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# Development of nuclear DNA markers to detect hybridization and assess phylogenetic relationships among subspecies of rainbow trout ( Oncorhynchus mykiss) and cutthroat trout (Oncorhynchus clarki)

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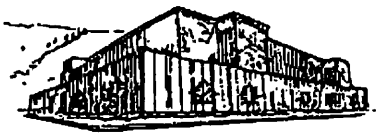

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# DEVELOPMENT OF NUCLEAR DNA MARKERS TO DETECT HYBRIDIZATION AND ASSESS PHYLOGENETIC RELATIONSHIPS AMONG SUBSPECIES OF RAINBOW TROUT (*Oncorhynchus mykiss*) AND CUTTHROAT TROUT (*Oncorhynchus clarki*)

by

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Development of Nuclear DNA Markers to Detect Hybridization and Assess Phylogenetic Relationships Among Rainbow Trout (*Oncorhynchus mykiss*) and Cutthroat Trout (*Oncorhynchus clarki*)

Director: Fred W. Allendorf

I identified 42 species-specific DNA markers for rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*O. clarki*) using the polymerase chain reaction (PCR) and nine combinations of primers complementary to the ends of interspersed nuclear elements. The species-specific markers were highly reproducible, anonymous fragments of DNA, which allowed identification of rainbow and cutthroat trout subspecies and detection of introgressive hybridization between the various subspecies. This non-lethal assay reliably identifies rainbow trout, cutthroat trout, and their hybrids in a manner concordant with other methods of species identification.

Six combinations of primers complementary to the ends of interspersed nuclear elements were used, in conjunction with PINE-PCR, to analyze the phylogenetic relationships between five subspecies of cutthroat trout (*O. clarki* sp) and two subspecies of rainbow trout (*O. mykiss* sp). The six primer combinations amplified 440 anonymous DNA fragments. Similarity index values, based upon fragment sharing between samples, were compiled into 1000 pseudoreplicate distance matrices. UPGMA cluster analysis and principal coordinate analysis (PCoA) established phylogenetic relationships. Bootstrap values were greater than 90% at all major nodes in the UPGMA cluster analysis. The first four axes of the PCoA clearly separated the rainbow trout from the cutthroat trout and accounted for 80% of the total variance. The results strongly support the separation of rainbow trout and cutthroat trout subspecies into separate, monophyletic evolutionary lineages.

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# INTRODUCTION

## 1.1. BACKGROUND

A critical threat to the persistence of native cutthroat trout (*Oncorhynchus clarki*) in the Pacific Northwest is hybridization with other introduced and non-native salmonids, principally rainbow trout (*O. mykiss*). Though it is expected that low-level hybridization has historically occurred between rainbow and cutthroat trout in regions of sympatry throughout the Pacific Northwest, these rates of hybridization are thought to have increased dramatically due to prolific stocking efforts over the past century. Consequently, as many of the native cutthroat trout populations are becoming increasingly endangered, there is a need for an efficient and non-lethal method of species identification to ensure that rescue and recovery efforts are appropriately expended.

At present, there are six recognized subspecies of *O. mykiss* and 14 recognized subspecies of *O. clarki* (Behnke 1992). Furthermore, depending on which data sets are considered, there is a marked discordance on how the various subspecies are related. For example, Leary et al. (1987) used protein electrophoresis to measure the genetic divergence between inland rainbow trout (*O. m. gairdneri*) and seven subspecies of cutthroat trout. Three of the cutthroat trout subspecies, Lahontan (*O. c. henshawi*), westslope (*O. c. lewisi*), and coastal cutthroat trout (*O. c. clarki*), were more similar to inland rainbow trout than they were to the other four subspecies of cutthroat trout analyzed: Colorado River (*O. c. pleuriticus*), finespotted (*O. c.* sp), greenback (*O. c. stomias*), and Yellowstone (*O. c. bouvieri*). In contrast, Gyllensten and Wilson (1987) analyzed mitochondrial DNA (mtDNA) and found that subspecies of cutthroat trout form a distinct group from the rainbow trout subspecies.

