonomy of speckled dace has ben traditionally based on phenotypic characters, of which few are able to distinguish among geographically-defined groups. This has resulted in grouping of all forms into a single, highly variable species (Hubbs et al., 1974). Many different genus and species names have been given to entities currently in the synonymy of *Rhinichthys osculus*: *Argyreus* (3 species names; Cope, 1883; Girard, 1856), *Agosia* (4 species names; Jordan, 1890; Evermann and Meek, 1981; Cope, 1883; Rutter, 1902), *Apocope* (4 species names; Cope, 1872; Cope and Yarrow, 1875; Gilbert, 1893; Hubbs and Kuhne, 1937), *Tigoma rhinichthyoides* (Cope, 1872), *Ceratichthys squamilentus* (Cope, 1872), and *Rhinichthys* (many species and subspecies names; Cope, 1874; Lugaski, 1972); most of these names were synonymized by Gilbert (1998). There have been as many as 12 separate species described in this complex (Jordan and Evermann, 1898). To further complicate the taxonomy, many distinct subspecies and many undescribed taxa within speckled dace have been acknowledged (14 taxa Eschmeyer, Catalogue of Fishes, Cal Acad; 15 taxa - Deacon and Williams, 1984; 22 taxa - Desert Fishes Council unpublished data).

Speckled dace are the most common fish in the Great Basin (Smith, 1978), a region of internal drainages in western North America divided into broad intervening valleys by rugged, north-south oriented mountain ranges. The Great Basin covers an arid expanse of about 190,000 square miles (492,000 square km) and is bordered by the Sierra Nevada range on the west, the Wasatch Mountains on the

east, the Columbia Plateau on the north, and the Mojave Desert on the south. This region contained up to 80 different lakes during pluvial times (Hubbs and Miller, 1948), characterized by extended periods of high rainfall, with the high water level of Lake Lahontan occurring 12,700 years ago. There were periods of repeated connection and isolation throughout these lakes that ended about 10,000 years ago (Hubbs and Miller, 1948).

Here, we focus on Southeastern Oregon's endorheic basins, which are the northern most drainages of the Great Basin. Warner Valley was filled with a large lake during pluvial times, but now has many separate bodies of water (Figure 1.2; Hubbs and Miller 1948). This includes Coleman Valley, Cowhead Lake, and the Warner Lakes. Historical connections between Coleman Lake and Cowhead Lake to Long Valley (NV) and Surprise Valley (CA) (respectively) in the south have been hypothesized (Hubbs and Miller, 1948). Long Valley and Surprise Valley are narrow north-south oriented basins that connected to each other and likely Lake Lahontan from their southern ends. To the north of Warner Valley is Lake Abert basin, which includes Summer Lake, the Chewaucan River, and Lake Abert; these bodies of water were all connected in a 'U' shape during pluvial times. West of Warner Valley, over the Warner Mountains, lies Goose Lake, which has been most recently connected to the Pit River system (Hubbs and Miller, 1948), although a weaker hypothesis posits a connection between Goose Lake and the Klamath system (Cope, 1883). Silver Lake (or Fort Rock Lake), to the north of Lake Abert, once contained a large lake but is now mostly dry with a great expanse of desert to the northwest (Hubbs and Miller, 1948). An outlet of this basin existed to

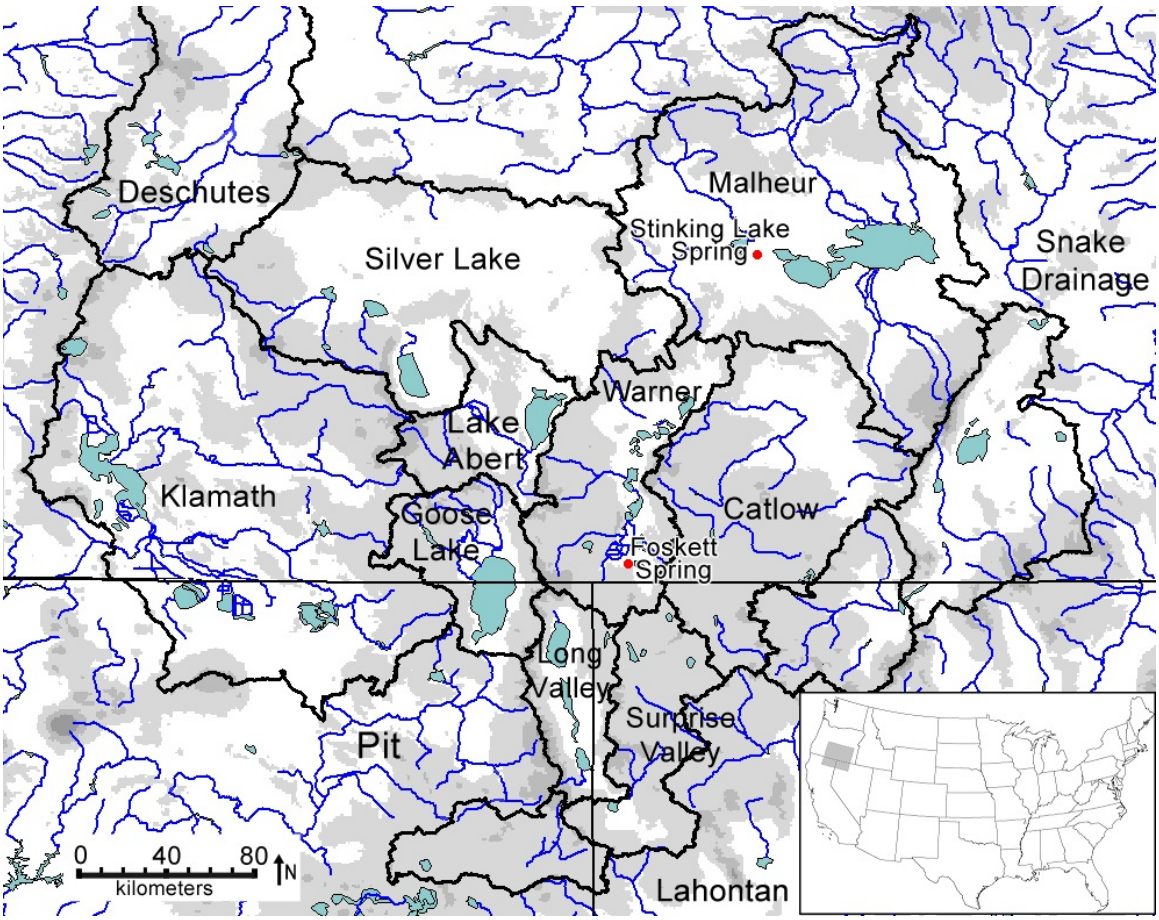

Figure 1.2: Eastern Oregons drainage basins. Two springs of interest are labeled, Foskett Spring and Stinking Lake Spring. Shaded background is elevation; white 0-1500m, lightest gray 1500-2000m, medium gray 2000-2500m, darkest gray 2500m-above.

the northwest to the Deschutes River, a Columbia River tributary. The northernmost basin in the Oregon desert is Malheur basin. Pluvial Lake Malheur probably spilled east to the Malheur River (Gehr and Newman, 1978) and a similar eastward drainage through Malheur or Crane Gaps may have occurred earlier, before late-Pleistocene emplacement of lava flows (Bisson and Bond, 1971). This biogeographic history of repeated connections and separations has had a fragmenting effect on the fishes in the Great Basin as there are many populations of wide-spread fish species now isolated in basins and springs.

One of these isolated fish populations is the Foskett Spring speckled dace (*R. osculus* ssp.), a putative undescribed subspecies. It is represented by a single population that inhabits Foskett Spring on the west side of Coleman Lake in Lake County, Oregon and was listed as threatened under the federal Endangered Species Act in 1985 because of its extremely isolated range, low numbers, and vulnerable spring habitat (US Fish and Wildlife Service, 1985). In 1997 the population estimate of Foskett Spring was around 30,000 fish (Dambacher et al., 1997), but in 2005 was only 3,000 fish (US Fish and Wildlife Service, 2009). The population crash is probably a result of the reduction of open water habitat, most likely due to the exclusion of livestock (Dambacher et al., 1997). Foskett Spring is a natural spring that rises from a springhead pool, flows through a narrow spring brook into a series of shallow marshes, and then disappears into the soil of the normally dry Coleman Lake. Coleman Valley is a small endorheic basin within Warner Basin that Hubbs and Miller (1948, p. 65) suggested was last flooded by an arm of Warner Lake at the end of the pluvial period (9,000-10,000 years ago). It is likely that

speckled dace became isolated in Foskett Spring at that time (Ardren et al., 2010).

Our current understanding of the taxonomy, morphology, and ecology of Foskett Spring speckled dace is limited. The only morphological description seems to be a personal communication from Oregon State University (OSU) Professor Carl Bond, dated 1990 and presented in the 1998 Recovery Plan (US Fish and Wildlife Service, 1998). Bond indicated that Foskett Spring speckled dace differed slightly from those found in the rest of the Warner Basin by possessing a shorter lateral line, larger eye, barbels present on most individuals (versus absent), and a more posterior dorsal fin origin. His initial assessment has not been reexamined, so the five-year review for the species recommended a "systematic assessment of morphological traits and life history... to determine whether or what subspecies classification is warranted" (US Fish and Wildlife Service, 1998). Here, we further investigate the morphological and genetic distinctiveness of these speckled dace to fulfill these recommendations.

Recently, Ardren et al. (2010) examined two mitochondrial genes (Cytochrome b and NADH subunit 2) in speckled dace from localities throughout the Warner and Goose Lake basins, including Foskett Spring. They found that the genetic differences between dace from Warner and Goose Lake basins were comparable to typical differences among cyprinid species (Smith et al., 2002), again indicating the presence of unrecognized taxonomic units within the speckled dace species complex. Figure 1.3 (reproduced from Ardren et al. (2010)) illustrates this deep divergence in a haplotype network where each ND2 haplotype is represented by a circle and 62 inferred haplotypes separate the Goose Lake and Warner samples.

Within Warner Basin, Foskett Spring dace were the most divergent population, although not reciprocally monophyletic (usually a requirement for species or subspecies status when using genetic data; (Zink, 2004)), so Ardren et al. (2010) concluded it was "difficult to justify subspecies status" using their data. This suggests incomplete sorting of the ancestral genotypes and supports their suggestion that isolation events in Warner Basin were recent.

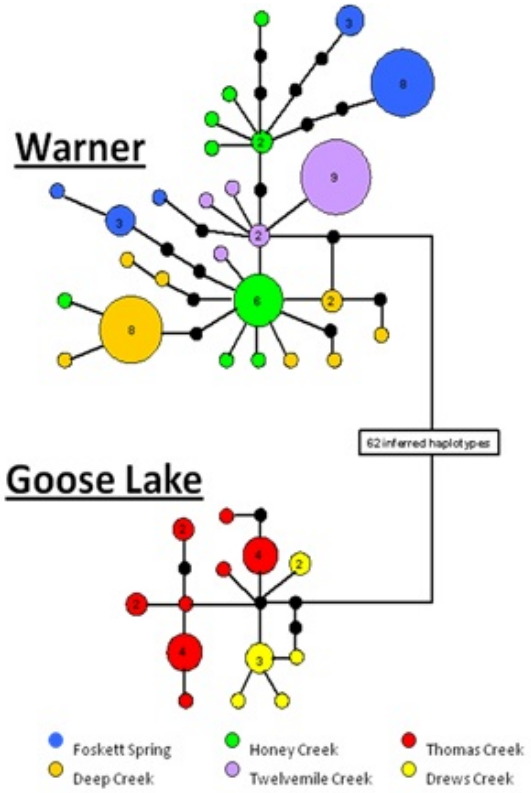

Figure 1.3: Haplotype network reproduced from Ardren et al. (2010), based on ND2 sequences. Circles are colored by the location of specimens, and black dots show inferred substitutions (62 black dots needed to connect Warner and Goose Lake basin fish are not shown). Numbers in colored circles indicate the number of specimens. A line between colored circles represents a single base pair change out of the 1312 base pairs sequenced.

Morphological data from Carl Bond (US Fish and Wildlife Service, 1998) suggests that fish from Foskett Spring are unique and fish from Warner and Goose Lake are similar, while the genetic data overwhelmingly suggest that Warner and Goose Lake basins are highly divergent and that within Warner Basin, Foskett Spring dace are only slightly differentiated (Ardren et al., 2010). These data suggest that the Foskett Spring morphology represents a rapid response to a small, predator-free, warm water, spring habitat, which could simply be a result of morphological plasticity. The data also suggests that Foskett Spring speckled dace are too recently separated from Warner basin dace to be genetically distinct. The conclusions drawn from the mitochondrial data do not preclude the possibility that other genetically based traits that are associated with morphological or life history differences could have occurred in the last 10,000 years as a result of rapid evolution in different selective environments. However, more morphological and genetic data are required to determine the morphological and genetic diagnosability of speckled dace from Foskett Spring, to determine what taxonomic status or protection this entity warrants.

1.2 Research Questions and Summary of Approach

Here we present research on the phylogenetics, population genetics, and morphometrics of speckled dace in Oregon's Great Basin region to answer two primary questions: 1) What is the pattern of genetic differentiation among populations of speckled dace? and 2) Do Foskett Spring speckled dace and dace from different

basins have identifiable morphologies? The genetic and morphometric data will aid in a taxonomic revision of speckled dace, inform management decisions for these fish, and provide a better picture of the biogeography of the region.

We investigated the phylogeography of speckled dace in Oregon's Great Basin to determine if there is evidence for genetic differentiation among populations or basins sufficient to merit formal taxonomic recognition. In particular, we evaluated whether Foskett Spring speckled dace is genetically distinct from other speckled dace populations and therefore if its taxonomic status is in need of revision. To accomplish these goals we collected genetic data from throughout the Warner basin and surrounding drainages (Goose Lake, Lake Abert, Silver Lake, and Malheur; Figure 1.2). Specifically, we sequenced the mitochondrial ND2 gene and the nuclear S7 ribosomal intron, and we genotyped microsatellites. The more rapidly evolving microsatellite markers are predicted to be particularly useful in testing whether Foskett Spring harbors a recently isolated but independently evolving group of fishes. This combination of markers provides a substantially more detailed analysis of the fine-scale genetic structure between these fish than previous studies using only one marker.

We studied the morphological variation of speckled dace in the same Great Basin region to test Carl Bond's assertion that Foskett Spring speckled dace have an identifiable morphology. Additionally, we wanted to determine if the putative taxonomic units identified by the phylogenetic analysis have unique morphologies. This was done by collecting geometric morphometric data, linear morphometric data, and lateral line scale counts in order to reconstruct the pattern of morpho-

logical variability in this region. We also used this data to help determine whether the morphology of speckled dace is affected by the different habitat types they are found in (springs and streams) by comparing the morphology of spring dace (including Foskett Spring) with stream dace. This comparison could indicate whether there is a "spring" morphology type shared by daces inhabiting different springs in Oregon.

1.3 Species Concepts and Criteria

Reevaluation of the taxonomic status of Foskett Spring speckled dace and dace throughout Oregon's Great Basin, requires an operational species concept. Scientists agree that evolutionary independence occurs when mutation, selection, gene flow, and drift operate on populations separately (De Queiroz, 2005). But species concepts have been debated since Darwin and Linnaeus' time (Darwin, 1859; Linnaeus, 1735). The Biological Species Concept states that a set of actually or potentially interbreeding populations is a species (Mayr, 1942), but this definition has been criticized for many reasons. It is problematic in this study system because speckled dace hybridize with longnose dace (*Rhinichthys cataractae*) in the wild (Smith, 1973) despite the fact that these species are more than 20% divergent at the Cytochrome b locus (Pfrender et al., 2004); they also hybridize with redside shiner (*Richardsonius balteatus*) which is in a different genus and possesses a very distinct morphology. Hybridization happens readily in many groups of fish (Verspoor and Hammart, 2006; Smith, 1973), even between species that have been

distinct for long evolutionary times, and that possess distinct ecology and recognizable morphology. The criterion of interbreeding is therefore inappropriate for most fish cases (Turner, 1999).

Important historically in designating fish species (Wiley, 1978), the Morphospecies Concept defines a species as a group of individuals that differs morphologically from others. This concept is useful in the cases of allopatric species, fossils, and asexual organisms, and was particularly useful before the advent of molecular methods. Most species today have been defined by this species concept; however it has been highly criticized since the advancement of genetic methods (Turner, 1999; Puorto et al., 2001; De Queiroz, 2007). Genetically distinct populations may look very similar due to convergence or plasticity, and conversely large morphological differences sometimes exist between very closely related populations (De Queiroz, 2007), as appears to be the case with speckled dace (Hubbs et al., 1974). It would not be responsible to use only morphological data in this case, because of the potential to misclassify morphologically cryptic species or highly variable species. But morphology can still be a useful criterion and can provide another character for defining species.

The Evolutionary Species Concept attempts to be an empirical, universal definition that has wide applicability. Simpson (1961) defines an evolutionary species as a lineage (an ancestral-descendant sequence of populations) evolving separately from others and with its own unitary evolutionary role and tendencies. This definition frames a testable hypothesis of whether populations are maintaining separate, identifiable evolutionary linages (Wiley, 1978; Wiley and Mayden, 2000). This def-

inition does not however provide a specific basis for what constitutes a separate lineage, whether it is ecological, biological, genetic, or morphological. So additional criteria are required to define a species.

The Phylogenetic Species Concept (PSC) is based on monophyly of a group of organisms (sharing one ancestor), usually based on genetic data (deQueiroz and Donoghue, 1990), and is very suitable for distinguishing morphologically similar species and discovering cryptic diversity. However, the PSC, owing to its reliance on monophyly and/or diagnosability, may be too insensitive to resolve recent speciation events. For example, if a widespread river fish species gains entry to a lake and evolves into a new species, there are now two species: one paraphyletic ancestral and one monophyletic daughter species, as in the case of the monophyletic Devils Hole pupfish, *Cyprinodon diabolis*, and the paraphyletic ancestral Amargosa pupfish, *C. nevadensis* (Echelle, 2008). There has not been enough time for the ancestral species to become reciprocally monophyletic with respect to the new lake species, but these are clearly different evolutionarily. This definition discounts the amount of adaptive variation that can accumulate rapidly when populations are exposed to different environments.

The PSC has also been challenged because of the ambiguity of its definition: when two lineages diverge and become monophyletic with respect to each other, the two populations are regarded as separate species (Nelson and Platnick, 1981), but there is little agreement on how much divergence is required. Using just the minimum divergence to generate monophyly as a requirement has been argued to produce excessive splitting of lineages (Turner, 1999), but requiring very deep di-

vergences could ignore newer species. A median approach using a particular level of divergence to define species would standardize this method, but typical genetic distances among species vary depending on what group of organisms is being discussed (insect, mammals, birds, fishes, different families of fishes, etc.) and what molecular marker is being used (mtDNA evolves more rapidly than nuclear DNA; Brown 1983; Avise 2009). We reference percent divergences observed in other cyprinid groups for the particular genes we have used to form a more accurate measure of the typical percent divergences among cyprinid species (Zardoya and Doadrio, 1998; Schmidt et al., 1998; Wang et al., 2002).

There is also the question of which genetic markers to use. Percent divergence at mtDNA loci is still the most common delimiter of fish species; the Barcode of Life Data system uses mitochondrial Cytochrome c oxidase to barcode over 5000 fish species (Ward et al., 2009). Mitochondrial DNA is also favored because the rate of nucleotide substitution in vertebrates is approximately five times greater than the nuclear genome, giving higher resolution for population-level studies (Brown, 1983; Moritz et al., 1987). However, inferring evolutionary relationships based solely on evidence from one gene tree is inadequate, as it provides only one independent estimate of the species tree (Pamilo and Nei, 1988; Moore, 1995; Maddison, 1997; Knowles and Carstens, 2007; Burbrink and Pyron, 2011), so it has become the standard to analyze nuclear DNA loci and check for congruence of phylogenetic relationships between nuclear DNA and mtDNA (Jacobsen et al., 2010; Sota and Sasabe, 2006; Benavides et al., 2007; Hackett et al., 2008). Herein, we use one mtDNA gene, one nuclear gene, and eight nuclear microsatellite loci to fulfill this

requirement.

Since the Endangered Species Act (ESA) also protects evolutionarily significant units (ESUs) it is important that we determine taxonomic status down to the ESU level. In 1991, NOAA proposed that a population be considered an ESU if it is 1) reproductively isolated from other conspecific populations, and 2) represents an important component in the evolutionary legacy of the species (Waples, 1991). With the increased prevalence of genetic data, Haig et al. (2006) added that at minimum, a unit being considered as an ESU be genetically discrete in relation to the remainder of the species, as well as biologically significant to the species (unique range or habitat or life history trait).

Our operational species concept will combine features of the phylogenetic and morphological species concepts as criteria for species status, within the theoretical framework of the evolutionary species concept (Figure 1.4). We will look for monophyly of lineages first, although paraphyly will not necessarily disqualify a taxonomic designation, for instance if the separation has been recent. If genetic divergences between these monophyletic lineages are consistent with divergences between other species of cyprinids, those populations will be strong candidates for species recognition. Groups of dace that are not reciprocally monophyletic based on nuclear and mitochondrial genes will not be considered distinct species. However, if a group has one or two markers with evidence of genetic distinction (such as microsatellites or mtDNA), they will be considered for ESU designation if they have diagnosable morphological differences because of their potential to become a species given sufficient time (Darwin's incipient species; Avise and Hamrick (1996,

p. 60)).

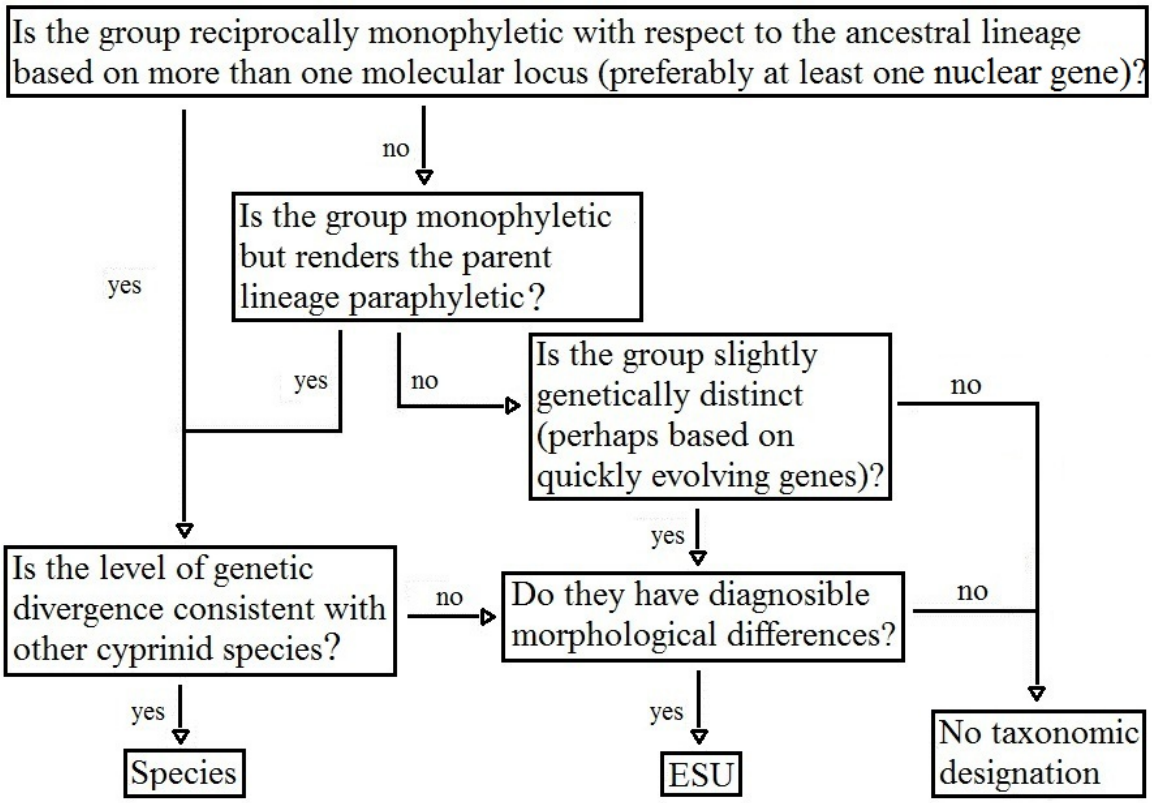

Figure 1.4: A flow chart illustrating our operational species concept and the criteria required for taxonomic designations.