## 1.2. STUDY OBJECTIVES

It is apparent that a large number of DNA markers, which can be obtained and analyzed via non-lethal sampling, are needed for the numerous subspecies of trout in the Pacific Northwest. A suite of such DNA markers would allow researchers to assess the hybrid status of specific populations and accurately classify individual fish to the subspecies level. Therefore, the primary objective of this study was to develop a non-lethal, PCR-based assay, using nuclear DNA markers, which discriminates rainbow from cutthroat trout subspecies and detects hybridization between the various subspecies as effectively as allozymes. The secondary objective was to use DNA markers, derived from the non-lethal PCR assay, to test if westslope, coastal, and Lahontan cutthroat trout are genetically more similar to inland rainbow trout than other cutthroat trout subspecies.

## 1.3. SUMMARY

This study illustrates the application of a novel nuclear DNA assay, Paired Interspersed Nuclear Element PCR (PINE or PINE-PCR), to the identification of cutthroat trout subspecies, rainbow trout subspecies, and their hybrids, as well as its utility in elucidating the phylogenetic relationships among various subspecies of Pacific trout. The technique uses a non-lethal sampling protocol, is efficient, cost-effective, and yields reproducible results that are as accurate, if not more so, than other methods of species identification such as meristic character counts and protein electrophoresis.

Furthermore, only six PINE-PCR reactions were required to amplify 440 anonymous DNA fragments, 98.4% of which were polymorphic for subspecies of

rainbow and cutthroat trout. This assay yields results that are, for the most part, concordant with other published views of the phylogenetic relationship of the genus *Oncorhynchus*, principally that the rainbow trout and cutthroat trout species complexes have separate monophyletic origins.

# DEVELOPMENT OF NUCLEAR DNA MARKERS TO DETECT INTROGRESSIVE HYBRIDIZATION AMONG CUTTHROAT TROUT (*Oncorhynchus clarki*) AND RAINBOW TROUT (*Oncorhynchus mykiss*)

**Abstract:** I identified 42 species-specific DNA markers for rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*O. clarki*) using the polymerase chain reaction (PCR) and nine combinations of primers complementary to the ends of interspersed nuclear elements. The species-specific markers were highly reproducible, anonymous fragments of DNA, which allowed identification of rainbow and cutthroat trout subspecies and detection of introgressive hybridization between the various subspecies. This non-lethal assay reliably identifies rainbow trout, cutthroat trout, and their hybrids in a manner concordant with other methods of species identification.

## 2.1. INTRODUCTION

A critical threat to the persistence of native cutthroat trout (*Oncorhynchus clarki*) in the Pacific Northwest is introgressive hybridization with other introduced and non-native salmonids, principally rainbow trout (*O. mykiss*). Trout are highly vulnerable to hybridization due to their poor reproductive isolating mechanisms and external fertilization (Campton 1987). Additionally, the resultant hybrid progeny are usually fertile. The positive feedback that a lack of hybrid sterility presents can perpetuate the hybrid condition, thus compromising the genetic integrity of native trout populations. The potential result is a hybrid swarm of fish in which all locally adapted gene complexes are disrupted (Allendorf and Leary 1988, Leary et al. 1995). Therefore, the maintenance of genetically pure populations is critical to preserve the genetic integrity of a particular species or subspecies and to facilitate the production of native brood stocks for use in population augmentation, stocking, and re-introduction programs. Furthermore, considering that many populations of native rainbow and cutthroat trout are further jeopardized by their small population sizes, there is an immediate need for researchers

assessing these populations to utilize non-lethal sampling techniques, such as fin clipping.

The traditional methods of species identification and hybrid detection are morphological and meristic character analyses, which involve sacrificing individual fish to be sampled for resolution of internal anatomical features (Campton 1987). Effective application of these methods assumes that hybrid individuals will be morphological intermediates of the parental types. This is an erroneous assumption (Neff and Smith 1979, Leary et al. 1983, 1985). Additionally, Leary et al. (1984) showed that when a population of westslope cutthroat trout (*O. c. lewisi*) is 10% introgressed with rainbow trout the meristic counts do not detectably differ from those of pure westslope cutthroat trout.

Protein electrophoresis, a common and dependable method of hybrid identification in trout (Leary et al. 1983, Campton 1987), analyzes tissue-specific enzymes to detect the requisite genetic variation used in species identifications. Though Carmichael (1986) and Leitner (1994) have shown it is possible to detect adequate genetic variation in largemouth bass (*Micropterus salmoides*) through non-lethal sampling techniques, the greatest power for species identification and hybrid detection in trout is attained through lethal sampling of eye, liver, and muscle tissues.