Chapter 2 – Genetic variation of *Rhinichthys osculus* from Oregon's Great Basin

2.1 Introduction

Speckled dace (*Rhinichthys osculus*) are a widespread group of fish that have a complicated taxonomic history which has led to many unanswered questions about genetic relationships among different populations. Speckled dace have deep genetic divergences among many of the Western North American basins they occupy (Oakey et al., 2004; Smith and Dowling, 2008; Pfrender et al., 2004) and vary morphologically in meristics, color, size, and body proportions. For these reasons, they have been referred to as a species complex (Page and Burr, 1998), where unrecognized variation across their range indicates more taxonomic diversity than currently recognized within the current concept of *Rhinichthys osculus*. Speckled dace in Oregon's Great Basin have experienced periods of repeated connection and isolation during the Pleistocene, which has created a confusing biogeographic pattern that has not been fully investigated. One interesting population of speckled dace is isolated in Foskett Spring, which is ESA-listed as an undescribed threatened subspecies, but the taxonomic status of this population has never been confirmed.

We analyzed phylogenetic and population genetic data from speckled dace in Oregons Great Basin to reconstruct the pattern of genetic variability across the

landscape. We also used genetic distance measures to determine if there is evidence for genetic differentiation among populations or basins sufficient to merit formal taxonomic recognition. In particular, we evaluated whether the genetic distinctiveness of Foskett Spring speckled dace supports revision or maintenance of its taxonomic status. Determining the taxonomic status of Foskett Spring speckled dace responds directly to the recommendation in the five-year review for Foskett Spring Dace "to determine whether or what subspecies classification is warranted" (US Fish and Wildlife Service, 2009).

This study had three major questions: 1) What is the pattern of genetic differentiation among populations of speckled dace and how does it relate to the geologic history of the area? 2) Are there any unrecognized units that warrant taxonomic status? 3) Should Foskett Spring speckled dace be recognized as a distinct taxonomic entity?

To answer these questions we collected specimens and associated genetic data from throughout the Warner basin and surrounding drainages (Goose Lake, Lake Abert, Silver Lake, and Malheur; Figure 1.2). Specifically, we sequenced the mitochondrial ND2 gene and the nuclear S7 ribosomal intron, and we genotyped microsatellite loci. This combination of markers provides a substantially more detailed analysis of the fine-scale genetic structure among these fish than previous studies using only one marker (Ardren et al., 2010). The more rapidly evolving microsatellite markers are predicted to be particularly useful in testing whether Foskett Spring harbors a recently isolated and independently evolving group of fishes, and were used successfully to test population structure in the Oregon Chub

(DeHaan et al., 2012).

2.2 Methods

2.2.1 Specimen Acquisition

Specimens were captured using a seine net, dipnet, or minnow trap, and euthanized in an aqueous solution of tricaine methane sulfonate (MS-222, approximately 2g/L), buffered with equal concentration of sodium bicarbonate. Following euthanization, small pieces of tissue (fin clips or small plugs of epaxial muscle weighing 1g or less) were taken from each individual and preserved in 95% ethanol or saturated salt (SED) buffer. These collections were performed under Animal Care and Use Protocol 4050 approved by Oregon State Universitys Institutional Animal Care and Use Committee (IACUC).

2.2.2 Sampling Locations

Sampling sites include the Warner Valley and all surrounding drainages containing speckled dace, include the Goose Lake, Lake Abert, Silver Lake, and Malheur basins (Figure 2.1), and likely include all possible close relatives of Foskett Spring speckled dace because of their geographic proximity. This geographic region is the northern portion of the Great Basin (see Figure 1.2). We sampled up to 30 dace from each of three streams in each basin (Figure 2.1), as well as 41 Foskett Spring speckled dace (Table 3.1). We also sampled spring dace from Stinking Lake Spring

in Malheur drainage, which was the only other spring we found with speckled dace in the basins of interest (based on museum records and input from district biologists).

Three nearby basins do not appear to harbor speckled dace. Long Valley in Nevada has little perennial water and no dace were found there during our investigations in the summer of 2011. We sampled the only location with flowing water and found no dace, and the district biologist of Washoe County (pers. comm. Matt Maples of NDOW) did not know of any springs or streams containing dace. We sampled in Surprise Valley of California on public lands (all within Modoc National Forest) and found no speckled dace. The water in these Warner mountain streams is heavily diverted for agriculture use so speckled dace or other native fish may be found in irrigation ditches on private property, but not in the higher elevation streams on public land. Catlow Valley to the east of Warner has no records of speckled dace.

2.2.3 DNA Extraction

DNA was extracted using the NaCl extraction protocol adapted from Lopera-Barrero et al. (2008). Fin clips or tissues were cut into small pieces and placed in 550 μL lysis buffer (50mM Tris-HCl, pH8.0, 50mM EDTA, 100mM NaCl) with 1% SDS and 7 μL of 200 μg/mL proteinase K. The samples were incubated in a 50°C water bath for 12 hours, and then 600 μL 5M NaCl was added to each sample and centrifuged for 10 min at 12000 rpm. The supernatant was transferred

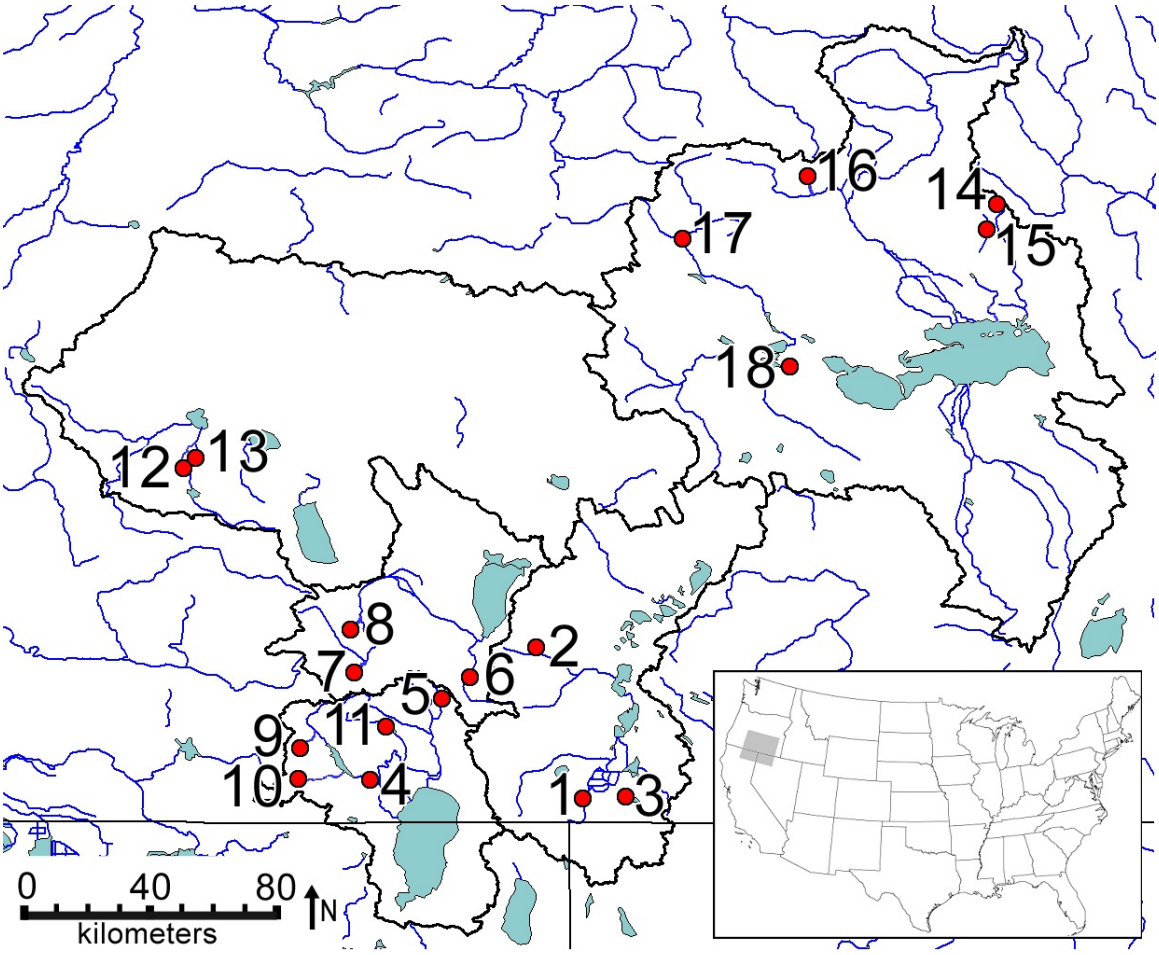

Figure 2.1: A map of the study area showing the Eastern Oregon endorheic basins investigated in this study. Sampling locations are indicated by dots: 1=Twenty Mile (Warner), 2=Snyder (Warner), 3=Foskett Spring (Warner), 4=Drew's Creek (Goose Lake), 5=Cox Creek (Goose Lake), 6=Crooked Creek (Lake Abert), 7=Little Coffee Pot Creek (Lake Abert), 8=South Creek (Lake Abert), 9=Cottonwood Creek (Goose Lake), 10=Dent Creek (Goose Lake), 11=Hay Creek (Goose Lake), 12=Silver Creek (Silver Lake), 13=West Fork Silver (Silver Lake), 14=Cow Creek (Malheur), 15=Rattlesnake (Malheur), 16=Sawtooth Creek (Malheur), 17=Nicoll Creek (Malheur), 18=Stinking Lake Spring (Malheur). Inset shows location of sample area within the United States

Table 2.1: Basin and stream locations (springs marked by asterisks) of the speckled dace collected for this study. The number of vouchers is the total number of specimens collected and used for morphometric analyses. The number in parentheses is the number of tissue samples taken for genetic analyses. Samples are deposited in the Oregon State Ichthyology Collection under the catalog numbers provided.

Basin	Location	N=vouchers(tissues)	Catalog number
Warner Valley	Twenty Mile Creek	17 (15)	OS 18125
	Snyder Creek	6 (6)	OS 18126
	*Foskett Spring	41 (41)	OS 18127
	(total)	64 (62)	
Goose Lake	Cox Creek	3 (3)	OS 18129
	Drews Creek	31 (15)	OS 18128
	Cottonwood Creek	11 (11)	OS 18400
	Dent Creek	30 (14)	OS 18393
	Hay Creek	30 (15)	OS 18394
	(total)	105 (58)	
Lake Abert	Crooked Creek	30 (15)	OS 18130
	Little Coffeepot Creek	30 (15)	OS 18391
	South Creek	30 (15)	OS 18397
	(total)	90 (45)	
Silver Lake	Silver Creek	8 (8)	OS 18401
	West Fork Silver	8 (8)	OS 18396
	(total)	16 (16)	
Malheur	Cow Creek	11 (11)	OS 18398
	Rattlesnake Creek	4 (4)	OS 18389
	Sawtooth Creek	16 (15)	OS 18390
	Nicoll Creek	12 (10)	OS 18392
	*Stinking Lake Spring	12 (12)	OS 18286
	(total)	55 (52)	

to a new tube, 700 μL of absolute cold ethanol was added to precipitate the DNA and the samples were stored at -20°C for 2 hours. The DNA samples were then centrifuged for 10min at 12000rpm and the supernatant was decanted, leaving a DNA pellet. The pellet was washed with 700 μL of 70% ethanol, centrifuged, and the supernatant was decanted. The pellet was resuspended in 80 μL TE buffer (10mM of Tris pH 8.0 and 1 mM of EDTA), treated with 30μg/mL of RNAse and incubated in a water bath for 40 min at 37°C. The DNA is stored at -20°C.

2.2.4 DNA Sequencing

Inferring evolutionary relationships based solely on evidence from one gene tree is inadequate, as it provides only one independent estimate of the species tree (Moore, 1995). So, it has become the standard to analyze nuclear and mitochondrial DNA loci and check for congruence of phylogenetic relationships between them (Jacobsen et al., 2010; Sota and Sasabe, 2006; Benavides et al., 2007; Hackett et al., 2008). The mitochondrial gene, NADH subunit 2 (ND2) was selected because it was used by Ardren et al. (2010) in their study of speckled dace. The nuclear intron S7 was selected because it is commonly used in ichthyological studies (Wang et al., 2002; Luca et al., 2008; Houston et al., 2010) and pilot sequencing indicated it would show enough genetic divergence to be informative.

The mitochondrial gene, NADH subunit 2 (ND2, 1001bp), was amplified using the primers ASN (5-CGCGTTTAGCTGTTAACTAA-3) and ILE (5-CCGGATCACTT TGATAGAGT-3) (Dr. Thomas Dowling, Arizona State University, pers. comm.).

The PCR reactions contained 1X PCR buffer, 1.5mM $MgCl_2$, 1μM of each primer, 0.2mM dNTPs, and 0.5U PlatinumTaq (Invitrogen). We used a standard temperature profile with an initial denaturation at 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1.5 minutes; followed by a final extension at 72°C for 10 minutes. The nuclear S7 intron (857bp) was amplified using S7RPEX1F (5-TGGCCTCTTCCTTGGCCGTC-3) and S7RPEX2R (5-AACTGTCTGGCTTTTCGCC-3) (Chow et al., 1998). The PCR reactions contained 1X Qiagen MasterMix and 0.5μM of each primer. The temperature protocol was as follows: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, annealing between 59-62°C for 1 minute, and extension at 72°C for 2 minutes; followed by a final extension at 72°C for 10 minutes.

Following amplification, all PCR products were purified using ExoSAP (Hanke and Wink, 1994) and then sequenced both directions using the BigDye Terminator v3.1 Cycle Sequencing (ABI). Resulting products were run on the ABI 3730 capillary sequence machine in the CoreLabs at Oregon State University. Forward and reverse sequences were edited using the program Geneious v4.8.2 (created by Biomatters); heterozygotes in the nuclear gene were labeled with IUPAC ambiguity codes.

2.2.5 Phylogenetics

Sequences of *Rhinichthys atratulus* (blacknose dace) and *R. cataractae* (longnose dace) were used as outgroups for all phylogenetic analyses because they are close

relatives of *R. osculus*. The *R. cataractae* individuals were collected from Big Wood River in Idaho and graciously provided by Tom Dowling (Arizona State University). *R. atratulus* sequences were downloaded from GenBank (accession numbers: GU134262.1, AY103163.1, JN569221.1 (Naugatuck River, CT), JN569262.1 (Scantic River, CT)).

The number of haplotypes per population for these genes is presented in Appendix 2. The number of parsimony informative sites for each alignment of each gene (ND2 and S7) were calculated in MEGA 5.05 (Tamura et al., 2011). Genetic distances based on a Neighbor-Joining tree (Tamura-Nei genetic distance model) were calculated between and within the clades (determined in the ML and Bayesian analyses) in MEGA 5.05 (Tamura et al., 2011). To look at the genetic structure within Warner Valley, a haplotype network of the ND2 haplotypes was created for the three Warner Valley locations using statistical parsimony in TCS v1.21 (Clement et al., 2000). A haplotype network was also created using the S7 data and all samples from Goose Lake, Silver Lake, Lake Abert, and Warner Valley.

Models of nucleotide substitution that best fit the sequence data were selected by jModelTest (Posada, 2008) after the likelihood scores were calculated using a BIONJ base tree. The Bayesian information criterion (BIC), corrected Akaike information criterion (AICc) and decision theory (DT) methods were used to select the best fit model for each gene. For the nuclear (S7) dataset, all three methods chose F81+G (all nucleotide substitutions are equal, base frequencies are allowed to vary), and for the mitochondrial (ND2) dataset all three methods chose TrN+G (Tamura-Nei plus gamma, variable base frequencies, equal transversion rates). Se-

lection of the best fit partitioning scheme for the alignment datasets was performed in PartitionFinder v1.0.1 (Lanfear et al., 2012). For the concatenated dataset (ND2 and S7 combined), partitions for each gene and each codon position and all combinations thereof were tested using the AIC, AICc, and BIC model selection criteria. The most consistently selected scheme was four partitions: ND2 codon partitions 1, 2, and 3 separately, and all S7 codon positions together.

The Maximum Likelihood (ML) phylogeny was inferred in RAxML v7.2.6 (Stamatakis, 2006). The datasets were partitioned based on the scheme selected by AIC, AICc, and BIC in PartitionFinder. A rapid bootstrap analysis and search for the best-scoring ML tree was performed with 1000 bootstrap replicates, the GTR+G model of rate heterogeneity, 25 distinct rate categories, random seed bootstrapping, and random number seed for MP starting tree computations. This was done for the concatenated dataset and for ND2 and S7 separately. The resulting trees were viewed in FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

A Bayesian phylogenetic analysis was run in MrBayes v3.2.1 (Ronquist et al., 2012). The datasets were partitioned as in the ML analysis and different models of evolution were applied to each partition based on the models selected by PartitionFinder. Because MrBayes does not implement TrN+G, (which is a special case of GTR+G), the ND2 codons were set to GTR+G (nst=6, rates=gamma), and S7 was set to F81+I+G (nst=1, rates=invgamma). A Metropolis coupled Markov chain Monte Carlo algorithm was applied with three heated chains and one cold chain, 1 million generations sampled every 500 generations, and two parallel trees. To assess the convergence of model parameters and tree topology after the initial

1 million runs, we examined whether the standard deviation of split frequencies between the two runs was less than 0.01. If needed, 500,000 additional generations were added until the standard deviation was below 0.01 and stationarity was achieved. Twenty-five percent of the generations were discarded as the burn-in. The clade credibility tree and phylogram for each dataset (concatenated, ND2, and S7) were viewed in FigTree v1.3.1.

We used the Bayesian BEAST algorithm (Heled and Drummond, 2010) to date clade divergences and coalescence times to the most recent common ancestor for different clades. We combined the data from ND2 and S7, then ran analyses using a relaxed molecular clock in BEAST 1.6.1 (Drummond and Rambaut, 2007). The datasets for each gene were imported into BEAUti 1.7 (Drummond and Rambaut, 2007) which we used to generate the input file for BEAST. The evolutionary model was set to TrN+G for ND2 and F81+G for S7, as selected by jModelTest (above), with estimated base frequencies and gamma model of site heterogeneity with 4 gamma categories and each codon position in a separate partition. The clock model was set to lognormal relaxed clock (uncorrelated) based upon a lognormal prior using the 'speciation birth death' process. Ucld.mean was a diffuse gamma distribution (shape 0.001, scale 1000), initial value set centrally within the prior distribution. BEAST runs consisted of 2.0×10^8 MCMC generations with sampling every 10000 generations. The output log file from each run was examined in Tracer (Rambaut and Drummond, 2007) and the posterior distributions were checked for stationarity and convergence. Effective sample sizes exceeded 200 for all parameters of interest. A maximum clade credibility tree (1% burnin) was

summarized in TreeAnnotator v1.5.4. The resulting trees were viewed in FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Our BEAST tree was calibrated at the split between *Rhinichthys cataractae* and *R. osculus*, with a prior uniform distribution having a mean of 6.3mya, a maximum of 8.1mya, and a minimum >4.5 mya. The oldest fossils assignable to *Rhinichthys osculus* were identified by Smith et al. (2002), and came from the Glenns Ferry Formations on the Snake River Plain (about 4.5mya). Smith and Dowling (2008) calculated the estimated time of origin for the *R. osculus* clade using Marshall (2008)'s method for bracketing divergence times, where a confidence interval is calculated using multiple fossils from multiple dates. There were only three time horizons containing *R. osculus*, so the 50% alpha value was quite large (1.8my, Smith and Dowling 2008), and resulted in an estimate of 6.3mya for the divergence of *R. osculus* from its sister taxon, *R. cataractae*. The fossil provides a strict minimum age for the group that contains this fossil (in this case *Rhinichthys osculus*). We used age priors with uniform distributions in which the minimum limit is set to the minimum age of the stage where the fossil is found (4.5mya) and the upper limit is set high enough to not restrict the upper bound (8.1mya, 2 C.I.s above the minimum).

2.2.6 Genotyping and Microsatellite Analysis

Eight microsatellite loci were genotyped for 223 speckled dace individuals using the primers listed in Table 2.2. PCRs were performed with 1X GoTaq Master

Mix (Promega) and 0.3-0.5 μM primer concentration (forward primer fluorescently labeled). The temperature protocol was as follows: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, annealing temp for 1 minute, and extension at 72°C for 2 minutes; followed by a final extension at 72°C for 10 minutes. These PCR products were genotyped on an ABI 3730xl genetic analyzer (Hatfield Marine Science Center, Oregon State University). GeneMapper v4.1 software (Life Technologies) was used to assign allele peaks and assignments were checked by eye.

Table 2.2: Primers, annealing temperatures (-0.5 indicates a drop down protocol of half a degree per cycle), number of alleles per locus, allele size ranges, and original sources for the 8 microsatellite primers used in this study. Additional information about alleles for each loci and population is presented in Appendix 2.

Locus	Primers	Annealing temp	# of alleles	Size range	Source
CypG3	F: AGTAGGTTTCCCAGCATCATTGT R: GACTGGACGCCTCTACTTTCATA	67(-0.5)	65	126-436	Baerwald & May, 2004
CypG9	F: GCAGTCACGTATTAAGGCGAGCAG R: GAGCGGACTCTCAGGCACCTACC	67(-0.5)	6	95-115	Baerwald & May, 2004
CypG13	F: ACACCCAGTTCCTGATGGA R: CATTTATTTCATACTGCACTACACA	55	60	164-456	Baerwald & May, 2004
CypG24	F: CTGCCGCATCAGAGATAAACACTT R: TGGCGGTAAGGGTAGACCAC	67(-0.5)	24	150-230	Baerwald & May, 2004
CypG27	F: AAGGTATTCTCCAGCATTTAT R: GAGCCACCTGGAGACATTACT	57	26	214-318	Baerwald & May, 2004
CypG33	F: TATGAGCTTTGGAAAGAGACACTG R: AATAGCCGGGAAATTATCAATAGA	67(-0.5)	3	84-92	Baerwald & May, 2004
Lco1	F: CACGGGACAATTTGGATGTTTTAT R: AGGGGGCAGCATACAAGAGACAAC	59	77	277-597	Turner et al., 2004
Lco4	F: ATCAGGTCAGGGGTGTCACG R: TGTTTATTTGGGGTCTGTGT	58	28	228-288	Turner et al., 2004

All loci were tested for possible null alleles and allelic dropout using MicroChecker 2.2.4 (van Oosterhout et al., 2004). To test for Linkage Disequilibrium amongst loci, we ran Fisher's exact tests with MCMC settings of 1000 dememoriza-

tion steps, 100 batches, and 1000 iterations per batch for all combinations of loci using GENEPOP v3.4 (Raymond and Rousset, 1995). Tests for deviations from Hardy-Weinberg equilibrium (HWE) were also performed in GENEPOP v3.4 with Fisher's exact tests with the same MCMC settings. Observed (H_O) and expected (H_E) heterozygosity were calculated in Arlequin 3.5.1.3 (Excoffier et al., 2005).

The most probable number of putative populations (K) that best explained the pattern of genetic variability was estimated using the program Structure v2.3.3 (Pritchard et al., 2000). An admixture model with correlated allele frequencies was chosen with a burn-in length of 500,000 and 5,000,000 MCMC steps, and the average admixture coefficient for individuals (alpha) was inferred with a uniform prior for alpha (initial value=1, max=10, SD=0.025). For each putative K (2-12), 20 independent runs were performed. The second order rate of change of the likelihood function with respect to K (deltaK) was plotted to aid in the selection of the optimal number of populations (following Evanno et al. 2005).

Genetic differentiation between pairs of populations identified in Structure was estimated with F_{ST} (Weir and Cockerham, 1984) in GENEPOP v3.4, and Nei's D genetic distance (Nei et al., 1983) was computed with the GENDIST package in Phylip (Felsenstein, 1993).

A Factorial Correspondence Analysis (FCA) was performed in GENETIX 4.03 (Belkhir et al., 1996). This is an exploratory data analysis that is a special case of a principal components analysis, but designed to analyze simple two-way contingency tables containing some measure of correspondence. It identifies relationships between individual genotypes when there are no *a priori* expectations as to the

nature of the relationships.

We measured the effective population size, N_e, using the linkage disequilibrium method in LDNe (Waples and Do, 2008), which measures the effective population size of one generation back from the sampled individuals. The LD method makes some simplifying assumptions (markers are selectively neutral and independent, population has discrete generations and is closed to immigration, and sampling is random). However, only discrete generations and random sampling, because we sampled opportunistically, are violated here. We used a pcrit (allele frequency cutoff to avoid inflation by rare alleles) of 0.05, and the 95% CIs were based on jackknifing of the loci.

2.3 Results

2.3.1 Phylogenetics

To determine the pattern of genetic differentiation among speckled daces in Oregon's Great Basin, we created phylogenetic trees using the S7 and ND2 sequence data. The Bayesian analysis and Maximum likelihood analysis of the concatenated dataset (ND2 and S7 together, 146 individuals) produced the same overall topology (Figure 2.2). There are five distinct clades of speckled dace from the area we sampled: those from streams in the Malheur basin, those from Stinking Lake Spring (within Malheur Basin), those from Goose Lake, from Silver Lake, and Warner Valley and Lake Abert forming a monophyletic group together. The

deepest divergences in this tree are the split between Malheur and everything and the split between Stinking Lake Spring and the other three clades (four basins). When branches with less than 75% bootstrap support are collapsed, these three most terminal clades form a polytomy of unknown relationships.

ML tree and Bayesian trees constructed from the nuclear S7 intron were congruent (Figure 2.3, 161 individuals) with only slight differences in the RAxML bootstrap values and MrBayes posterior probabilities. This gene tree also shows reciprocal monophyly of Malheur basin, Stinking Lake Spring, and the other four basins combined. However, the relationships between these three main clades is different, with Malheur stream dace and Stinking Lake Spring dace being sister to each other, rather than Malheur being sister to all other dace as in the concatenated dataset. The S7 phylogenetic tree does not resolve any relationships between the four basins in the last clade, but the haplotype network (Figure 2.4) shows some genetic structuring of S7 haplotypes. The most common haplotype is shared by Goose Lake, Silver Lake, and Lake Abert individuals, but these three basins do not share any other haplotypes and show slight clustering. This indicates that these basins are far from panmictic but have not had sufficient time to achieve reciprocal monophyly at the S7 locus. Warner Valley individuals share some haplotypes with both Goose Lake and Lake Abert, however they mostly appear nested within the Lake Abert haplotypes.

The Bayesian analysis and Maximum likelihood analysis of ND2 (Figure 2.5, 197 individuals) both show that Warner Basin fish (including those from Foskett Spring) are paraphyletic relative to fish from the Lake Abert system, which form

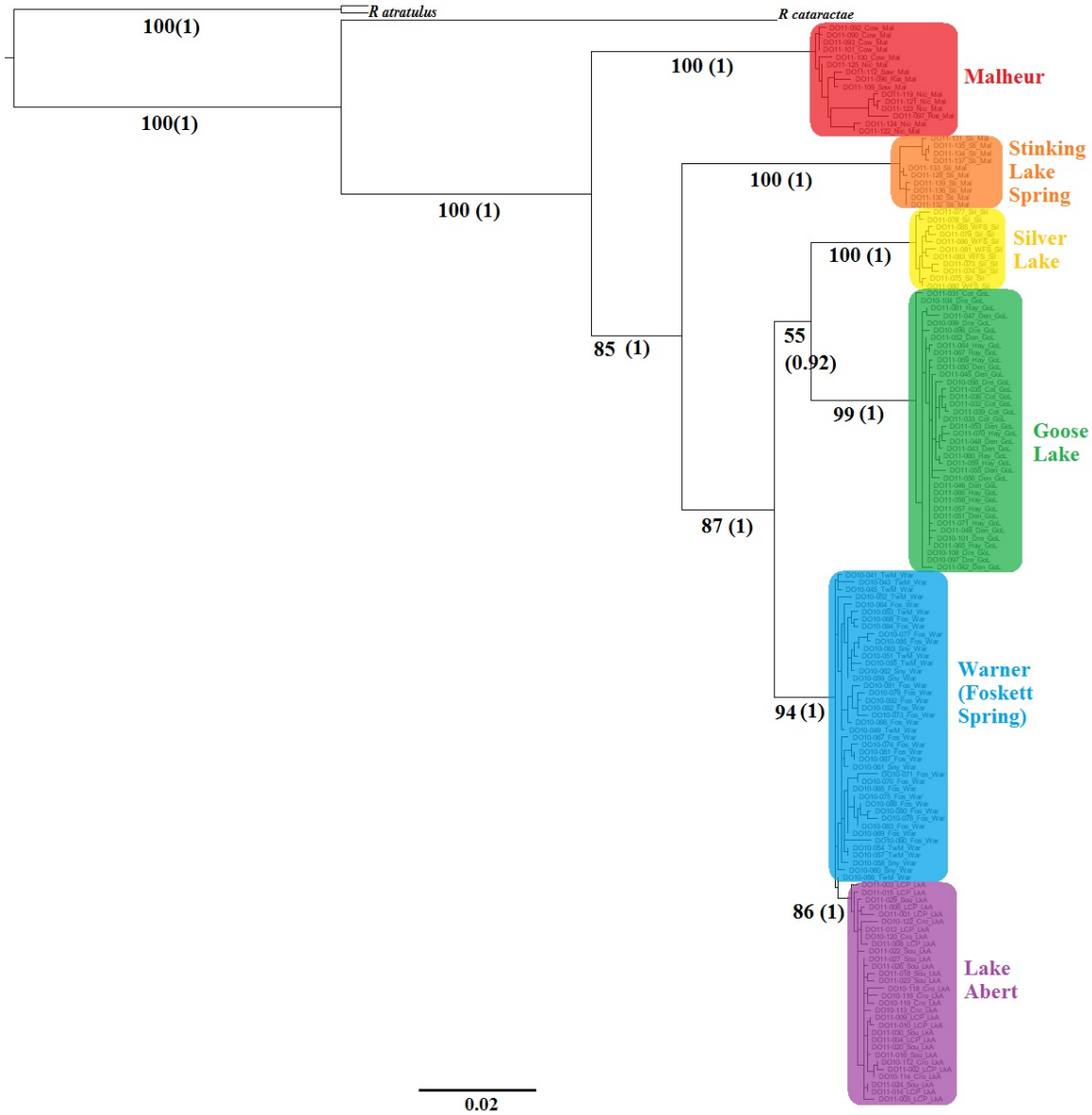

Figure 2.2: Maximum likelihood (RAxML) phylogeny based on the concatenation of ND2 and S7 sequences from 146 individuals of *Rhinichthys osculus* collected from Eastern Oregons Great Basin. Bootstrap values (and Bayesian posterior probabilities from MrBayes in parentheses) are presented on the branches. The scale bar shows the number of substitutions per sequence position.

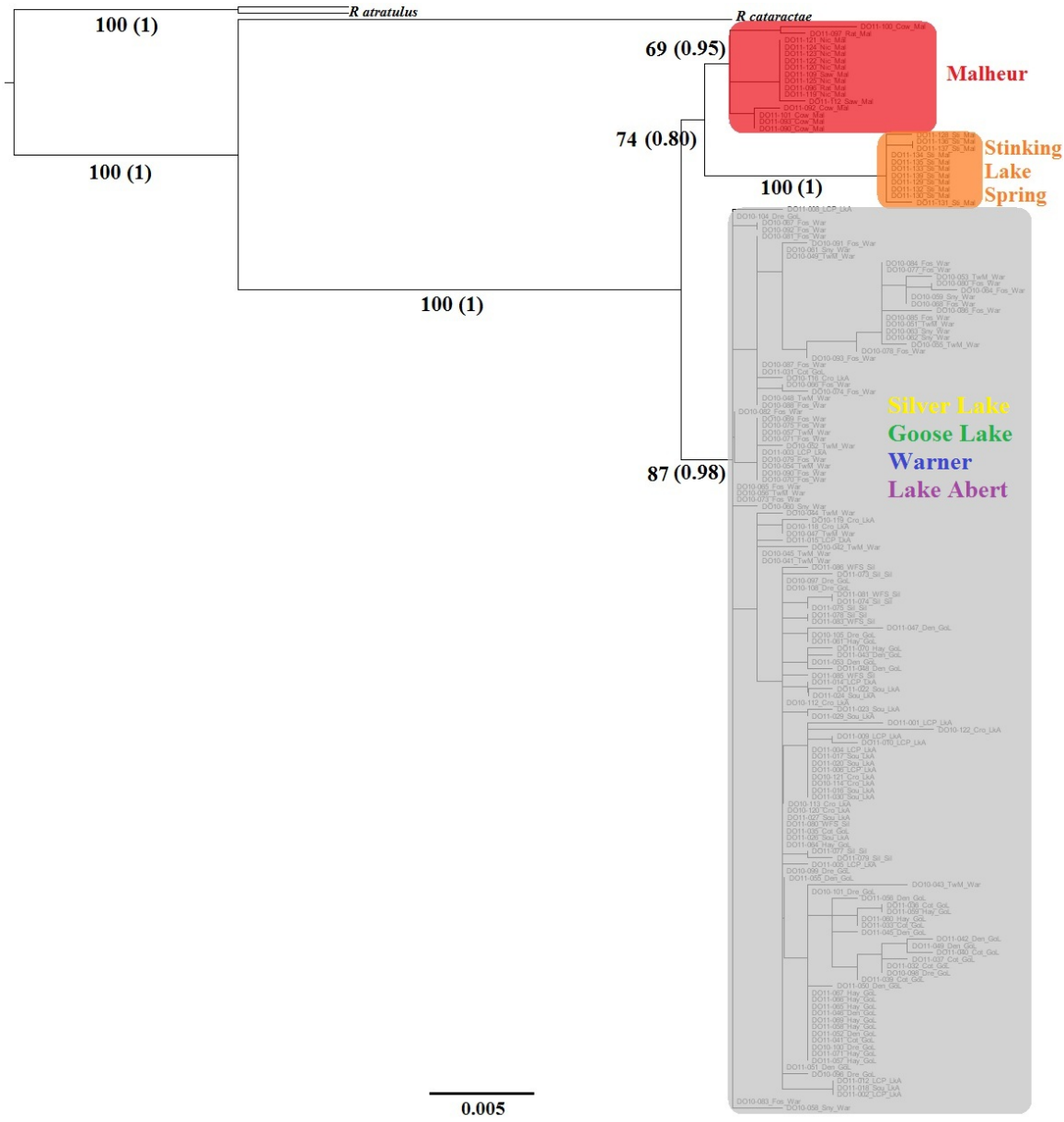

Figure 2.3: Maximum likelihood (RAxML) phylogeny based on S7 sequences from 161 individuals of *Rhinichthys osculus* collected from Eastern Oregon's Great Basin. Bootstrap values (and Bayesian posterior probabilities from MrBayes in parentheses) are presented at branches. The scale bar shows the number of substitutions per sequence position.

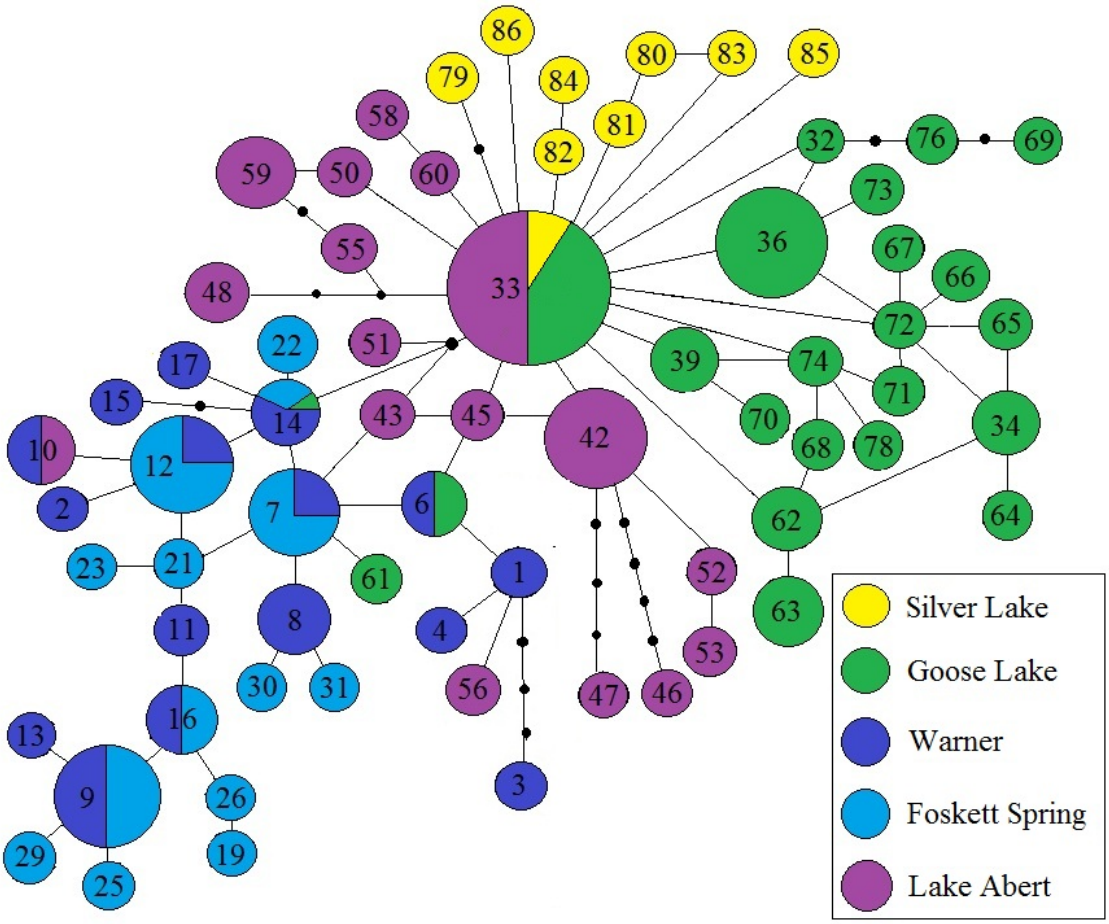

Figure 2.4: Haplotype network of Goose Lake, Silver Lake, Lake Abert, and Warner Valley individuals based on S7 sequences (gray clade in Figure 2.3). Circles are colored by the location of specimens, and black dots show inferred substitutions. Each line represents a single base pair change out of the 857 base pairs sequenced. Numbers in colored circles indicate the arbitrary haplotype number. The relative size of the dots indicates the number of individuals with a given haplotype.

a monophyletic clade in the maximum likelihood topology. With the exception of Warner, the phylogeny shows monophyly of all of the major drainage basins and deep divergence of Malheur basin from all other clades. There is no further structuring within each basin based on ND2 phylogenetic tree, as the individual streams do not form clusters. The haplotype network of Warner Valley individuals (Figure 2.6) shows that Foskett Spring speckled dace are not monophyletic, but rather are scattered amongst individuals from Warner streams. This does highlight, however, that Foskett Spring speckled dace share no ND2 haplotypes with dace from other locations.

The fact that the ND2 topology matches the tree from the concatenated dataset indicates that the fewer variable sites in the S7 sequence data give a weaker signal; 292 out of 1001bp of ND2 are parsimony informative, whereas only 73 out of 857bp of S7 are parsimony informative. One key difference between the two gene trees is the sister relationship of the two clades from Malheur seen in the S7 tree, as opposed to the ND2 tree which shows Malheur dace as sister to all other speckled dace sampled here.

BEAST returned a very similar tree topology as the ML and MrBayes analyses (Figure 2.7). The 95% HPD (highest posterior density) bars for the approximate branching dates are large, spanning about 2.5 million years at each node, likely because there was only one fossil calibration. Despite the limited fossil record for these minnows and weak calibration, this tree still provides approximate dates for divergences. Malheur stream fish diverged from all the other fish in this study between 1.91-5.38mya, and Stinking Lake Spring dace diverged from the other

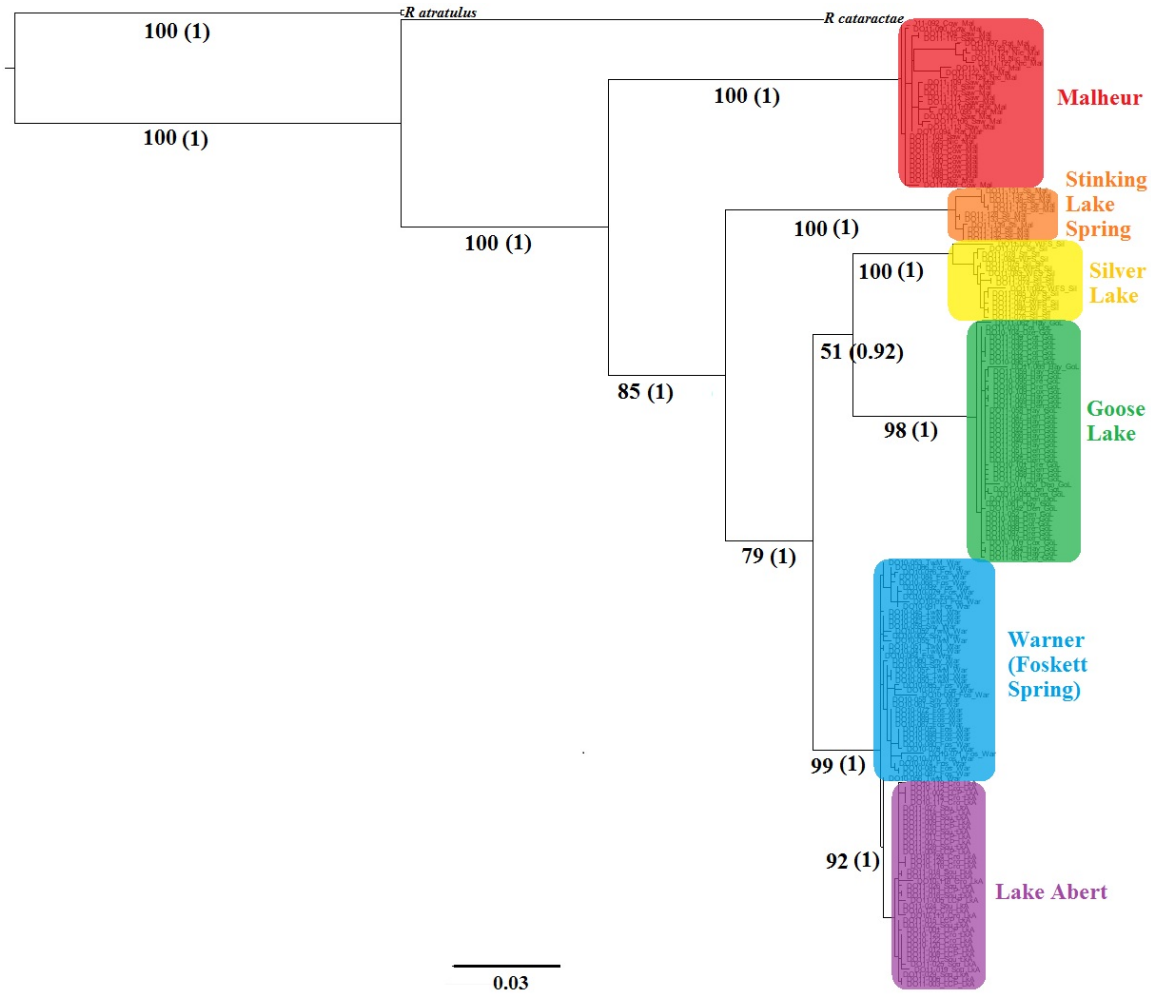

Figure 2.5: Maximum likelihood (RAxML) phylogeny based on the ND2 sequences from 197 individuals of *Rhinichthys osculus* collected from Eastern Oregons Great Basin. Bootstrap values (and Bayesian posterior probabilities from MrBayes in parentheses) are presented at branches. The scale bar shows the number of substitutions per sequence position.

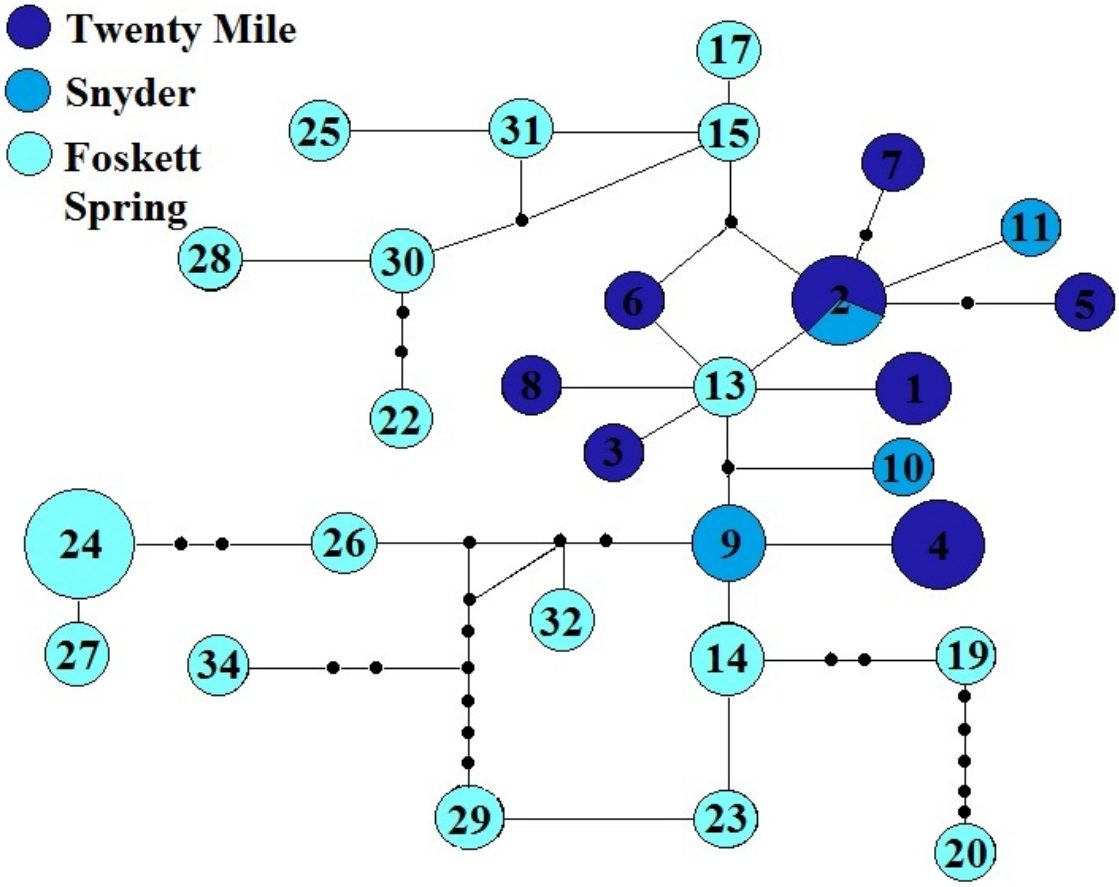

Figure 2.6: Haplotype network of Warner Valley individuals based on ND2 sequences. Circles are colored by the location of specimens, and black dots show inferred substitutions. Numbers in colored circles indicate the arbitrary haplotype number. The relative size of the dots indicates the number of individuals with a given haplotype. A line between colored circles represents a single base pair change out of the 1001 base pairs sequenced.

basins between 1.31-3.97mya. The Warner/Lake Abert clade split from the Silver Lake/Goose Lake clade between 0.71-2.33 mya. Lake Abert split from Warner around 0.23mya and Goose Lake and Silver Lake split around 1.09mya.