The advent of the polymerase chain reaction (PCR) (Saiki et al. 1988) facilitated the development of a number of non-lethal species identification and hybrid detection techniques that are based upon DNA analysis. For example, restriction fragment length polymorphisms (RFLPs) of both the mitochondrial DNA (mtDNA) and nuclear (nDNA) genomes have proven useful in species identifications and hybrid detection (Avise and

Saunders 1984, Chow et al. 1993, Hansen and Loeschcke 1996, Hayes et al. 1996, Williams et al. 1996, Wilson et al. 1996, Baxter et al. 1997). However, RFLP analysis is laborious as the genomic sequences flanking the target sites have to be discovered for PCR primer synthesis and PCR products have to undergo restriction digestion. Furthermore, RFLP analysis of mtDNA, which is maternally inherited, assesses only the directionality of hybridization unless it is used in conjunction with another species identification technique. Thus, in situations where "sneaker males" are invading salmonid spawning beds and causing hybridization, their genetic contribution will not be detected.

Repetitive DNA sequences, e.g. simple sequence repeats (SSRs) and variable number of tandem repeats (VNTRs), have been used to distinguish species and detect hybrids (Heath et al. 1993, Gupta et al. 1994). However, repetitive sequences have a high mutation rate and primers based on tandemly repeated elements often generate high levels of background noise because they can anneal to any repeat within a given repeat array.

Randomly amplified polymorphic DNA (RAPD or (RAPD-PCR) (Williams et al. 1990) is another PCR-based tool that is used to identify species and detect hybridization between species (Bardacki and Skibinski 1994, Naish et al. 1995, Riesberg and Gerber 1995, Smith et al. 1996). Elo et al. (1997) used RAPDs to develop markers that would detect hybridization between brown trout (*Salmo trutta*) and Atlantic salmon (*S. salar*). Although it incorporates non-lethal sampling, there are drawbacks to this method. The small size of a RAPD primer, usually a decamer, coupled with low annealing temperatures, allows for potentially non-specific annealing throughout the genome being

amplified. Furthermore, sequence heteroduplexes, generated by the annealing of differentially sized allelic RAPD products, have been reported to form during RAPD-PCR (Riedy et al. 1992, Ayliffe et al. 1994).

Short interspersed elements (SINEs) have been used to identify and classify numerous species, including salmonids (Kido et al. 1991, Murata et al. 1993, Takasaki et al. 1994, Murata et al. 1996, Greene and Seeb 1997, Hamada et al. 1997, Takasaki et al. 1997). During episodic bouts of amplification, multiple copies of a particular SINE element are generated and inserted at random into the genome via RNA intermediates (Deininger 1989, Tachida 1996). The only means of removal of a SINE is thought to be a recombination event (Kordis and Gubensek 1995, Cook and Tristem 1997). To date, no one has been able to illustrate a case in which independent SINE insertions have occurred at the same location within distinct genomes (Takasaki et al. 1997, Eyre-Walker 1999). Therefore, SINEs constitute a class of DNA markers that are very useful for species identification.

Thus far, five SINEs have been identified within the salmonids. Kido et al. (1991) described *Fok* I, an element specific to the genus *Salvelinus*. Pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*) share the *Sma* I element (Kido et al. 1991). Furthermore, Spruell and Thorgaard (1996) discovered that a portion of the 5′ ends of *Fok* I and *Sma* I were found throughout the salmonids. *Hpa* I is found within all members of the family Salmonidae (Kido et al. 1991) as is the *Hpa* I-like *Ava* III element (Kido et al. 1994). Ginatulin and Ginatulin (1996) described another SINE element, *Pvu* II, specific to pink salmon. Additionally, Winkfein et al. (1988) identified *RSg*-1, a long interspersed nuclear element (LINE) found within rainbow trout.

In addition to these SINE families, Goodier and Davidson (1994) estimated that there are approximately 50,000 copies of the *Tc* 1 element within the pink salmon genome. Though the *Tc* 1 elements are technically classified as transposons, which are able to move freely throughout the genome, these elements have lost their mobility and are now considered well incorporated within the genomes of host species. Thus, they have characteristics analogous to those of SINEs.