In order to help identify clades that might deserve taxonomic recognition, we calculated the average sequence divergence. The three clades identified by the S7 gene tree are the clades that have the highest genetic differentiation based on the mitochondrial gene; Malheur, Stinking Lake Spring, and the other four basins (Table 2.3). The percent sequence divergence for S7 between these clades small, but is still significantly different (2-sample t-test, $p<0.0001$) from the intra-basin differentiation. Dace from Stinking Lake Spring average a 1.56% and 1.40% difference from fish from the four basins and from Malheur, respectively, and Malheur fish differ on average by 1.17% from the other four basins. The ND2 gene distances show substantial divergences between each of the five clades, from as low as 4.23% between Goose Lake and Warner to as high as 13% between Malheur and the other four basins. Stinking Lake Spring dace are 10-13% different from each other basin.

The phylogenetic trees we produced also provided more information about the genetic distinctiveness of Foskett Spring speckled dace. Based on both gene trees and the concatenated tree, we determined that these speckled dace are not monophyletic, but rather appear scattered within a larger clade also including specimens from the Warner basin (Figure 2.5).

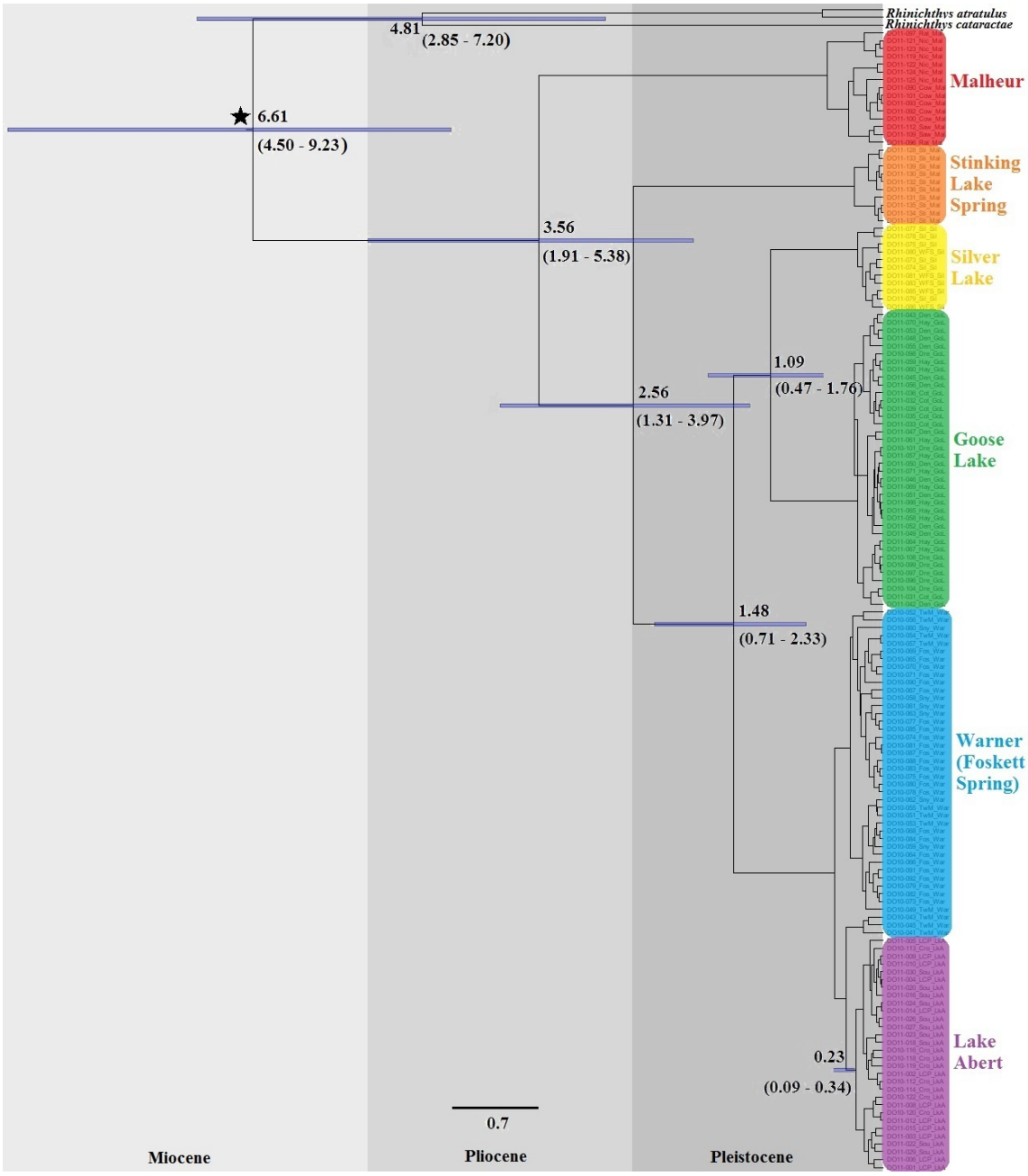

Figure 2.7: BEAST v1.4.7 Bayesian tree using the concatenated dataset (ND2 and S7) of *Rhinichthys osculus* collected from Southeast Oregon. The dates at branching points are in millions of years before present, blue bars indicate 95% HPD of divergence dates. Geologic epochs are highlighted. The fossil calibrated node is indicated with a star.

Table 2.3: Percent sequence divergence of ND2 based on average number different base pairs per 1000 total base pairs. Percentages in italics along the diagonal represent intra-basin variation.

	Warner	Lake Abert	Goose Lake	Silver Lake	Stinking Lake Spring	Malheur
Warner	*1.67*	1.89	4.23	5.72	10.00	12.99
Lake Abert		*1.28*	4.27	5.68	9.95	12.94
Goose Lake			*2.53*	4.96	9.97	12.96
Silver Lake				*8.13*	12.84	13.12
Stinking Lake Sp					*0.45*	10.83
Malheur						*4.75*

2.3.2 Microsatellites

No locus or population had signals of null alleles or allelic drop out. None of the pairs of loci displayed evidence of linkage disequilibrium. Some populations deviated from HWE for some loci, but none were consistently problematic. All of the deviations from HWE resulted from heterozygote deficiency, even though there was no signal of allelic drop out. Other explanations for this pattern, including inbreeding or the Wahlund effect due to population substructure (Wahlund, 1928), are difficult to assess with this limited data. Though, Loc1 and Loc4 have been noted to be affected by null alleles in a previous study (Salgueiro et al., 2003), which makes allele dropout a more plausible explanation.

The observed and expected heterozygosities (H_E) ranged from 0.74 in Lake Abert to 0.88 in Silver Lake (Table 2.4). The high levels of genetic diversity detected in these populations are consistent with the high census population sizes that are maintained by cyprinids. These values are higher than those found in many

other cyprinid species with high genetic diversity, such as the Cape Fear shiner ($H_E = 0.70$) reported by Saillant et al. (2004), *Anaecypris hispanica* ($H_E = 0.68$) reported by Salgueiro et al. (2003), and splittail (*Pogonichthys macrolepidotus*, $H_E = 0.66$) reported by Baerwald et al. (2007).

Table 2.4: Summary statistics for the microsatellite data: Sample size, observed and expected heterozygosities with standard deviation, and effective population size (with 95% confidence intervals) as calculated by LDNe.

	N	H_O	S.D.	H_E	S.D.	N_e	95% CI
Malheur	40	0.7205	0.2717	0.7611	0.2671	11.3	6.3-19.2
Stinking Lake Spring	12	0.8496	0.1621	0.8447	0.0975	31.6	34.2-inf
Silver Lake	16	0.9280	0.0667	0.8847	0.1166	604.4	39.4-inf
Goose Lake	49	0.7331	0.2801	0.8004	0.2768	95.3	51.2-330.0
Warner	18	0.7374	0.2770	0.7608	0.3034	197.9	67.0-inf
Foskett Spring	41	0.7866	0.1987	0.7520	0.1994	110.5	27.1-inf
Lake Abert	39	0.7314	0.3141	0.7413	0.3155	326.1	148.0-inf

Structure inferred that there were seven populations in our sample area, using the Evanno et al. (2005) method (Figure 2.8); the five basins and both spring populations were all identified as distinct. Most interestingly, Foskett Spring speckled dace represents a distinct population unit without recent geneflow between that spring and the surrounding Warner Basin. Some individuals do not assign to their geographic population, primarily Goose Lake, Silver Lake, and Warner, but individuals from the Stinking Lake, Malheur, and Foskett populations of speckled dace show much higher assignability to their groups.

Pairwise F_{ST} and Nei's D are shown in Table 2.5. The highest pairwise F_{ST}s between populations are found among Malheur and Foskett, Lake Abert, and Stinking Lake; between Stinking Lake and Foskett Spring and Lake Abert; and between

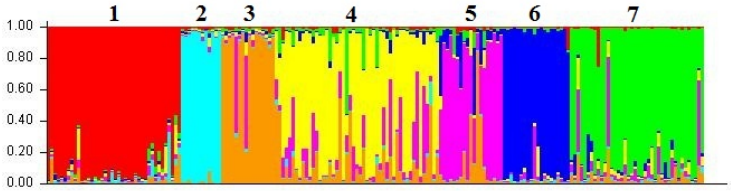

Figure 2.8: Structure bar plot results obtained on the microsatellite dataset of *Rhinichthys osculus* collected from Southeast Oregon at K = 7. 1 (red) is Malheur, 2 (light blue) is Stinking Lake Spring, 3 (orange) is Silver Lake, 4 (yellow) is Goose Lake, 5 (purple) is Warner, 6 (blue) is Foskett spring, and 7 (green) is Lake Abert. Each vertical bar represents one individual and the color of the bar represents the population assignment.

Foskett Spring and Lake Abert. Population pairs with low F_{ST} s include Silver Lake and Goose Lake, and Warner and Foskett Spring. Both distance measures shows similar patterns; high differences between Malheur and Stinking Lake, Foskett, and Lake Abert, and very low difference between Goose Lake and Silver Lake. Interestingly, Silver Lake and Goose Lake are more similar than Foskett, Warner, and Lake Abert are to each other.

Table 2.5: F_{ST} estimates between populations (below the diagonal) and Nei's D (above the diagonal)

	Malheur	Stinking Lake Spring	Silver Lake	Goose Lake	Warner	Foskett Spring	Lake Abert
Malheur	–	0.543	0.448	0.343	0.478	0.574	0.540
Stinking Lake	0.109	–	0.421	0.473	0.454	0.531	0.462
Silver Lake	0.088	0.081	–	0.262	0.401	0.501	0.310
Goose Lake	0.069	0.086	0.004	–	0.360	0.442	0.281
Warner	0.097	0.090	0.076	0.067	–	0.300	0.318
Foskett Spring	0.114	0.109	0.097	0.083	0.060	–	0.497
Lake Abert	0.117	0.101	0.066	0.061	0.071	0.108	–

The FCA shows three distinct and completely separable clusters; Malheur, Stinking Lake Spring, and the other basins combined (Figure 2.9). Stinking Lake Spring dace are separated from the cluster of Goose Lake, Silver, Warner, and Lake Abert along axis 1. Malheur dace are separated from everything along the second and third FCA axis. This mirrors the phylogenetic results from the S7 sequence data, in which three clear clades were identified.

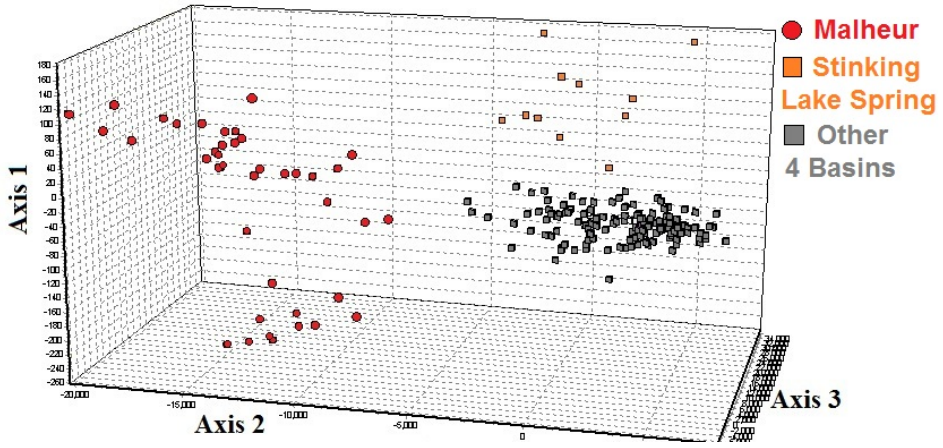

Figure 2.9: Factorial correspondence analysis plot based on microsatellite data of the *Rhinichthys osculus* collected from Southeast Oregon. Red circles represent Malheur, Orange squares represent Stinking Lake Spring, and Gray squares represent the other four basins (Goose Lake, Silver Lake, Warner, and Lake Abert).

The effective population size results are also presented in Table 2.4. Only two of the populations had non-infinite confidence intervals, Malheur and Goose Lake, likely because these two had the largest sample sizes. Goose Lake has between 51 and 330 effective individuals. Malheur stream dace have an extremely low N_e between 6 and 19 individuals. The higher pcrit of 0.01 gives a wider N_e estimate, between 55 and 186 individuals, however that is still unrealistically low.

2.4 Discussion

2.4.1 Pattern of Genetic Variability

We found genetic differentiation at different scales throughout Oregon's Great Basin region, depending on the molecular marker or test being used. Deep divergences among lineages were revealed by the sequence data, and have been separated for millions of years. More recently diverged clades were also supported by the mtDNA, but not by the nuclear sequence data. Fine scale population structure was revealed by the microsatellite data. All of these data help paint a picture of the genetic variability in this region that has previously been unclear.

The deepest genetic divergences were revealed by all of the genetic markers and show strong support for three clades within our sample area (Figure 2.3, 2.5, 2.9); Malheur stream dace, Stinking Lake Spring dace, and dace from the other four endorheic basins (Silver Lake, Goose Lake, Warner, and Lake Abert). The concatenated sequence dataset and ND2 alone show that Malheur stream dace are the most distantly related and are sister to a clade containing the other speckled dace populations. If branches are collapsed at commonly-used support values (75% bootstrap, 0.95 posterior probability), the nuclear sequence data shows a polytomy between these three clades. The clustering of Goose Lake, Silver Lake, Warner Valley, and Lake Abert fishes into one indistinguishable clade indicates that fish in these basins diverged from each other more recently.

Finer genetic divisions were revealed by the mitochondrial sequence data, which was expected as this is a faster evolving locus. There are three subclades within

the endorheic basin clade. Goose Lake and Silver Lake dace are distinct from each other and have a sister relationship (Figure 2.5, 2.2). Warner and Lake Abert dace form a clade, sister to Goose Lake and Silver Lake, but are not reciprocally monophyletic. Lake Abert forms a distinct clade, but Warner Valley dace are paraphyletic with respect to this group and have no further structuring based on the mitochondrial sequence data (Figure 2.6).

Population level genetic structuring, the finest-scale detected in this study, was revealed by the microsatellite Structure analysis: Malheur, Stinking Lake Spring, Silver Lake, Goose Lake, Warner, Foskett Spring, and Lake Abert form populations (Figure 2.8). This indicates that there has been no recent gene flow between the basins and springs populations, and particularly interesting is the separation of Foskett Spring and Warner dace. The separation of Foskett from the rest of Warner is consistent with the geological history of the area as there have been no water connections between basins or between springs and streams within basins since the end of the Pluvial period (10,000 years ago).

The smaller genetic distance measures based on the microsatellite data found between Silver Lake and Goose Lake (Table 2.5) gives evidence that dace from these basins are more closely related to each other than Foskett Spring, Warner, and Lake Abert dace are to each other. In contrast, the mitochondrial sequence data shows a closer genetic relationship of Foskett Spring, Warner, and Lake Abert dace, where Lake Abert forms a clade within Warner and Foskett Spring is not distinct from Warner (Figure 2.5). This could reflect more recent hydrological connections or bait bucket transfers providing migrants, the latter seeming more

likely because of the common use of speckled dace for bait fish and the lack of recent connections between these particular basins. Chen et al. (2009) found a similar pattern in tui chub (*Gila bicolor*) from the same area: individuals from Goose Lake were closely related to Summer Lake (subbasin of Lake Abert) and Warner individuals, even though the geologic history suggests long isolation between these basins. In particular, the Summer Lake tui chubs are thought to be human transfers from Goose Lake because the population there has been subject to multiple eradications (Chen et al., 2009), which gives more evidence for recent human transfers of speckled dace into the Goose Lake populations to explain the admixture signals.

2.4.1.1 Effective Population Size

The effective population size measurements were overall uninformative; five out of the seven measurements had infinite confidence intervals, probably because the linkage disequilibrium method (LDNe) requires high sample sizes and many highly polymorphic loci, and cyprinids have large census sizes. Malheur stream dace have an inferred N_e between 6 and 19 individuals, which does not seem accurate given the large census sizes and high number of streams that they are located in (Stephanie Miller, ODFW, pers. comm.). In comparison, an endangered silvery minnow (*Hybognathus amarus*) had a similar N_e estimate, between 62-102 based on the temporal method (Alo and Turner, 2005). However, the normal N_e, pre-exploitation, which seems closer to the large, widespread Malheur population, was

on the order of 10,000 - 1 million (Alo and Turner, 2005). With the data we have currently, we cannot accurately determine the effective size of these populations, which would shed light on the stability of the isolated populations.

2.4.2 Phylogeography

The three main clades, Malheur, Stinking Lake Spring, and the other four basins all split from each other before the start of the Pleistocene; the Malheur stream dace may have separated from everything else as early as the Miocene-Pliocene boundry (Figure 2.7). This early separation makes sense, geologically, because Malheur drainage was not connected to these other drainages during the Pluvial period (Hubbs and Miller, 1948), and was more likely recently connected to the Snake drainage. So although it is clear that these dace are distinct from those inhabiting the rest of Oregon's Great Basin, they may be closely related to dace in the adjacent Snake drainage. Therefore, the Malheur stream dace may be part of a taxonomic unit that includes populations in different drainages not included in this study. One possibility is that *Rhinichthys osculus carringtoni*, Bonneville speckled dace, which is a subspecies from Bonneville and the upper Snake drainage, is the same taxonomic entity as the Harney-Malheur Basin populations, as hypothesized by Smith et al. (2002).

The dace from each of Oregon's other Great Basin drainages are also distinct from each other (except for those from Warner and Lake Abert), but they may be closely related to dace in adjacent drainages due to ancient watershed connections

(Figure 1.2). Silver Lake is thought to have been connected to the north to the Deschutes and Columbia systems, or with less support, to the Klamath system (Hubbs and Miller, 1948). Goose Lake is hypothesized to have drained to the Pit River system before becoming isolated (Hubbs and Miller, 1948). This connection is supported by evidence from the tui chub (*Gila bicolor*), which also has a widespread distribution throughout the Great Basin. Chen et al. (2009) found that tui chub from Goose Lake and Pit River were indistinguishable from each other. Evidence from Ardren et al. (2010) (cytochrome b sequence data) suggests that Goose Lake dace are closely related to one of the linages found in Klamath basin, and although Ardren et al. (2010) did not sample Silver Lake or the Pit River system, this provides some support for close linkage among the Klamath, Goose Lake, and Silver Lake basins.

Silver Lake and Goose Lake split during the Pleistocene, so these two basins may have been connected by high waters of the Pleistocene Pluvial period, although the geologic high water evidence suggests there was no interchange during that time (Hubbs and Miller, 1948). The microsatellite data indicate that Silver Lake and Goose Lake dace are closely related, however, which could indicate that these two basins were connected in more recent history, or were subject to anthropogenic transfers.

Goose Lake/Silver Lake split from Warner/Lake Abert during the Pleistocene, around 1.5mya, which is much later than the Warner Mountain uplift around 4.5mya (Minckley et al., 1986). That barrier would have been significant enough to prevent genetic mixing between Goose Lake and Warner Valley, and the se-

quence data indicates it took until 2.33mya at the earliest for reciprocal monophyly to occur between Goose Lake and Warner. Additional sequence data beyond the mtDNA and one nuclear gene may give better information; Ardren et al. (2010), using CytB mutation rates, calculated this split at 2.83–4.27mya, so different molecular markers likely show different histories.

Lake Abert basin dace separated from the Warner Valley dace around 230,000 years ago, much more recent than the split of these two basins from the Goose Lake/Silver Lake clade. However, Lake Abert basin is located between Silver Lake and Goose Lake, and has low elevation barriers separating them (800ft to Silver Lake and 900ft to Goose Lake), but is separated from Warner Valley by a large ridge called the Abert Rim, which rises 2500ft above the valley floor (4000ft to 6500ft), seemingly showing disagreement between the geologic and genetic data. However, Abert Rim fault has had recent movement, around 175,000 years ago (Pezzopane and Weldon, 1993), so the tectonic division between Lake Abert and Warner Valley appears to correspond with the date of the genetic division.

Warner Valley was connected through Coleman Valley (containing Foskett Spring) in the south to Surprise Valley (NV) during the high water time of the Pleistocene, which in turn was connected to Long Valley (CA) and the Pleistocene Lake Lahontan. These biogeographic patterns suggest that dace in surrounding drainages, such as Klamath, Deschutes, Snake, or Lahontan, are the closest relatives of the speckled dace in Oregon's Great Basin, but only a widespread, range-wide phylogeographic study of speckled dace will fully resolve these systematic questions.

The dates from the BEAST analysis are likely overestimates of the actual geological divergence time between these basins because they reflect the age to the most recent common ancestor (MRCA) and population isolation may well have occurred subsequent to the origin of haplotype clades. Additionally, population structure may contribute to the maintenance of divergent lineages and increase the coalescence time (Hoelzer et al., 1998). It seems clear that the biogeographic patterning of speckled dace populations distributed among major river basins in this region reflects vicariant and dispersal events that occurred during the Pliocene and Pleistocene. The Warner Valley, Foskett Spring, and Lake Abert dace clade and Goose Lake/Silver Lake divergences all occurred during the Pleistocene.

2.4.3 Unrecognized Taxonomic Entities

Three clades were consistently observed in all of the molecular datasets; Malheur dace, Stinking Lake Spring dace, and dace from the other four basins combined (Figure 2.2, 2.9). The percent sequence difference between these clades is quite high; Malheur basin dace are about 12% different from other clades on average (ND2), Stinking Lake Spring dace are about 10% different. For the same gene region, ND2, European *Barbus* species differ by an average of 7.97% (Zardoya and Doadrio, 1998) and species in the North American cyprinid genus *Lythrurus*, differ by an average of 10.3% (Schmidt et al., 1998). The level of sequence divergence for S7 that we observed between the three main clades (1.17-1.56%) is close to the levels observed between other different species within the family Cyprinidae (Wang

et al., 2002). The mitochondrial and nuclear data indicate that the divergence between the Malheur clade, Stinking Lake Spring clade, and the four basin clade are on the order of species-level differences noted in other studies of cyprinids (McPhail and Taylor, 1999). Although dace from each of the four other basins are monophyletic (except Warner), the levels of divergence are lower than typical between-species differences and the nuclear data do not distinguish among these basins.

2.4.4 Foskett Spring

We found no genetic evidence that speckled dace from Foskett Spring warrant subspecies or species status. Foskett Spring speckled dace are not monophyletic, but rather appear within a larger clade also including specimens from the Warner basin (Figure 2.2, 2.3, 2.5). The apparent lack of genetic distance between Foskett Spring and Warner basin dace based on both nuclear and mitochondrial DNA sequence data indicates that this population has not been isolated long enough to diverge significantly (10,000 years, Ardren et al. 2010). Our results confirm Ardren et al. (2010)'s finding that Foskett Spring dace are not distinguishable based on mtDNA sequence data. However, we did find evidence that Foskett Spring dace are a distinct population, separate from the rest of Warner Valley dace (Figure 2.8). This provides evidence that Foskett Spring and Warner stream dace have not experienced genetic admixture recently and are reproductively isolated.

2.4.5 Conclusion

We found clear evidence for the presence of three distinct taxonomic entities: dace from Malheur, dace from Stinking Lake Spring, and dace from the other four endorheic basins (Goose Lake, Silver Lake, Lake Abert, and Warner). Further population structure was recovered from the Structure analysis and the mtDNA sequence data, which distinguished among each sampled basin and spring. The populations not distinct from the nuclear sequence data (Goose Lake, Silver Lake, Lake Abert, Warner, and Foskett Spring) have not had enough evolutionary time to diverge along the slower evolving nuclear intron marker, or to have diagnosable microsatellite genotypes, but are clearly distinct populations.

The apparent lack of genetic differentiation between Foskett Spring and Warner basin dace based on both nuclear and mitochondrial DNA sequence data indicates that this population has not been isolated long enough to diverge significantly (only 10,000 years). However, the microsatellite analysis suggests a lack of recent gene flow (reproductive isolation) between Foskett Spring dace and dace in the rest of Warner basin. The lack of reciprocal monophyly indicates that Foskett Spring Dace is not a distinct species, but the evidence of recent genetic isolation and the unique habitat of the population qualifies it for consideration as an evolutionarily significant unit on a unique evolutionary path. If this population has distinguishable morphological features, we would recommend Foskett Spring speckled dace for ESU status.

Chapter 3 – Morphological variation of *Rhinichthys osculus* from Oregon's Great Basin

3.1 Introduction

Speckled dace (*Rhinichthys osculus*) are a widespread group of fish that have had a complicated taxonomic history and have been referred to as a species complex. This confusion stems from the variation in meristics, color, size, proportions, and morphological traits over their range (Hubbs et al., 1974; Hubbs and Miller, 1948; Woodman, 1992; Scott and Crossman, 1998). Describing the morphological variation of speckled dace from Oregon's Great Basin will help inform any taxonomic revisions and provide some clarity to the confusing taxonomy of this fish. To reconstruct the pattern of variability across Oregons Great Basin, we investigated whether speckled dace from different basins, habitat types, or genetically-defined populations differ in morphology. To evaluate morphologic variation, we collected geometric and linear morphometric data and lateral line scale counts.

Our analysis was motivated primarily by the need to evaluate the taxonomic status of Foskett Spring speckled dace, a federally listed undescribed, threatened subspecies from Eastern Oregon. Currently, there is no morphological diagnosis by which to distinguish this taxon from other dace distributed throughout the state. A brief examination by OSU Professor Carl Bond, dated 1990 and presented in the

1998 Recovery Plan (US Fish and Wildlife Service, 1998), suggested that Foskett Spring speckled dace differed slightly from those found in the rest of the Warner basin by having a larger eye and a more posterior dorsal fin origin, but this has never been confirmed. Does Foskett Spring in fact have a distinct morphology and was Carl Bond correct? By answering these questions, we respond directly to the recommendation in the five-year review for Foskett Spring Dace for a "systematic assessment of morphological traits and life history to determine whether or what subspecies classification is warranted" (US Fish and Wildlife Service, 2009).

We also investigated whether the morphology of two populations of spring dace in the study area, Foskett Spring and Stinking Lake Spring, differ consistently from stream dace. If the environment affects speckled dace morphology, daces from geographically isolated but environmentally similar springs would possess similar morphologies, either due to an ecophenotypic response to small, predator-free, spring habitats, or rapid genetic adaptation to similar environmental conditions. Conversely, morphological variation may be unaffected by habitat and only reflect shared evolutionary history, in which case spring dace would tend to resemble their closest genetic relatives among stream-dwelling dace.

3.2 Methods

3.2.1 Specimen Acquisition

Specimens were captured using a seine net, dipnet, or minnow trap, and euthanized in an aqueous solution of tricaine methane sulfonate (MS-222, approximately 2g/L), buffered with equal concentration of sodium bicarbonate. Following euthanization, the specimens were preserved in 10% formalin. After one week in formalin, the specimens were transferred to a 95% ethanol solution for permanent storage. All specimens collected and used in this paper are catalogued in the OSIC (Appendix 1). These collections were performed under Animal Care and Use Protocol 4050 approved by Oregon State Universitys Institutional Animal Care and Use Committee (IACUC).

3.2.2 Sampling Locations

The chosen sampling sites represent all drainages surrounding the Warner Valley (Figure 1.2), and likely include all possible close relatives of Foskett Spring speckled dace because of their geographic proximity. This geographic region is the northern portion of the Great Basin. Foskett Spring is located in the Coleman Lake subbasin of Warner basin. We aimed to sampled 30 dace from three streams in each basin (Figure 1.2), as well as 40 Foskett Spring speckled dace (for actual numbers see Table 3.1). We also sampled spring dace from Stinking Lake Spring in Malheur drainage, which was the only additional spring we found with speckled dace in the

basins of interest (based on museum records and input from district biologists). In total we collected speckled dace from Warner Valley, Goose Lake basin, Lake Abert basin, Silver Lake basin, and Malheur basin.

Long Valley, Surprise Valley, and Catlow Valley were not included in this study because speckled dace do not appear to inhabit these drainages. Long Valley in Nevada has little perennial water and no dace were found there during our investigations in the summer of 2011 (we sampled at the only location with flowing water and no dace were found). The district biologist of Washoe County (pers. comm. Matt Maples of NDOW) did not know of any springs or streams containing dace. We sampled in Surprise Valley of California on public lands (all within Modoc National Forest) and found no speckled dace. The water in these Warner mountain streams is heavily diverted for agricultural use so speckled dace or other native fish may be found in irrigation ditches on private property, but not in the higher elevation stream on public land. Catlow Valley to the east of Warner has no records of speckled dace (Hubbs and Miller 1948, pers. comm. Shannon Hurn, ODFW).

3.2.3 Geometric Morphometrics

Geometric morphometric analyses involved placing 16 homologous landmarks (adapted from Nacua 2010) that describe the overall body shape of each individual (Figure 3.1). Insect pins were inserted in the landmark locations on all 330 specimens collected and pictures were taken with a Nikon D5000 digital camera. The land-

Table 3.1: Basin and stream locations (springs marked by asterisks) of the speckled dace collected for this study. The number of vouchers is the total number of specimens collected and used for morphometric analyses. The number in parentheses is the number of tissue samples taken for genetic analyses. Samples are deposited in the Oregon State Ichthyology Collection under the catalog numbers listed.

Basin	Location	N=vouchers(tissues)	Catalog number
Warner Valley	Twenty Mile Creek	17 (15)	OS 18125
	Snyder Creek	6 (6)	OS 18126
	*Foskett Spring	41 (41)	OS 18127
	(total)	64 (62)	
Goose Lake	Cox Creek	3 (3)	OS 18129
	Drews Creek	31 (15)	OS 18128
	Cottonwood Creek	11 (11)	OS 18400
	Dent Creek	30 (14)	OS 18393
	Hay Creek	30 (15)	OS 18394
	(total)	105 (58)	
Lake Abert	Crooked Creek	30 (15)	OS18130
	Little Coffeepot Creek	30 (15)	OS 18391
	South Creek	30 (15)	OS 18397
	(total)	90 (45)	
Silver Lake	Silver Creek	8 (8)	OS 18401
	West Fork Silver	8 (8)	OS 18396
	(total)	16 (16)	
Malheur	Cow Creek	11 (11)	OS 18398
	Rattlesnake Creek	4 (4)	OS 18389
	Sawtooth Creek	16 (15)	OS 18390
	Nicoll Creek	12 (10)	OS 18392
	*Stinking Lake Spring	12 (12)	OS 18286
	(total)	55 (52)	

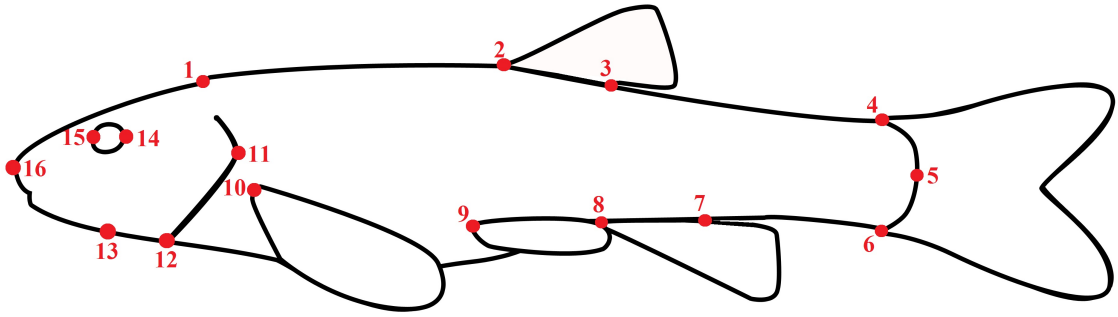

Figure 3.1: Sixteen landmarks used in the geometric morphometric analysis of speckled dace (*Rhinichthys osculus*): (1) tip of the supraoccipital, (2) origin of dorsal fin, (3) insertion of dorsal fin, (4) dorsal base of caudal fin, (5) midpoint of posterior edge of the hypural plate, (6) ventral base of caudal fin, (7) insertion of anal fin, (8) origin of anal fin, (9) origin of pelvic fin, (10) origin of pectoral fin, (11) posterior margin of opercle, (12) ventral conjunction of contralateral fleshy margins of opercular cover, (13) anterior tip of isthmus (inset) (14) posterior edge of orbit, (15) anterior edge of orbit, (16) anterior tip of upper snout.

marks were digitized in tpsDig2 (Rohlf, 2010) and then loaded into the MorphoJ 1.05d (Klingenberg, 2008) software platform, where non-shape variation was removed using generalized Procrustes superimposition (Rohlf and Slice, 1990), which translates all specimens to a common location, scales them to unit size, and rotates them to minimize deviation from a consensus configuration.

We regressed Procrustes coordinates of all of the specimens against log centroid size in MorphoJ (Klingenberg, 2008) to see whether the specimens display allometry. Allometry occurs in most fishes, where certain body proportions change over time as growth rates change, which can conflate true differences in morphology with shape differences due to size differences among specimens. In order to correct for allometry, a principal components analysis (PCA; Jolicoeur 1963) was per-

formed in MorphoJ 1.05d (Klingenberg, 2008) on the residuals from the allometric regression, rather than on the Procrustes scores. This produced a size-standardized morphospace permitting meaningful comparisons of the morphology of specimens of different sizes.

The PCs which summarized substantially more variation than the next most variable axis (PC1 31.29%, PC2 13.55%, PC3 8.03%) were tested for significant morphological differences among groups of dace, defined by the molecular Structure analysis. Pairwise Hotelling's comparisons of MANOVAs were performed in PAST (Hammer et al., 2001) and Bonferroni corrected. Tukey Honestly Significant Difference (HSD) tests were done on each PC in the statistical package R v2.15.2 (R Core Team, 2012). Only the PCs that had significant Tukey HSD comparisons between groups were examined further for morphological variation among groups.

We also checked for sexual dimorphism. Thirty-two individuals from Foskett Spring were sexed by dissection. A PCA on the allometric regression residuals had three principal components summarizing more than 5% of the variation. A MANOVA comparing these PCs between males and females was not significant (p=0.516), indicating that, for Foskett Spring, the configuration of the selected morphometric landmarks is not sexually dimorphic. Populations from South Creek (n=29) and Dent Creek (n=30) were sexed as well and the MANOVA of PC1, PC2, and PC3 by sex was not significant (p=0.1330), so sexual dimorphism for these landmarks appears absent in stream populations as well. Sexual dimorphism was not taken into account in any further analyses.

Canonical Variate Analyses (CVA) on the same residuals (MorphoJ 1.05d; Klin-

genberg 2008), with specimens grouped by genetically-defined populations (see Chapter 2), were used to determine the aspects of morphology that best discriminate among *a priori* groups. CVA finds the axes of variation that optimize among-group differences relative to within-group variation, thereby finding the shape features that best distinguish among groups of specimens.

3.2.4 Linear Morphometrics

Linear morphometric measurements were taken with digital calipers for all of the fish collected in this study (N=330). There were 37 measurements taken (Armbruster (2012), Table 3.2), three times per fish, and averaged, to reduce the influence of human error. A regression of these measurements against the standard length was performed to correct for allometry, and the residuals were log transformed, because the residuals were larger for larger standard lengths.

A principal components Analysis on the log transformed residuals was performed in the program PAST (Hammer et al., 2001). Only the PCs that summarized substantially more variation than the next most variable axis were evaluated further. The loadings of each PC were investigated to determine what linear measurements were changing on the different axes. Loadings with absolute magnitude greater than 0.2 were considered to be important (following Sidlauskas et al. (2011)). To determine significant differences between groups, Pairwise Hotellings comparisons of MANOVAs were performed in PAST (Hammer et al., 2001) and Bonferroni corrected. Tukey Honestly Significant Difference (HSD) tests were done

Table 3.2: Principal Component (PC) loadings from the linear morphometric analysis. Loadings larger than 0.2 are indicated by an asterisk.

#	Measurement	PC1	PC2	PC3
	Percent variance explained	19.23	15.45	10.27
1	Premaxilla to Anal fin Origin	0.068	-0.002	-0.083
2	Premaxilla to Dorsal fin Origin	0.120	0.046	-0.124
3	Premaxilla to Pelvic fin Origin	0.098	-0.003	-0.082
4	Premaxilla to Pectoral fin Origin	0.080	-0.013	0.054
5	Supraoccipital Crest to Dorsal fin Origin	0.182	0.025	-0.187
6	Supraoccipital Crest to Pelvic fin Origin	0.186	-0.021	-0.100
7	Supraoccipital Crest to Pectoral fin Origin	*0.236	-0.010	0.062
8	Dorsal fin Origin to Caudal fin	-0.081	0.022	*0.201
9	Dorsal fin Origin to Pectoral fin Origin	0.120	0.052	*-0.207
10	Dorsal fin Origin to Pelvic fin Origin	0.160	*0.207	0.149
11	Dorsal fin Origin to Anal fin Origin	0.031	0.170	0.200
12	Dorsal fin Origin to Anal fin Insertion	-0.057	0.176	0.104
13	Dorsal fin Base Length	*-0.221	*0.569	-0.007
14	Dorsal fin Insertion to Pectoral fin Origin	0.055	0.175	-0.220
15	Dorsal fin Insertion to Pelvic fin Origin	0.072	*0.249	-0.066
16	Dorsal fin Insertion to Anal fin Origin	0.085	0.150	0.120
17	Dorsal fin Insertion to Anal fin Insertion	0.007	0.043	*0.240
18	Dorsal fin Insertion to Caudal fin	-0.053	-0.110	*0.276
19	Anal fin Base Length	-0.064	*0.476	-0.017
20	Anal fin Origin to Caudal fin	-0.058	-0.045	0.190
21	Anal fin Insertion to Caudal fin	-0.062	-0.157	*0.376
22	Pelvic fin Origin to Anal fin Origin	0.007	0.049	-0.028
23	Pelvic fin Origin to Caudal fin	-0.046	-0.003	0.140
24	Pelvic fin Origin to Pectoral fin Origin	0.121	0.127	*-0.352
25	Greatest Body Depth	0.180	0.121	*0.238
26	Greatest Body Width	0.165	*0.211	*0.229
27	Minimum Caudal Peduncle Depth	0.155	0.036	0.114
28	Head Length	0.192	0.040	0.067
29	Jaw Length	*0.450	-0.180	-0.111
30	Preopercle Length	0.190	0.049	0.023
31	Snout Length	*0.309	-0.032	0.053
32	Head Depth at Posterior Opercle	*0.214	0.031	0.174
33	Head Depth at Posterior Preopercle	*0.224	-0.010	0.134
34	Snout Depth at Anterior part of Eye	*0.243	-0.077	0.072
35	Eye Diameter	0.140	0.102	-0.155
36	Bony Interorbital	*0.220	0.189	0.142
37	Premaxilla to Supraoccipital Crest	0.133	0.128	0.024

in the statistical package R v2.15.2 (R Core Team, 2012). Only the PCs that had significant Tukey HSD comparisons between groups were examined further for morphological variation among groups.

To determine if any of the genetically distinct populations were diagnosibly different using morphology, discriminant function tests were run on the logged residuals from linear morphometric analysis in PAST (Hammer et al., 2001). Each population was compared to all other clades of speckled dace in this study, and frequencies of the discriminant scores were graphed. If there was no overlap between the scores of the population in question and the rest of the samples, then this population was considered completely diagnosable. Equality of the means of the two groups was tested by Hotelling's T-squared.

3.2.5 Lateral Line Scales

Carl Bond also noted that the lateral line of Foskett Spring speckled dace contained fewer pored scales than in other Warner Valley speckled dace (US Fish and Wildlife Service, 1998). To test his assertion, we counted the lateral line scales of all fish collected for this study (N=330) three times, averaged the counts, and performed a Tukey HSD test, which tests for pairwise significant differences between groups and corrects for experiment-wise error rate when there are multiple comparisons being made.

3.3 Results

3.3.1 Overall Variation

The principal components analyses of the geometric and linear morphometric data describe the overall shape variation observed across the entire sample of speckled dace in the drainages and springs of Eastern Oregon. PCA on the geometric morphometric data produced five principal components. The wireframes in Figure 3.2 visualize the shape change on the axes of PC1, PC2, and PC3 for the geometric morphometric analysis. The shape change along PC1 is overall body curvature. PC2 describes variation in caudal peduncle length, dorsal and pelvic fin positions, and head size, with the positive PC2 values having longer caudal peduncles, more anterior fin positions, and smaller heads. Variation along PC3 relates to body depth with higher values having deeper bodies.

Table 3.2 shows the loadings for each PC for the different measurements; the loadings larger than 0.2 indicate which morphological measurements are most important on each axis (Sidlauskas et al., 2011). Larger PC1 values relate to larger measurements for jaw and snout length, and various head depth measurements. PC2 loadings indicate differences in dorsal fin size and position with positive scores indicating wider dorsal fins, located more posteriorly. Larger PC3 values relate to longer measurements length-wise across the caudal peduncle (dorsal to caudal, anal to caudal), but smaller measurements from the dorsal and pelvic fins to the pectoral fins, as well as greater body depth and width.

The geometric and linear morphometric datasets show similar patterns of vari-

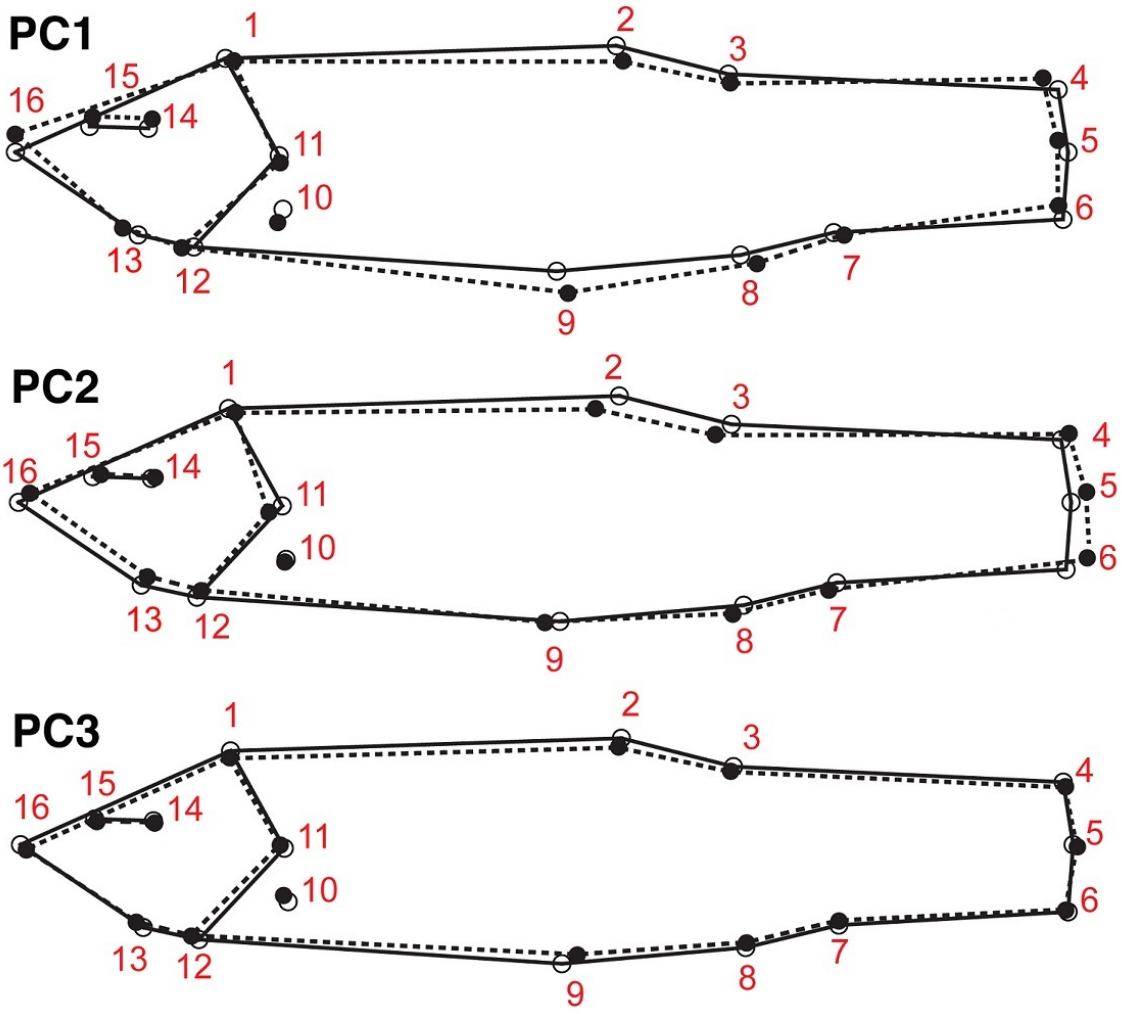

Figure 3.2: Wireframe visualization of variation along the principal components (PC1, PC2, PC3) from geometric morphometric analysis. Hollow circle landmarks and the solid lines represent the configuration of the average specimen. The filled-in circle landmarks and dotted lines represent one approximate extreme of observed variation on that axis, which on PC1 represents 0.04 units, on PC2 represents 0.04 units, and on PC3 represents 0.02 units.

ation in speckled dace morphology. Caudal peduncle length, dorsal fin position, head size, and body depth are all features that show variability in both datasets. The linear dataset also showed variation in body width.

3.3.2 Overall Variation

The MANOVA of the first three principal components of the geometric morphometric landmark data and first three principal components of the linear morphometric data indicated many differences among the populations of speckled dace (Table 3.3). The linear data shows significant differences between every pair of populations. The geometric data showed differences between Warner and everything else, but Lake Abert, Goose Lake, Silver Lake, and Malheur did not have many significant differences. The Foskett Spring, Stinking Lake Spring, and Silver Lake populations did not differ from each other.

The Tukey HSD tests showed which axes are responsible for the overall significance of the MANOVA. From the geometric data, PC2 (8 comparisons $p<0.05$) and PC3 (4 comparisons $p<0.05$) had significant differences among groups of dace (Table 3.4), but PC1 had no significant differences among groups and is not considered further. The scatterplot in Figure 3.3 shows the variation along PC2 and PC3 for the different populations identified by the molecular data. The linear data showed variation along the first and third PCs (PC1: 16 out of 21 comparisons $p<0.05$; PC3: 10 out of 21 comparisons $p<0.05$; Table 3.5), but do not differ on the PC2 axis. Figure 3.4 shows the variation along principal components 1 and 3

for the different populations identified by the molecular data.

Table 3.3: P-values for pairwise Hotelling's comparisons from MANOVA on scores for the first three PC axes for the geometric morphometric analysis (above diagonal) or the first three PC axes for the linear morphometric analysis (below diagonal). Statistically significant p-values are following a sequential Bonferroni correction at a table-wide alpha level of 0.05 and are indicated by an asterisk.