Spruell et al. (1999a) performed PCR, with primers derived from SINE elements, to detect hybridization and characterize fish sampled from bull trout (*Salvelinus confluentus*) and brook trout (*Salvelinus fontinalis*) populations in Montana. Their primers were fluorescently-labeled oligonucleotides designed to anchor within the ends of interspersed nuclear elements and direct amplification away from the element, thus amplifying the anonymous intergenic region between two SINEs. Using primers for two different SINEs, they were able to maximize both the clarity and the number of fragments amplified per reaction, thus enhancing their ability to detect diagnostic markers. The protocol is known as paired interspersed nuclear element-PCR (PINE-PCR) and is analogous to techniques used in studies by Nelson et al. (1989), Peek et al. (1997), and Greene and Seeb (1997).

### 2.1.1. Objectives

Non-lethal sampling techniques are beneficial when assessing population identity and examining hybridization events for threatened species or species existing in low population numbers. The objective of this study was to develop a non-lethal, PCR-based assay that uses species-specific DNA markers to effectively discriminate rainbow from cutthroat trout subspecies and detect interspecific hybridization between the various

subspecies. This assay was compared to protein electrophoresis to validate the efficacy of the nuclear DNA markers.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Reference Populations

Species-specific DNA fragments were identified by screening reference samples of rainbow and cutthroat trout subspecies from populations throughout the Pacific Northwest. Sampled subspecies included the following: coastal cutthroat trout (*O. c. clarki*), westslope cutthroat trout, Yellowstone cutthroat trout (*O. c. bouvieri*), coastal rainbow trout (*O. mykiss irideus*), and inland rainbow trout (*O. m. gairdneri*). The sampled populations had their genetic identity previously confirmed via protein electrophoresis (Robb Leary, pers comm). Sampled populations were selected to represent a wide geographic range for each subspecies (Table 1). Three fish were randomly chosen from each sampled population to be examined for diagnostic PINE markers. Only one population of Yellowstone cutthroat trout was sampled due to the low level of genetic variation among populations of this fish (Leary et al. 1987, Allendorf and Leary 1988).

### 2.2.2. Nuclear DNA Isolation

Tissue samples from the field consisted of either whole fish transported on ice, or small caudal fin clips, approximately 1.0 $cm^2$, in 95% ethanol. Whole fish were stored at -40°C until sampled, while fin clips were stored at room temperature in 95% ethanol until sampled. DNA extractions were performed on muscle cores from frozen fish or subsamples of fin clips using the Puregene® kit (Gentra Systems Inc.) as per the

**Table 1. Five subspecies of western North American trout analyzed for species-specific DNA markers and the locations of populations sampled.**

| Subspecies | Strain | Source (Sponsor) | Location |
|---|---|---|---|
| *Oncorhynchus mykiss irideus* | Arlee | Jocko River Trout Hatchery (MTFWP) | Arlee, MT |
| | Wild | Little Sandy River, OR | 45.43°N, 122.21°W |
| *Oncorhynchus mykiss gairdneri* | Shasta | Dworshak National Fish Hatchery (USFW) | Ahsahka, ID |
| | Wild | Wolf Creek, MT | 48.23°N, 115.29°W |
| | Kamloops | Ennis National Fish Hatchery (USFW) | Ennis, MT |
| *Oncorhynchus clarki clarki* | Wild | Gierin Creek, WA | 48.12°N, 123.056°W |
| | Wild | Emil Creek, OR | 45.56°N, 121.59°W |
| | Wild | Lady Creek, OR | 45.31°N, 121.84°W |
| *Oncorhynchus clarki lewisi* | Fish Lake | McCall Fish Hatchery (IDFG) | McCall, ID |
| | Wild | Marshall Creek, MT | 46.89°N, 113.92°W |
| | Hungry Horse | Washoe Park Trout Hatchery (MTFWP) | Anaconda, MT |
| *Oncorhynchus clarki bouvieri* | McBride | Yellowstone River Trout Hatchery (MTFWP) | Big Timber, MT |

manufacturer's instructions. The DNA concentration for all samples was estimated through visualization on agarose gels stained with ethidium bromide (Maniatis et al. 1982).