	Warner	Foskett Spring	Lake Abert	Goose Lake	Silver Lake	Malheur	Stinking Lake Spring
Warner		*0.0001	*0.0004	*0.0043	0.2275	*0.0418	0.2283
Foskett Spring	*0.0000		*0.0021	*0.0016	*0.0017	*0.0001	*0.0004
Lake Abert	*0.0000	*0.0000		0.2479	0.1812	*0.0058	*0.0176
Goose Lake	*0.0000	*0.0000	*0.0000		0.6474	0.0669	*0.0276
Silver Lake	*0.0004	*0.0000	*0.0000	*0.0230		0.6888	0.5593
Malheur	*0.0000	*0.0000	*0.0000	*0.0000	*0.0002		*0.0337
Stinking Lake Sp	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000	*0.0000	

Table 3.4: Tukey HSD p-values for comparisons of principal component (PC) scores between groups from the geometric morphometric analysis. PC2 is above the diagonal, PC3 below. Significant p-values are marked with an asterisk.

	Malheur	Stinking Lake Spring	Silver Lake	Goose Lake	Lake Abert	Warner	Foskett Spring
Malheur		0.265	0.907	*0.033	*0.005	0.995	*0.000
Stinking Lake Sp	0.388		0.931	1.000	0.982	0.705	*0.011
Silver Lake	0.997	0.794		0.717	0.321	0.999	*0.000
Goose Lake	1.000	0.112	0.945		0.972	0.336	*0.000
Lake Abert	1.000	0.180	0.978	1.000		0.096	*0.009
Warner	0.075	0.994	0.338	*0.008	*0.017		*0.000
Foskett Spring	0.351	*0.001	0.145	0.357	0.303	*0.000	

Many populations showed significant morphological differences based on the geometric and linear data. Malheur basin dace (excluding Stinking Lake Spring dace) differed significantly from Goose Lake and Lake Abert dace, having a longer caudal peduncle and more anterior dorsal and pelvic fin positions than these two basins on average (PC2 geometric, Figures 3.3, 3.2). Warner dace have narrower

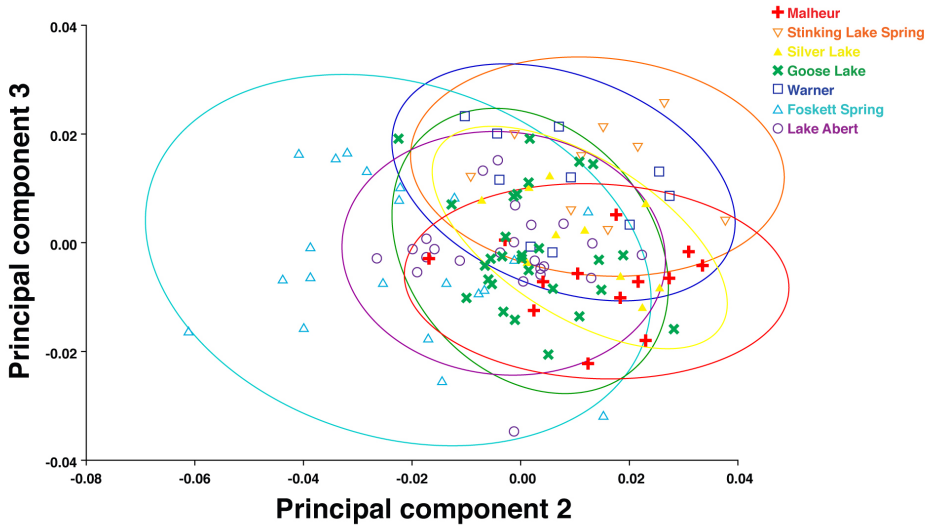

Figure 3.3: Scatterplot of principal components two and three from geometric morphometric analysis. Ellipses indicate 95% confidence intervals for the distribution of each population.

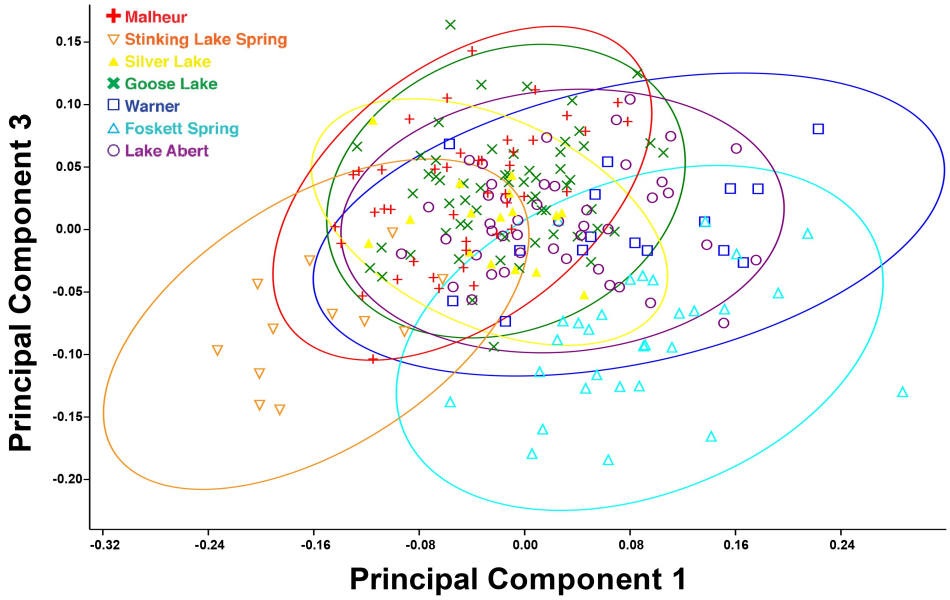

Figure 3.4: Scatterplot of principal components one and three from the linear morphometric analysis. Ellipses indicate 95% confidence intervals for the distribution of each population.

bodies than Foskett Spring, Lake Abert, and Goose Lake daces, as indexed by the geometric PC3 (Figure 3.3). The Lake Abert population is significantly different from the Goose Lake, Malheur, and Silver Lake populations, and the Warner population differs from those in Goose Lake, Malheur, and Silver Lake along the linear PC1 axis, which indicates differences in head size and shape. Despite the statistically significant linear and geometric morphological differences between population pairs, the ranges of observed morphologies still overlap substantially.

Table 3.5: Tukey HSD p-values for comparisons of Principal component (PC) scores between groups from the linear morphometric analysis. PC1 is above the diagonal, PC3 below. Significant p-values are marked with an asterisk.

	Malheur	Stinking Lake Spring	Silver Lake	Goose Lake	Lake Abert	Warner	Foskett Spring
Malheur		*0.000	0.916	0.102	*0.000	*0.000	*0.000
Stinking Lake Sp	*0.000		*0.000	*0.000	*0.000	*0.000	*0.000
Silver Lake	0.619	*0.000		0.990	*0.036	*0.000	*0.000
Goose Lake	1.000	*0.000	0.493		*0.010	*0.000	*0.000
Lake Abert	0.281	*0.000	1.000	0.137		0.072	*0.001
Warner	0.521	1.000	*0.000	0.395	1.000		1.000
Foskett Spring	*0.000	0.993	*0.000	*0.000	*0.000	*0.000	

Foskett Spring speckled dace differ significantly from all other dace populations by having shorter caudal peduncles, more posterior insertions of the dorsal and pelvic fins, and larger heads (geometric PC2, TukeyHSD $p<0.01$, Figures 3.3, 3.2). They also have a wide variety of body depths (geometric PC3) but are still significantly different from Stinking Lake Spring dace. Foskett Spring and Warner daces have a similar morphotype that is different from all other populations (linear PC1, $p<0.001$, Figure 3.4), which indicates that these fish have larger jaws and head sizes than other populations (Table 3.2).

3.3.3 Diagnosibly Different Populations

To determine whether or what populations are diagnosable from each other, we used the CVA on the geometric morphometric data. Only the first three CVs provided effective discrimination among groups (Figures 3.5, 3.6). Malheur dace (both stream and Stinking Lake Spring) and Foskett Spring dace are distinct from each other along the CV1 axis, where Malheur dace have more negative CV1 values and Foskett Spring dace have higher CV1 values (Figure 3.5). The higher CV1 values indicate that Foskett Spring dace have shorter caudal peduncles, larger heads, and more posterior dorsal fin origins, with Malheur dace having the opposite morphology (Figure 3.7). Warner basin dace had consistently higher CV2 values (Figure 3.5), which relates to overall shallower bodies. The other main endorheic basins did not clearly distinguish from one another based on the geometric morphometric data.

Three populations were completely distinguishable based on the frequency plots of discriminant function scores using linear measurements (Figures 3.8, 3.9, 3.10): Foskett Spring (p=2.15E-28), Stinking Lake Spring (p=8.94E-20), and Warner Valley (p=1.01E-10). Foskett and Warner were also found to be distinct by the CVA on the geometric data. Three other populations have significantly different discriminant function scores from the rest of the samples: Goose Lake (p=7.73E-12), Lake Abert (p=2.47E-7), and Malheur (p=3.88E-14) but were not fully diagnosable. Only Silver Lake was not significantly different (p=0.237).

The measurements which had the greatest effect on the discriminant scores for

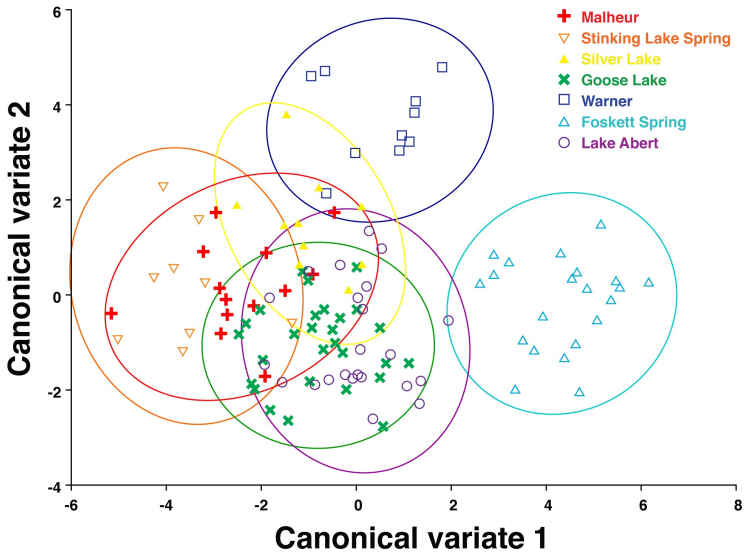

Figure 3.5: Scatterplot of Canonical Variates one and two from geometric morphometric analysis. Ellipses indicate 95% confidence intervals for the distribution of each population.

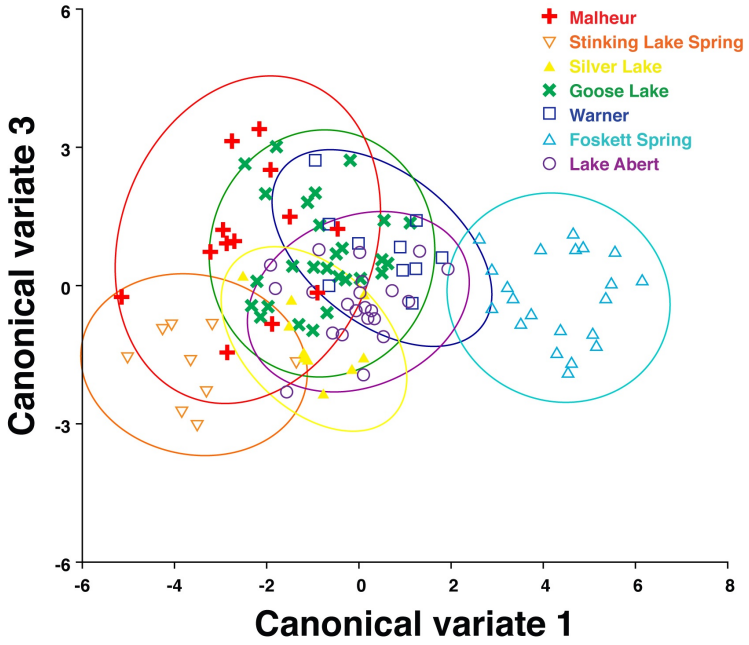

Figure 3.6: Scatterplot of Canonical Variates one and three from geometric morphometric analysis. Ellipses indicate 95% confidence intervals for the distribution of each population.

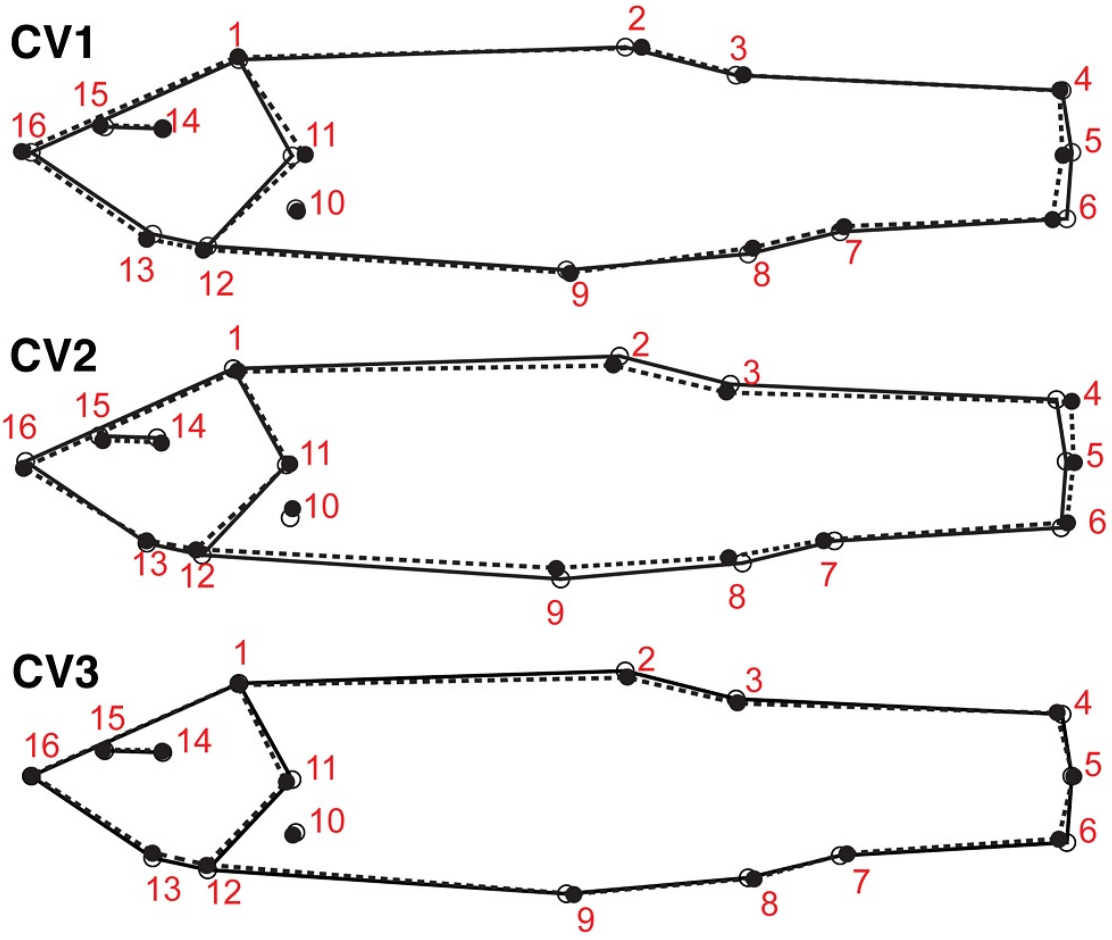

Figure 3.7: Wireframe visualization of variation along the Canonical Variates (CV1, CV2, CV3) from geometric morphometric analysis. Hollow circle landmarks and the solid lines represent the configuration of the average specimen, filled-in circle landmarks and dotted lines represent one approximate extreme of variation on that axis. The deformation on CV1 represents 4.0 units, that on CV2 represents 4.0 units, and that on CV3 represents -2.0 units.

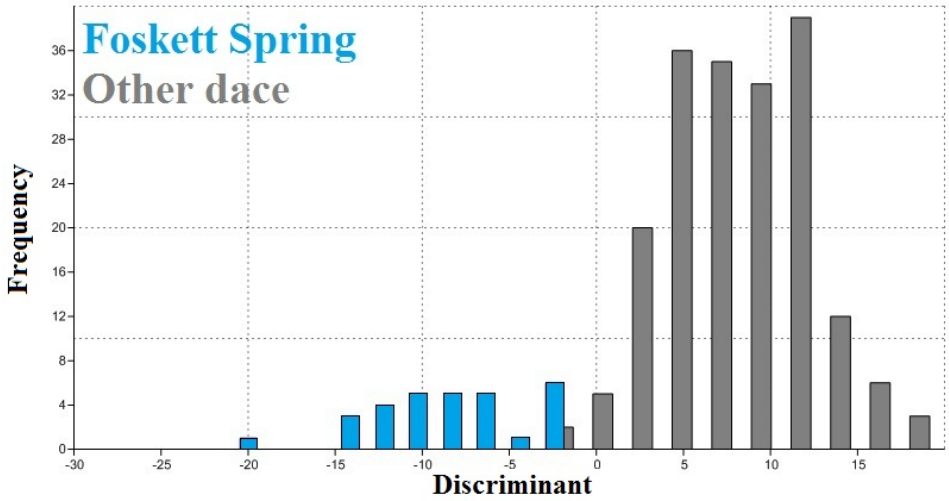

Figure 3.8: The discriminant function scores for Foskett Spring dace compared to all other dace in our dataset based on the linear morphometric measurements.

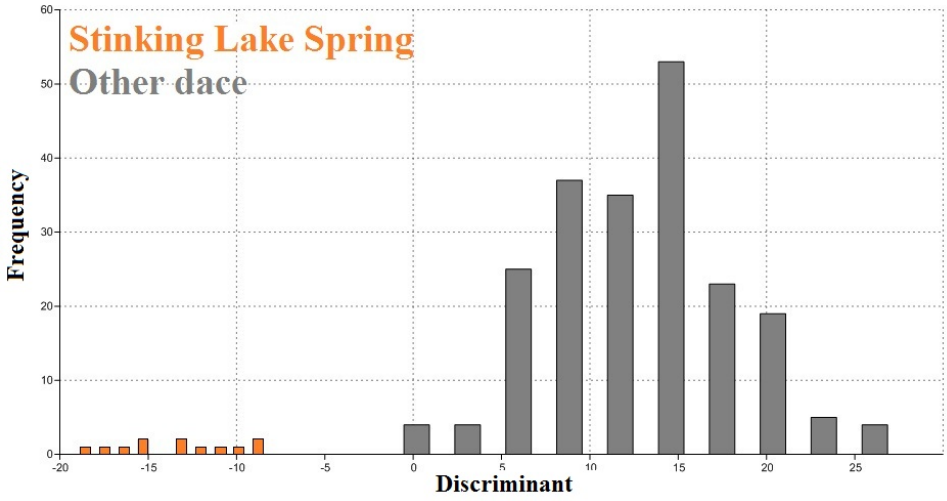

Figure 3.9: Discriminant function scores for Stinking Lake Spring dace compared to all other examined dace based on the linear morphometrics.

Foskett Spring dace were the premaxilla to the dorsal fin origin (which is consistently 0.9mm longer on average for Foskett Spring dace, Figure 3.12A), minimum caudal peduncle depth, and preopercle length (0.21mm longer on average, Fig-

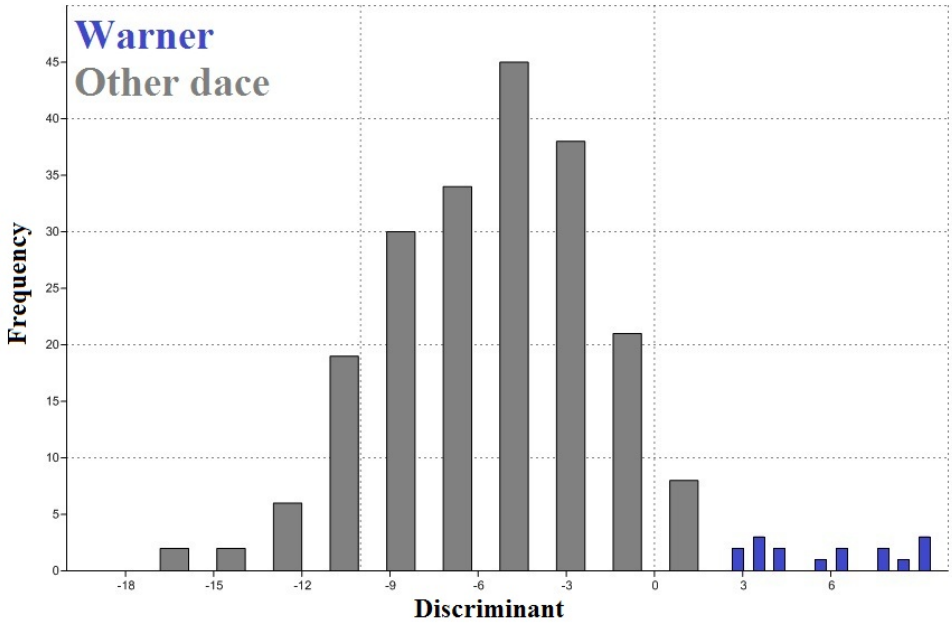

Figure 3.10: Discriminant function scores for Warner Valley dace compared to all other examined dace based on the linear morphometrics.

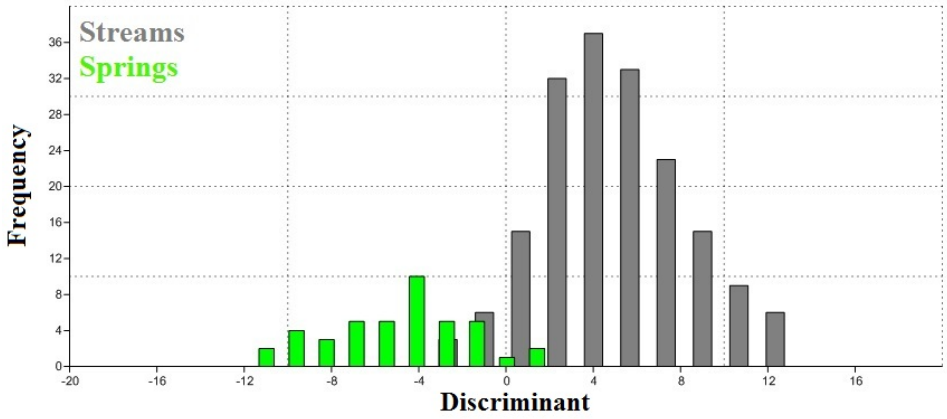

Figure 3.11: Discriminant function scores for the two spring dace populations (Foskett and Stinking Lake) compared to all stream dace based on the linear morphometrics.

ure 3.12B). Stinking Lake Spring dace were diagnosable from all other dace, with high loadings for measurements relating to head size (Table 3.2, measurements 30, 32, 37: smaller heads). At any standard length below 40 mm (we did not find larger dace in Stinking Lake Spring), the head depth at preopercle measurement is around 0.7mm shorter in Stinking Lake Spring dace than in the rest of the populations, including Foskett Spring dace (Figure 3.13). For Warner basin, the linear measurement with the largest effect on the discriminant function analysis is from the dorsal fin insertion to the anal fin origin. This and other cross-body measurements approximate body depth and are consistently smaller in Warner dace (Figure 3.14).

3.3.4 Diagnosability of Springs Versus Streams

A discriminant function analysis comparing the two spring populations to the stream populations did not show complete diagnosability of spring dace, although the difference in dace between these two habitat types was significantly different (Figure 3.11, p=4.94E-23). The measurement with the highest loading was the premaxilla to dorsal origin (2, Table 3.2), which was the highest loading for Foskett Spring (see above) and had longer measurements in the spring fish (on average 0.9mm longer in fish of the same SL), indicating a more posterior dorsal fin origin (as well as measurements 14 and 15).