### 2.2.3. Paired Interspersed Nuclear Element PCR

PINE-PCR was employed to maximize the number of diagnostic fragments between rainbow and cutthroat trout. Primers used in this study (Table 2) were synthesized from the *Fok* I, *Hpa* I, and *Sma* I SINE families (Kido et al. 1991). Three additional primers were synthesized from the *RSg*-1 LINE family (Winkfein et al. 1988), the *Tc* 1 element (Goodier and Davidson 1994), and Jeffreys 33.6 core fragment (Jeffreys et al. 1985). Jeffreys' core fragment was selected for it has been effective as a probe in fingerprinting studies of rainbow trout (Spruell et al. 1994) and sockeye salmon (Thorgaard et al. 1995).

DNA fragments were amplified via PINE-PCR from approximately 25.0 ng of genomic DNA in a 10 μL PCR reaction containing 1X Stoffel buffer (100 mM KCL, 100 mM Tris-HCL, pH 8.3), 5.0 mM $MgCl_2$, 0.20 mM dNTPs, 0.25 μM of each primer, and 1.0 U AmpliTaq® DNA Polymerase Stoffel Fragment (Perkin-Elmer). Amplifications were performed in MJ Research PTC-100 thermal cyclers using the following profile: 1 cycle of 95°C/1.5 min, 30 cycles of 91°C/1 min, 60°C/1 min, 72°C/1.5min, 1 cycle of 72°C/1.5 min, and 12°C until termination. The resulting DNA fragments were electrophoretically separated in 4.5% denaturing polyacrylamide gels and visualized using a Hitachi FMBIO-100 fluorescent imager. Fragments were scored relative to MapMarker ladder (BioVentures) with version 6.0 of the FMBIO-100 data analysis software (Hitachi Software).

**Table 2. Primers used in PINE-PCR to amplify species-specific DNA fragments for rainbow and cutthroat trout.**

| Primer | Sequence (5′ → 3′) | Reference |
|---|---|---|
| *Fok* I 5′ | CCAACTGAGCCACACGGGAC | Kido et al. 1991 |
| *Hpa* I 3′ | TGAGCTGACAAGGTACAAATC | Kido et al. 1991 |
| *Hpa* I 5′ | AACCACTAGGCTACCCTGCC | Kido et al. 1991 |
| *RSg*-1 5′ | GGCAGCAGAGAACTGGAAGG | Winkfein et al. 1988 |
| *Sma* I 5′ | AACTGAGCTACAGAAGGACC | Kido et al. 1991 |
| *Tc* 1 5′ | TGATTGGTGGAGTGCTGCAG | Goodier and Davidson 1994 |
| 33.6 | TGGAGGAGGGCTGGAGGAGGGC | Jeffreys et al. 1985 |

### 2.2.4. Diagnostic Fragment Identification

DNA fragments produced during PINE-PCR must adhere to certain criteria to be considered diagnostic. They need to be reproducible, robust, and easily discernible from other fragments. Fragments that were faint and irreproducible were therefore not characterized as diagnostic fragments. In theory, diagnostic DNA fragments should be homologous across sampled individuals, i.e. generated from SINE elements located in the same location in each individual. They also should be found in all individuals sampled from a species or subspecies. For example, a fragment common to all fish screened from the three westslope cutthroat trout reference populations, but no other sampled fish, was deemed diagnostic for the westslope cutthroat trout subspecies (Figure 1). Likewise, a fragment that was found in all rainbow trout screened, both coastal and inland, and none of the cutthroat trout, was deemed diagnostic for the rainbow trout species.

### 2.2.5. Identification and Classification of Wild Populations

Once the diagnostic or species-specific markers were identified, they were tested using samples from wild populations. Tissue samples of 291 fish from a larger study at the Wild Trout and Salmon Genetics Lab (WTSGL) at the University of Montana were analyzed (Table 3). Populations were sampled from the following areas: the John Day River basin, Hood River basin, and lower Columbia River Gorge.