The spring dace (Foskett and Stinking Lake) were statistically different from stream dace along the linear PC3 ($p<0.0002$, Figure 3.4), the measurements with

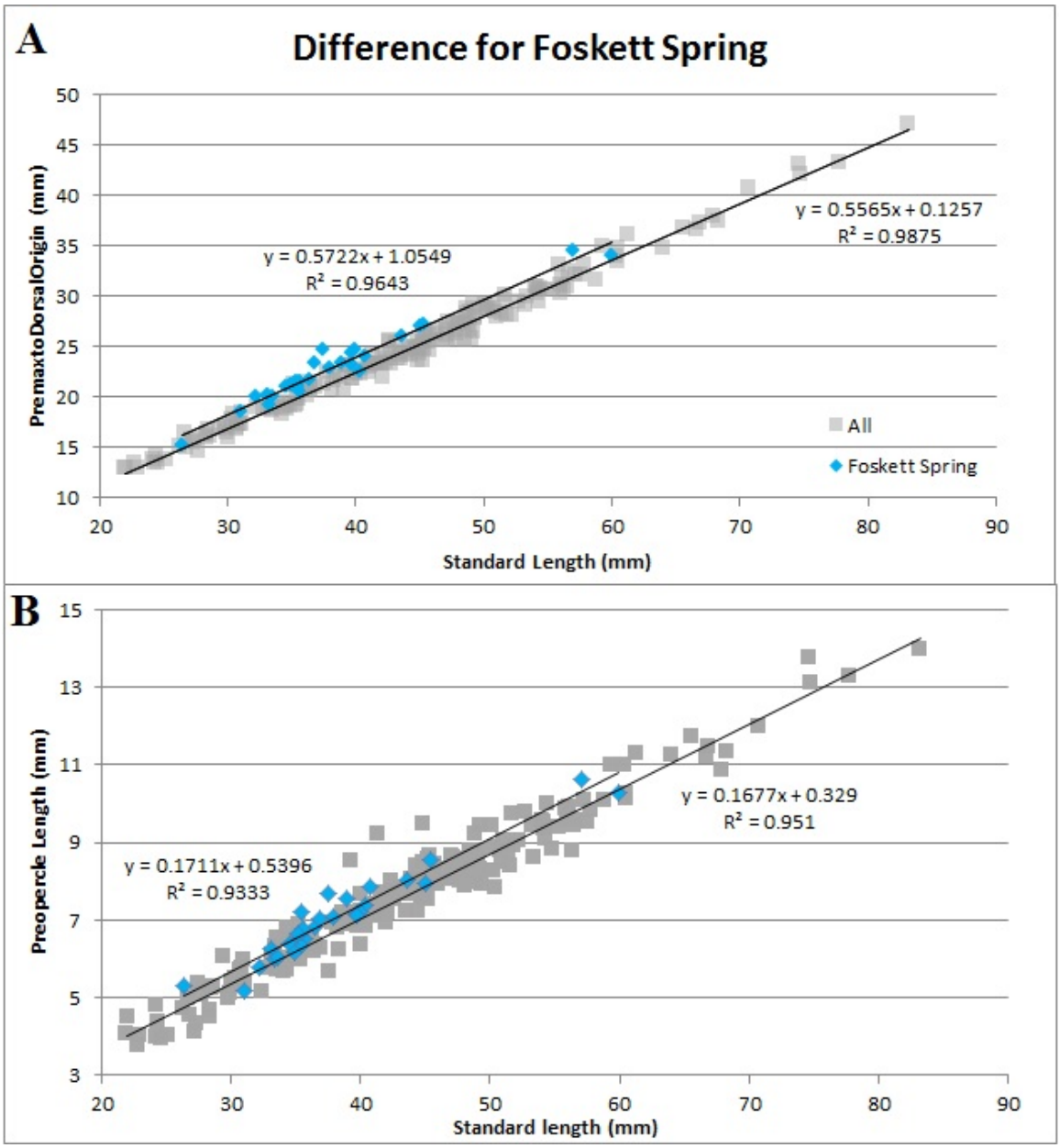

Figure 3.12: A: A plot of the distance from the premaxilla to the dorsal fin origin against standard length for Foskett Spring dace and other daces. The slopes of these two lines are parallel, but differ 0.9mm in their intercepts. B: A plot of preopercle length measurements against standard length for Foskett Spring dace and the other dace. The slopes of these two lines are parallel, but differ 0.2mm in their intercepts.

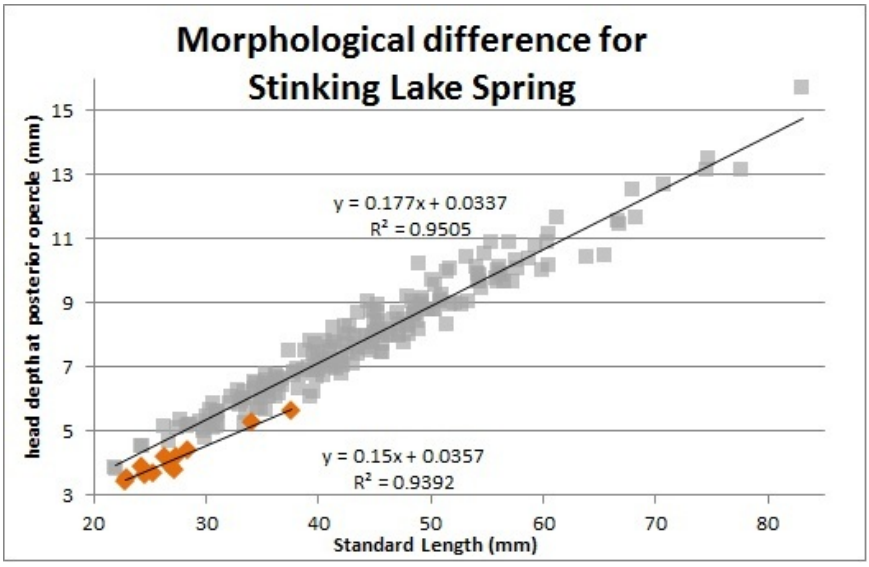

Figure 3.13: A plot of head depth at posterior opercle against standard length for Stinking Lake Spring dace (orange diamonds) and the other dace (gray squares). The slopes of these two lines are almost parallel, but differ 0.9mm in their intercepts.

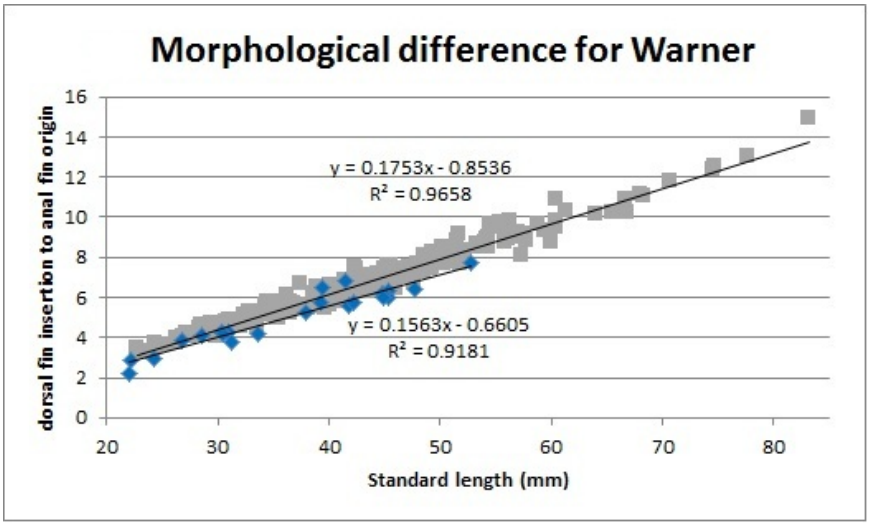

Figure 3.14: A plot of the distance from dorsal fin insertion to anal fin origin versus standard length for Warner dace (orange diamonds) and the other daces (gray squares). The slopes of these two lines are nearly parallel, but differ 0.2mm in their intercepts.

large loadings relating to a more posterior dorsal fin (measurements 8, 9, 14, 17, 18, 21; Table 3.2) and narrower body size (25, 26). The meristic counts of lateral line scale counts showed that the spring dace (Foskett Spring and Stinking Lake Spring) have significantly fewer scales than their stream counterparts ($p<0.001$, Figure 3.15). The springs did not differ from each other in lateral line counts ($p=1.0$). On average the spring dace had 8.3 fewer lateral line scales than the stream dace.

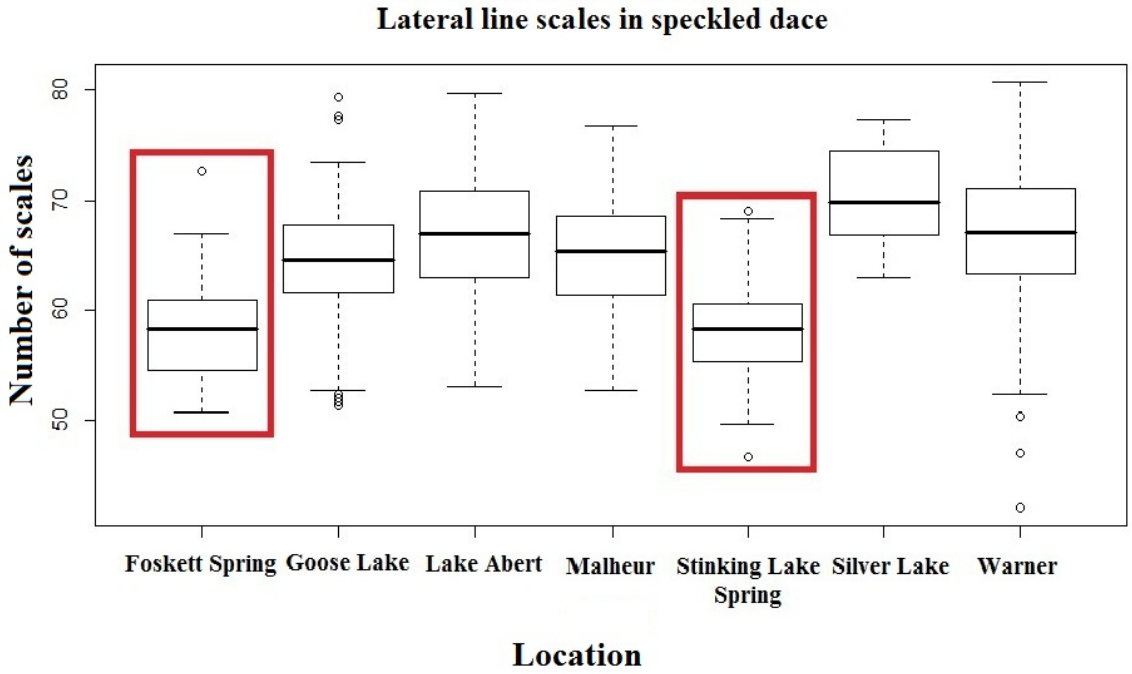

Figure 3.15: A box plot of the lateral line scale counts from the speckled dace collected from Eastern Oregon. Red boxes indicate the spring populations, which are significantly different from stream populations.

The two springs are also distinct from each other in head size and caudal peduncle length, as evidenced by both the linear and geometric datasets. Stinking

Lake Spring dace have more negative linear PC1 values than do Foskett Spring dace and all other groups of dace ($p<0.0001$). The large loadings that contribute to PC1 all relate to head size, with Stinking Lake Spring dace having smaller head depths (measurements 7, 32, 33, 34, 36; Table 3.2) and smaller mouths (measures 29 and 31) than the other populations of dace. Foskett Spring dace have higher CV1 scores from the geometric data (Figure 3.6), while streams have median scores, and Stinking Lake Spring dace have the lowest scores on average. The morphological change associated with the CV1 axis (Figure 3.7) indicates that Foskett Spring dace have larger heads and slightly longer caudal peduncles, with Stinking Lake Spring having the opposite pattern.

3.4 Discussion

We found that speckled dace from different populations in Oregons Great Basin region display interesting and somewhat unexpected patterns of morphological variation. The spring dace differed from the stream dace, but Foskett Spring and Stinking Lake Spring were also found to be distinguishable from each other. The stream dace populations from Malheur, Silver Lake, Goose Lake, and Lake Abert did not show diagnosable differences among groups; there was substantial overlap in morphologies of these populations (Figure 3.3, 3.4, 3.5). A few statistically significant differences were found among populations, but nothing that could completely distinguish the different basin populations.

Speckled dace from Malheur streams are the most genetically distinct clade (see

Chapter 2), but are not morphologically distinct. Perhaps these dace share a morphology with the other endorheic basins because the Great Basin habitat creates a selection regime that has optimized the same morphotype over the entire region. In such a scenario, the functional genes relating to morphology would have evolved convergently in all these basins, even though the neutral genes have diverged between Malheur and the other basins over time. Another explanation could be that these morphological features are plastic and controlled by the environment, which is likely very similar throughout the Great Basin.

Although Warner Valley dace are not very distinct genetically (see Chapter 2), this was the only morphologically distinct stream population. Warner dace have shallower bodies than the other dace (more positive CV2 values, Figure 3.5, 3.7, shorter distance from dorsal fin insertion to anal fin origin, Figure 3.14). In addition to the body shape difference, there is evidence that both Warner dace and Foskett Spring dace (located within Warner Valley), share the trait of larger heads and jaws (PC1, Figure 3.4). There is potentially some factor in the Warner Valley environment that has induced the narrower bodies and larger heads of Warner Valley dace, which could either be phenotypic response from a very plastic species or a functional genetic change that has happened rapidly. These two factors are nearly impossible to parse out without a common garden experiment or genomic data from morphologically functional genes.

Foskett Spring speckled dace are only slightly distinct genetically (see Chapter 2), but are very distinct morphologically. We found evidence that Foskett Spring have shorter caudal peduncles, more posterior dorsal fins (Figure 3.3, 3.6, 3.4), and

larger heads (Figure 3.5). The measurements that greatly impacted the discriminant function analysis can be used to create a key to determine if an unknown dace is from Foskett Spring; the most helpful measurement is the premaxilla to dorsal fin origin, which is consistently 0.9mm larger for Foskett Spring dace. A measurement that distinguishes head size would be the preopercle length, which is 0.21mm longer on average in Foskett Spring dace (Figure 3.12).

Carl Bond noted that Foskett Spring speckled dace have a larger eye than the other Warner dace (US Fish and Wildlife Service, 1998), but we did not corroborate this in our morphological analyses. We did, however, find evidence that Foskett Spring dace have a more posterior dorsal fin, as Bond originally described ("having the dorsal fin origin well behind the pelvic fin but before the anal fin origin," US Fish and Wildlife Service (1998)). The shorter lateral line (discussed below) was also noted by Bond and observed in our own lateral line counts.

3.4.1 Spring Dace Morphotype

Speckled dace from both springs (Foskett and Stinking Lake) had significantly fewer lateral line scales than the stream dace and were not statistically different from each other (Figure 3.15). Lateral line scale counts have been shown to be a thermally labile trait (Taning, 1952), so the shorter counts found in the spring dace are likely temperature induced rather than genetically adaptive. The temperatures experienced by dace in the springs studied here are very similar to each other because they are all fed by thermal aquifers (Foskett Spring averages 18.3°C all

year round, temperature has not been measured at Stinking Lake Spring). The stream temperatures that the rest of the dace experience vary throughout the whole year, potentially reaching highs of 24°C in low water flows of the summer, and lows of 2°C in the winter months (Paul Scheerer, ODFW, pers. comm.).

The spring dace in our study (Foskett and Stinking Lake) are statistically different from the stream dace, although not fully diagnosable, and have shorter caudal peduncles and more posterior dorsal fins (Figure 3.11, 3.4) than stream fish. These morphologies might better suit these fish in the spring habitat. There is little water flow in these springs, plenty of vegetation to hide in, and constant temperatures. This environment would not require constant swimming, but rather short bursts to evade predators (fish or birds) or capture food. A more posterior dorsal fin is typical of fish with this type of swimming behavior (Pavey et al., 2010). For example, Spoljaric and Reimchen (2007) found that populations of sticklebacks with posterior dorsal spines were found in small, shallow, sustained ponds, whereas populations with anterior dorsal spines were found in large lakes, and demonstrated continual swimming.

The typical cyprinid has a fusiform body type that is robust and made for cruising in streams (Claytor et al., 1991; McLaughlin and Grant, 1994), which is closer to the stream dace morphology. In studies with sticklebacks, individuals in small lakes (no water flow, more vegetation) have less robust bodies than do anadromous, continually swimming fish (Taylor and McPhail, 1986), which mirrors the pattern seen between the spring and stream speckled dace. The streamlined shape of three-spined sticklebacks inhabiting large lakes or rivers is conducive to

lowering pressure drag during steady swimming and should therefore increase open water foraging performance (Webb, 1984), which would also be essential for stream-dwelling speckled dace.

3.4.2 Spring Dace Differences

The difference in morphology between the two different springs, Foskett and Stinking Lake, indicates the presence of two "spring types": one with small heads and one with large heads. Stinking Lake Spring dace are also morphologically different from Foskett Spring dace in that they have smaller heads and jaws than all other dace populations in this study. At any given standard length below 40 mm, the preopercle depth measurement is around 0.7mm shorter in Stinking Lake Spring dace than in the rest of the populations, including Foskett Spring dace (Figure 3.13). This difference appears to change at different lengths of dace, because the regression lines appear to have different slopes, which may be because the sample of speckled dace from Stinking Lake Spring were all very small, less than 40mm SL. A more reliable key for diagnosing this spring dace would benefit from measurement of larger specimens.

The presence of two spring types appears to result from differences in the two spring habitats rather than inter-basin differences or genetic effects. If morphological change was proportional to neutral genetic distance, Warner and Foskett Spring (close genetic relatives) would have very similar head shapes, and Malheur and Stinking Lake Spring (distant genetic relatives) would have very different head

shapes. If the between-spring difference resulted from differences in basin habitat, we would expect to see the head differences mirrored in the Warner and Malheur stream dace. The latter seems like a better explanation because Warner and Malheur dace do have slightly different head morphologies, with Warner being more similar to Foskett Spring and Malheur being closer to Stinking Lake Spring. Both stream dace have more median head sizes, though, so the head morphology is not entirely basin driven.

The larger heads found in Foskett Spring dace and the smaller heads found in Stinking Lake Spring dace may reflect the different prey of these species; dace from Foskett Spring seem better suited to eat larger food items than are Stinking Lake Spring dace. Spoljaric and Reimchen (2007) found that sticklebacks with small heads lived in open water habitats of low productivity, and populations with large heads lived in higher productivity, structurally complex habitats. This pattern may be reflected in these spring populations of speckled dace, with Foskett Spring living in a more productive habitat than Stinking Lake Spring, however, very little is known about the prey or preferred food items of dace in these springs or about the ecology of these populations as a whole. Investigations about the stomach contents of these fish, the aquatic invertebrate composition, or the algae levels would be necessary to determine if there is a dietary difference between these two spring dace. One known difference between these two springs is the presence of other fish in Stinking Lake Spring (tui chub, *Gila bicolor*) and the absence of such fish in Foskett Spring. The head shape may be an effect of different predation risks or competitive exclusion from tui chubs in Stinking Lake Spring. The new data

presented here demonstrating the uniqueness of these spring dace has prompted investigations by ODFW, planned for summer 2013, to study the population size and ecology of these two populations.

3.5 Conclusion

We investigated the morphological variation in speckled dace from Oregon's Great Basin to determine if speckled dace from different basins or from different habitat types have different morphologies. Although there were statistically significant pairwise differences between speckled dace from stream habitats in some basins, these morphological characters do not provide a way to distinguish stream dace from different basins with certainty except for dace from Warner basin. However, we identified two spring types from Foskett Spring and Stinking Lake Spring that differ from each other in head size, and together, differ from stream dace in caudal peduncle size, dorsal fin position, and lateral line scale counts. We have now provided a basis on which to distinguish Foskett Spring speckled dace from other dace distributed throughout the state, which responds directly to the recommendation in the five-year review for Foskett Spring Speckled Dace (US Fish and Wildlife Service, 2009).

Chapter 4 – Discussion and Conclusions

4.1 Overview

We researched the morphometrics and phylogenetics of speckled dace (*Rhinichthys osculus*) in Oregon's Great Basin region to answer two questions: 1) What is the pattern of genetic differentiation among populations? and 2) Do Foskett Spring speckled dace and dace from different basins have identifiable morphologies? The genetic and morphometric data was used to identify any distinguishable populations of speckled dace and identify unrecognized taxonomic diversity. This new data will aid a much needed taxonomic revision of speckled dace, inform management decisions and the potential ESA listing status for these fish, and provide a better description of the biogeography of the region.

4.1.1 Phylogenetics

In order to determine if there is evidence for genetic differentiation among populations or basins sufficient to merit formal taxonomic recognition, we collected genetic data from throughout the Warner basin and surrounding drainages (Goose Lake, Lake Abert, Silver Lake, and Malheur; Figure 1.2). We discovered three previously unrecognized taxonomic entities: Malheur stream dace, Stinking Lake Spring dace, and a clade spanning the other four endorheic basins (Goose Lake,

Silver Lake, Lake Abert, and Warner), which split from each other before the start of the Pleistocene. The Malheur stream clade may have separated from the other dace clades as early as the Miocene-Pliocene boundary (Figure 2.7). The nuclear sequence data (S7, Figure 2.3) and the FCA of the microsatellite dataset (Figure 2.9) both identify these three clades as distinguishable entities. The Structure analysis (Figure 2.8) and the mtDNA sequence data (Figure 2.5) distinguish these three groups as well, but also suggest finer scale divisions of each basin in this sample area.

The different levels of distinguishability from the different molecular datasets and analyses likely results from the different rates of evolution displayed by nuclear introns, mtDNA, and microsatellites. The five main basins and Stinking Lake Spring are identified as clear groups based on mtDNA, but only Malheur and Stinking Lake Spring are distinguished from the other four basins by the nuclear sequence data. That difference in resolution can be explained by the much slower mutation rate of nuclear introns (Brown et al., 1979; Moritz et al., 1987). So Goose Lake, Silver Lake, Lake Abert, and Warner may have not had enough evolutionary time to diverge along the slower evolving nuclear intron marker.

The two main analysis methods used on the microsatellite data show different resolution and power. The Structure analysis identifies the endorheic basins and each spring as separate populations, whereas the FCA only distinguishes the three main clades. Structure uses the presence of linkage disequilibrium within a sample of genotypes and introduces population structure to minimize disequilibrium (Pritchard et al., 2000), so this method relies on allele and genotype frequencies.

The FCA method uses allele identity to place an individual in factorial space (Belkhir et al., 1996), and will find diagnosable differences in genotypes rather than merely allele frequency differences. These differences in method likely lead to the higher resolution from Structure, which shows recent genetic divisions, and the diagnosable differences identified by the FCA, which are important for taxonomic identification.

4.1.2 Morphology

We investigated whether speckled dace from different basins or from different habitat types within Oregon's Great Basin differ in morphology. Speckled dace in different basins did not distinguish from each other, except for dace from Warner basin, although there were statistically significant differences between some basins. However, we identified two spring types from Foskett Spring and Stinking Lake Spring that differ from each other in head size, and together, differ from stream dace in caudal peduncle size, dorsal fin position, and lateral line scale counts. We have now provided a basis on which to distinguish Foskett Spring speckled dace from other dace distributed throughout the state, which responds directly to the recommendation in the five-year review for Foskett Spring speckled dace (US Fish and Wildlife Service, 2009).

4.2 Stinking Lake Spring

The cryptic diversity identified in Stinking Lake Spring is quite interesting, because the presence of a spring population that is genetically and morphologically distinct from the stream fish in the same drainage basin tells a unique evolutionary story. Stinking Lake Spring dace are genetically much more distinct from Malheur dace than Foskett dace are from the rest of Warner, despite the similar geologic history. Both Malheur and Warner basin were filled with large Pleistocene lakes which have been desiccating for about the same amount of time (about 10,000 years), so the dace trapped in Foskett Spring and Stinking Lake spring have likely been isolated within their respective locations for the same amount of time (at least within a few thousand years of each other, which is relatively short on the evolutionary timescale). We have a few hypotheses that might explain the greater evolutionary divergence of Stinking Lake Spring speckled dace.

Stinking Lake Spring dace may have become isolated around the same time as dace in Foskett Spring, but evolved more rapidly because of rate heterogeneity. If the rates of molecular evolution in Stinking Lake Spring were elevated, this would cause more rapid genetic divergence. Small springs will usually have smaller population sizes than large streams, and small effective population sizes have been demonstrated to result in accelerated rates of DNA sequence evolution (Ohta, 1992; Johnson and Seger, 2001; Woolfit and Bromham, 2003). Perhaps Foskett Spring has maintained larger population sizes, or Stinking Lake Spring has experienced numerous bottlenecks. Also, spring environments often have high temperatures

either because of warm water aquifers or from less water movement. High temperatures will result in increased metabolic rates among poikilotherms, which may result in elevated rates of mutation stemming from the by-products of oxidative respiration (Rand, 1994; Martin, 1999; Gillooly et al., 2005). Higher metabolic rates are also correlated with faster generation times, which can contribute to faster evolution. This seems the less likely mechanism as Foskett Spring is known to have constant, warm temperatures, but does not appear to demonstrate rate heterogeneity.

The deeper genetic divergence found between Stinking Lake Spring and Malheur dace than between Foskett Spring and Warner dace might also be a result of increased selection pressure. Stinking Lake Spring and Malheur streams might present very different selective environments, which could cause genes under selection to diverge more rapidly. ND2 (NADH subunit 2) is a functional, protein-coding gene that may be affected be different selection pressures. However, S7 is a non-coding intron and is thought to be neutral (Chow et al., 1998), so would not be affected by selection. This neutrally evolving marker still shows the pattern of deep divergence between the Malheur populations, though, implying that differential selection pressure is not the main cause of the unique evolutionary pattern found in Stinking Lake Spring speckled dace.

Another possibility that seems more likely given the tumultuous past of the Great Basin region is population replacement of the Malheur stream dace. There may have once been a widespread population of speckled dace in Malheur when the basin was covered with a large lake. As the lake dessicated, individuals from this

population may have been isolated in small springs, including Stinking Lake Spring. The streams flowing in the northern Ochoco and southern Steens ranges could have seen extinction and subsequent replacement of their populations of speckled dace by the nearby Malheur River in the Snake River basin during high water events. This would result in two very distinct lineages that have evolved separately for 2-5 million years (Figure 2.7), but live in geographically close proximity. An anthropogenic induced version of this scenario is known to have occurred in the case of the Mohave tui chub (*Siphateles bicolor mohavensis*), which was displaced by introduced arroyo chubs (*Gila orcutti*) throughout most of the Mohave River (Chen et al., 2013). Relict populations of the Mohave tui chub exist in two isolated ponds, but have been replaced completely by competition elsewhere in their native range. Since the mtDNA, nuclear sequence, and microsatellites show the same highly-divergent pattern, it does not appear to be a case of population introgression and mitochondrial replacement (as in *Cyprinodon* in Mexico, Carson and Dowling 2006).

4.2.1 Spring Cyprinids

The Great Basin region contains many isolated populations of fish evolving separately in endorheic basins and spring environments. It is therefore interesting to compare the case of Foskett Spring and Stinking Lake Spring speckled dace to other cyprinid fish that have become isolated. One well known and unique case is Devils Hole pupfish, *Cyprinodon diabolis*, which are isolated in a warm (33°C), aquifer-

fed pool, and are ESA listed as Endangered based on the risk of ground water depletion. They are genetically distinct and monophyletic, but their ancestor, the Amargosa pupfish (*C. nevadensis amargosae*) is paraphyletic (Echelle, 2008). Not enough time has passed for the two species to become reciprocally monophyletic, but they are clearly different evolutionarily and morphologically (Turner, 1974), which, in our framework, provides enough evidence to confirm the species status.

Devils Hole opened up 60kya, but the estimated time of divergence for the mtDNA of *C. diabolis* is 0.5mya, based on a Bayesian method implemented by Echelle (2008) using mtDNA sequence data. Echelle (2008) supposes that lineage sorting prior to colonization of Devils Hole could be responsible for the earlier genetic divergence date. Another explanation for the unexpectedly high mitochondrial divergence is the extremely small population size in Devils Hole, between 50-500 individuals (Turner, 1974), which could have resulted in accelerated rates of evolution (Ohta, 1992). Since pupfish in Devils Hole are clearly direct descendants of pupfish in the surrounding Amargosa basin, this case seems different than the evolutionary story of Stinking Lake Spring dace, which are reciprocally monophyletic and have been genetically separated much longer from Malheur stream dace (2-5my) than the geographic separation (reduction of Pluvial lakes 10,000years ago). Conversely, Devils Hole pupfish are more distinct from Amargosa pupfish than Foskett Spring speckled dace are from Warner dace, which are not monophyletic and have only been separated for 10,000 years (Ardren et al., 2010).

The morphological plasticity experiments of the Devils Hole pupfish is another

interesting facet to consider. Pupfish in Devils Hole have a smaller body, larger head and eyes, and no pelvic fins, compared to Amargosa pupfish and pupfish from Devils Hole that have been transplanted to establish refuge populations. In common garden experiments, pupfish with less food, and therefore lower growth rates, showed proportionally larger head and eyes, smaller body depth, and reduction in pelvic fin development, the latter also being affected by elevated temperature (Lema and Nevitt, 2006). The phenotypic plasticity observed in pupfish could be mirrored in speckled dace, which could indicate a mechanism underlying the distinct morphologies of closely related Foskett Spring and Warner stream dace.

Hutton Spring tui chub (*Gila bicolor* spp.) from the Alkali subbasin in Eastern Oregon has been isolated from Lake Abert basin for 25-32,000 years and once occupied a large lake which has been dry for about 10,000 years (Bills, 1977), leaving only HUtton Springs behind. This chub is morphologically distinct by having a larger head size, similar to Foskett Spring speckled dace in this study. Based on mtDNA (cytB) tui chub from Hutton Spring are not monophyletic and occur within a clade also including chub from Lake Abert (Harris, 2000). This is a similar genetic and geological pattern to Foskett Spring speckled dace, and again shows that longer than 10,000 years of isolation is required for cyprinids to reach species status.

4.2.2 Another Spring Population of Speckled Dace

Kendall Warm Springs dace (KWS, *Rhinichthys osculus thermalis*) are a subspecies of speckled dace from a warm spring (constantly 29°C) in Wyoming which is separated from the Green river by a 3m waterfall (Hubbs and Kuhne, 1937). The waterfall, perhaps several thousand years old (Hubbs and Kuhne, 1937), is thought to prevent any Green river dace from moving upstream, and the cold water temperatures and voracious trout population of the Green River likely prevents effective downstream movement of KWS dace (Kaya et al., 1992). This spring is similar to Foskett Spring in that there is a warm spring pool that flows into a brook and has no other fish species, but KWS is not overgrown by tules or cattails and is threatened instead by human usage and a road culvert transecting the stream.

KWS dace are different morphologically from the Green River dace (*R. o. yarrowi*) by having fewer lateral line scales (as observed in spring dace in this study) and different pharyngeal teeth morphology. The former has been shown to be thermally labile (Taning, 1952; Barlow, 1961), but the pharyngeal teeth difference is thought to be a taxonomically-informative character (Gould and Kaya, 1991), and should be investigated in Foskett Spring dace. Common garden experiments have been attempted to test for phenotypic plasticity of the morphology, but Kaya et al. (1989) was unsuccessful in rearing speckled dace in captivity.

KWS dace are listed as a separate subspecies because the genetic analysis of five out of twelve restriction endonuclease sites from twelve individuals (Kaya et al., 1992) showed that KWS dace have different mtDNA haplotypes from Green

river dace. Similarly we found no shared mtDNA haplotypes (ND2) between Foskett Spring dace and Warner stream dace, but this slight genetic divergence was not sufficient to produce monophyly. KWS dace have never been shown to be monophyletic, but are slightly genetically distinct based on only mtDNA and have diagnosable morphological features, which qualifies KWS dace for ESU status according to our criteria. Given the similar environments and geological separation time between these two spring populations, it appears as if 10,000 years of isolation does not provide sufficient time for speckled dace to evolve into new species.

4.3 Taxonomy

Our operational species concept combines features of the phylogenetic and morphological species concepts as criteria for species status, within the theoretical framework of the evolutionary species concept (Figure 1.4). We first consider the monophyletic lineages and evaluate the genetic divergence; lineages with genetic divergences consistent with other species of cyprinids will warrant species recognition. Groups of dace that are not reciprocally monophyletic and with only slight evidence of genetic distinction will not be considered distinct species. If these groups have significant morphological differences, however, they will be considered for ESU designation because of the potential to become a species given sufficient time (Darwin's incipient species; Avise and Hamrick 1996, p. 60).

It is clear that three taxonomic units are consistently identified and diagnosably different, no matter the genetic marker or analysis method used. Malheur

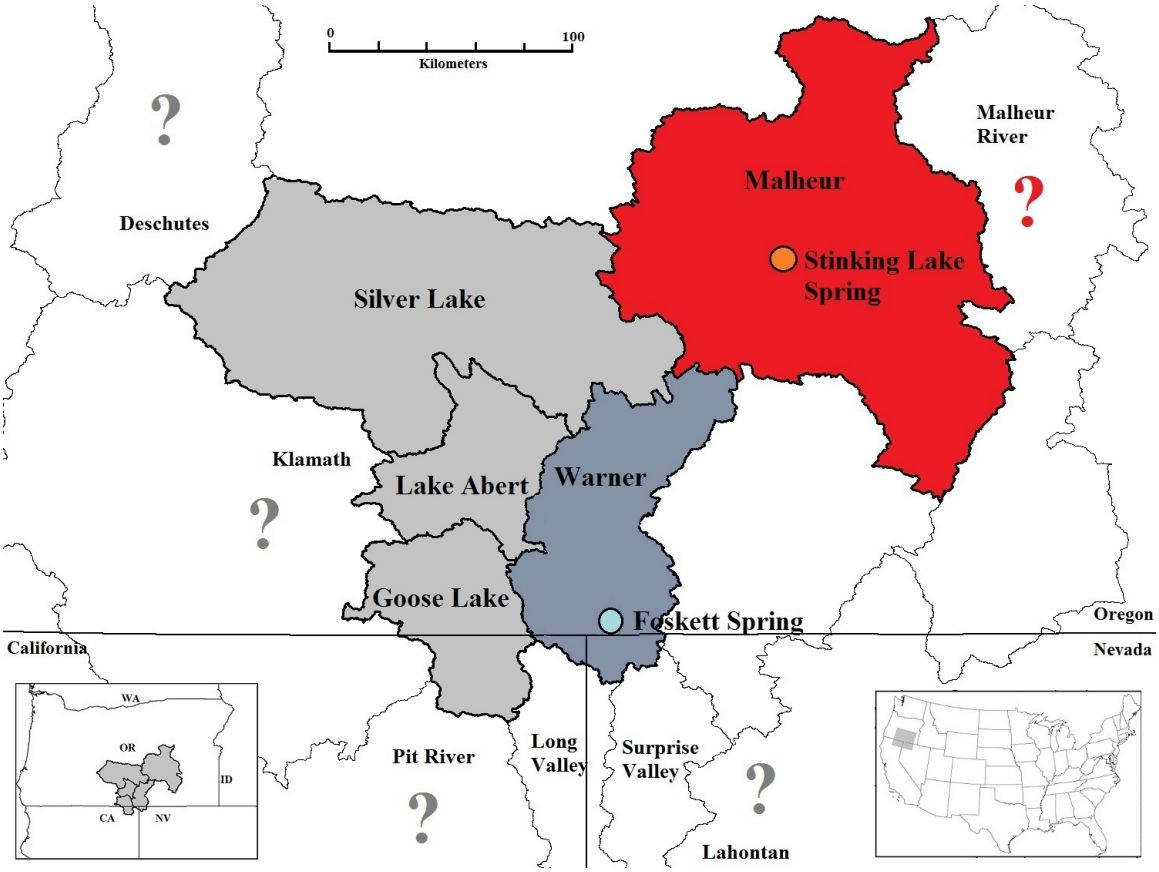

Figure 4.1: A map of the Eastern Oregon endorheic basins colored according to taxonomic status of speckled dace recommended herein. Red indicates the Malheur species, which may also include the Snake River drainage. Orange represents Stinking Lake Spring. Gray is the other species found in this region and may also include dace from the Deschutes, Klamath, Pit, or Lahontan basins, which were not sampled. Dark gray is the Warner basin ESU and grayish blue is the Foskett Spring ESU. Bold basin lines indicate distinct populations. Right inset shows the location of the map within the United States. Left inset shows the basin locations in Oregon.

stream dace, Stinking Lake Spring dace, and dace from the other four endorheic basins collectively are reciprocally monophyletic in phylogenies derived from nuclear DNA and mtDNA, thus fulfilling a major requirement for species status. Also, the percent divergence between these three clades is higher than levels commonly seen between cyprinid species (Zardoya and Doadrio, 1999; Schmidt et al., 1998), indicating that these entities are separate species (Figure 4.1). Although the four other basins are monophyletic based on mtDNA (except Warner/Foskett Spring) and were identified as separate populations by the microsatellite-based Structure analysis, the levels of divergence are lower than typical between-species differences, and the nuclear data does not distinguish among these basins, so they are considered to be part of the same species.

The morphological distinction of speckled dace from Stinking Lake Spring presents further evidence that this is a distinct species. Currently we are working on a species designation and key for this new species, which has never been previously named or recognized. However, we are not working on species designations for the other two potential species we identified. As the biogeographic data indicates, the Malheur stream dace and the four other basins may be part of taxonomic units that include dace in surrounding basins. A widespread phylogeographic study will be required to determine where species boundaries occur outside of the region we investigated.

The other four endorheic basins (Goose Lake, Silver Lake, Lake Abert, and Warner/Foskett Spring) form three less distinct clades based on the mtDNA, or five populations based on the Structure analysis. These units would be considered

ESUs according to our species concept if they have significant morphological differences. The only basin population of dace with unique and diagnosable morphology is Warner basin, which have shallower bodies than other dace. The morphological distinction of Warner dace suggests the presence of a Warner Valley speckled dace ESU within the species that includes Goose Lake, Silver Lake, Lake Abert, and potentially dace from neighboring basins.

4.3.1 Foskett Spring

The apparent lack of genetic distance between Foskett Spring and Warner basin dace based on both nuclear and mitochondrial DNA sequence data indicates that this population has not been isolated long enough to diverge significantly. But Foskett Spring speckled dace were identified as a population from the Structure analysis and also exhibit diagnosibly different morphology from other dace in this region. There is no evidence that this is a distinct species, but the evidence of genetic isolation, distinct morphology, and the unique habitat of the population qualifies Foskett Spring dace for consideration as an evolutionarily significant unit on a unique evolutionary path.

Given the high degree of genetic similarity between Foskett Spring dace and dace from Warner Basin, the morphological differences between spring and stream fish in the Warner basin are unlikely to have resulted from randomly accumulated mutations over a prolonged period of time. Rather, they likely result either from ecophenotypic induction or rapid local adaptation in certain morphologically func-

tional genes to the unusual habitat of Foskett Spring. These two effects, rapid genetic adaptation and phenotypic plasticity, are difficult to distinguish without the use of genomic, next-generation sequencing to identify functional genes under selection.

4.4 Management Implications

The management of speckled dace in Oregon should be changed. First, the presence of a new species in the Malheur drainage has necessitated further ecological and population investigations to determine the number of individuals in Stinking Lake Spring as well as to gather information about their life history. ODFW and the Malheur National Wildlife Refuge, on whose land Stinking Lake Spring is located, are already planning this research. There may be other populations of this same spring fish located in nearby springs; there are museum records of spring dace collected in the 1970s with the same morphology (OSIC unpublished data), but searches in these springs in 2011 did not find speckled dace. There may also be a need to list this species on the Endangered Species list if state and federal agencies (ODFW, BLM, USFWS) determine that Stinking Lake Spring dace are in jeopardy from environmental fluctuations affecting the small, isolated spring.

The taxonomic designation of Foskett Spring speckled dace should be revised from an undescribed subspecies to an Evolutionarily Significant Unit of speckled dace (see above). In addition to the genetic and strong morphological evidence that Foskett Spring dace are unique, the spring habitat that these fish are adapted to

is very unique. Foskett Spring has much higher mineral concentrations than other springs in Warner Valley (Si 39ppm, Na 63ppm, B 0.6ppm, K 8.6ppm; Mauger, 2000). This spring also has constant high temperatures (18°C) and low dissolved Oxygen (6mg/L), which is unlike the habitat of the stream dwelling dace, which provides further evidence of its evolutionary significance.

Foskett Spring should remain protected as threatened under the ESA because the isolated habitat and potential for habitat destruction from cattle is still a threat. Although BLM acquired the land in 1985 and fenced it to exclude cattle, there are still ungulate prints seen in the spring brook from time to time, potentially from cattle or native ungulates like deer or pronghorn (Paul Scheerer, ODFW and Kendra Hoekzema, pers. comm.). One unforeseen consequence of the exclusion of cattle is that the tule and cattail marsh has become significantly overgrown; where once there was open water which harbored the majority of the population, now it is almost impossible to even set a minnow trap. This reduction in habitat is likely the cause of the population drop over the last 15 years (Dambacher et al., 1997). In addition, the attempts to start another safety net population in man-made springs nearby have been unsuccessful.

4.5 Future Directions

This work has clarified many questions about the taxonomy and morphological and genetic variation of speckled dace from Eastern Oregon. The presence of three previously unrecognized species will require further work to describe, name, and

delimit these species. A range-wide phylogeographic study of speckled dace will be needed fully resolve which basins or populations outside of our study area are part of these three new species, and will likely identify even more species boundaries that need to be addressed. Research into the phenotypic plasticity of speckled dace morphology, especially as it relates to spring habitats, will be essential in future species identification. In particular, the need to find taxonomically informative characters that are not easily affected by habitat should be identified; a likely candidate is the difference in pharyngeal teeth found in KWS dace (Gould and Kaya, 1991) that should be investigated in Foskett Spring and Stinking Lake Spring dace. Next-generation sequencing can be used to find functional genes under selection that may be affecting the spring dace morphology, which can help distinguish between rapid genetic adaptation and phenotypic plasticity.

APPENDICES

Appendix A – Materials Examined

Rhinichthys osculus: All specimens from Oregon, USA

LAKE COUNTY:

OS 18125, 17 specimens, Twenty Mile Creek, Warner Valley (42°04.074'N 119°57.844'W).

OS 18126, 6 specimens, Snyder Creek, Warner Valley (42°30.076'N 120°05.779'W).

OS 18127, 41 specimens, Foskett Spring, Warner Valley (42°04.180'N 119°50.380'W).

OS 18129, 3 specimens, Cox Creek, Goose Lake Basin (42°21.213'N 120°22.165'W).

OS 18128, 31 specimens, Drews Creek, Goose Lake Basin (42°07.172'N 120°34.830'W).

OS 18400, 11 specimens, Cottonwood Creek, Goose Lake Basin (N 42°16.273' W 120°31.743').

OS 18393, 30 specimens, Dent Creek, Goose Lake Basin (N 42°12.783' W 120°46.728').

OS 18394, 30 specimens, Hay Creek, Goose Lake Basin (N 42°07.484' W 120°46.917').

OS 18130, 30 specimens, Crooked Creek, Lake Abert Basin (42°24.670'N 120°17.49'W).

OS 18391, 30 specimens, Little Coffee Pot Creek, Lake Abert Basin (N 42°33.208' W 120°38.002').

OS 18397, 30 specimens, South Creek, Lake Abert Basin (N 42°25.713' W 120°37.307').

OS 18401, 8 specimens, Silver Creek, Silver Lake Basin (N 43°02.498' W 121°05.259').

OS 18396, 8 specimens, West Fork Silver Creek, Silver Lake Basin (N 43°00.977' W 121°07.147').

HARNEY COUNTY:

OS 18398, 11 specimens, Cow Creek, Malheur Basin (N 43°46.905' W 118°46.105').

OS 18389, 4 specimens, Rattlesnake Creek, Malheur Basin (N 43°42.633' W 118°47.916').

OS 18390, 16 specimens, Sawtooth Creek, Malheur Basin (N 43°51.739' W 119°18.896').

OS 18392, 12 specimens, Nicoll Creek, Malheur Basin (N 43°40.870' W 119°40.443').

OS 18386, 12 specimens, Stinking Lake Spring, Malheur Basin, Oregon Lakes Drainage, Malheur National Wildlife Refuge (N 43°18.810' W 119°22.005').

Appendix B – Molecular Supplementary Materials

B.1 Electronic material

The following data can be found in the attached CD:

A Nexus file for each ND2 (ND2_alignment.nex) and S7 (S7_alignment.nex) of all of the sequences in alignment format.

A Fasta file for each ND2 (ND2_haplotype_sequences.fasta) and S7 (S7_haplotype_sequences.fasta) of each unique haplotype for each gene, numbered arbitrarily.

A table for each ND2 (ND2_haplotype_table.xlsx) and S7 (S7_haplotype_ table.xlsx) of each variable site in the sequence alignment, numbered by haplotype number, with each site numbered by basepair location in the alignment.

A list of each haplotype (numbered as in previous files) and which individuals have that given haplotype from this dataset. One file for ND2 (ND2_haplotypes.html) and S7 (S7_haplotypes.html).

A treefile for the phylogenetic tree created for each gene and the concatenated dataset for both Maximum Likelihood and Bayesian methods. (Concat_RAxML.tre; Concat_MrBayes.tre; ND2_RAxML.tre; ND2_MrBayes.tre; S7_RAxML.tre; S7_Mr Bayes.tre)

A spreadsheet of genotypes for each individual and locus was included (microsatellites.xlsx).

Table B.1: Number of haplotypes per population for ND2 and S7, and the number of alleles for the 8 microsatellite loci by population (Allelic richness by rarefaction in parenthesis).

Gene (length)	Total # haplotypes	Malheur	Stinking Lake Spring	Silver Lake	Goose Lake	Lake Abert	Warner	Foskett Spring
ND2 (1001bp)	120	25	6	11	25	17	12	23
S7 (857bp)	98	7	5	9	28	21	8	18
Microsatellite (Repeat motif)	Total # Alleles							
CypG3 (TAGA)	65	21 (13.4)	10 (11.3)	15 (13.0)	25 (13.6)	21 (11.3)	14 (7.4)	10 (11.6)
CypG9 (CAGA)	6	5 (3.0)	3 (4.4)	5 (3.0)	3 (2.0)	4 (3.0)	2 (3.3)	3 (4.0)
CypG13 (TAGA)	60	32 (15.3)	11 (10.5)	23 (18.0)	45 (17.8)	33 (15.2)	21 (11.5)	15 (15.7)
CypG24 (CAGA)	24	10 (6.7)	8 (8.0)	12 (10.6)	15 (8.5)	7 (10.3)	13 (6.6)	9 (5.4)
CypG27 (TAGA)	26	9 (13.5)	14 (13.2)	17 (13.9)	23 (13.5)	21 (13.8)	18 (9.8)	12 (13.4)
CypG33 (CTGT)	3	2 (1.9)	1 (1.0)	1 (1.0)	2 (2.0)	2 (1.9)	2 (2.0)	2 (1.3)
Lco1 (GATA)	77	25 (14.1)	17 (17.0)	18 (15.1)	39 (17.4)	37 (16.1)	20 (14.3)	20 (17.4)
Lco4 (GTGA)	26	6 (4.4)	4 (4.0)	7 (6.6)	23 (12.4)	13 (7.9)	9 (5.7)	6 (7.3)

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