Samples from the Hood River basin and the lower Columbia River Gorge were selected to illustrate the power of PINE-PCR at detecting differing species within a single population. Samples from the John Day River basin were being tested via allozymes as part of a concurrent study and thus facilitated a comparison of the efficacy of PINE-PCR versus allozymes. All samples were field-identified as either a rainbow or a cutthroat

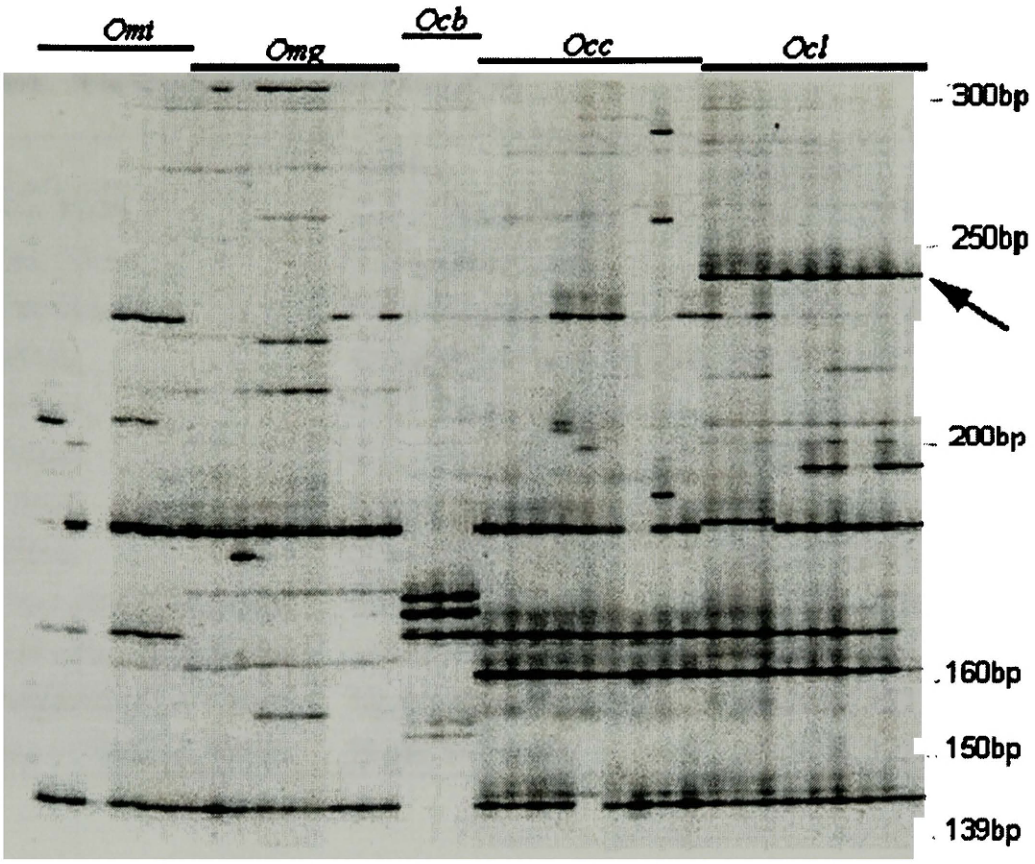


**Figure 1. Polyacrylamide gel section showing electrophoresed products of a *Fok* I/*Hpa* I 3′ PINE-PCR reaction. Black arrow on the right indicates a diagnostic marker for westslope cutthroat trout. Size standards, in base pairs (bp), are indicated on the right side. Across the top, subspecies designations are as follows: *Omi* - coastal rainbow trout; *Omg* - inland rainbow trout; *Ocb* - Yellowstone cutthroat trout; *Occ* - coastal cutthroat trout; *Ocl* - westslope cutthroat trout.**

**Table 3. Populations sampled from the John Day River basin, Hood River basin, and the lower Columbia River Gorge for validation of species-specific PINE markers. N is the number of fish sampled.**

| Basin | Creek | Location | N* |
|---|---|---|---|
| John Day River | Lower Dixie Creek | 44.27°N, 118.42°W | 33 |
| John Day River | Upper Dixie Creek | 44.34°N, 118.44°W | 29 |
| John Day River | Roberts Creek | 44.34°N, 118.57°W | 30 |
| Hood River | Green Point Creek, N. Fork | 45.35°N, 121.41°W | 30 |
| Hood River | Hood River, W. Fork | 45.36°N, 121.37°W | 9 |
| Hood River | Rimrock Creek | 45.29°N, 121.33°W | 20 |
| Hood River | Lower Dog River | 45.27°N, 121.33°W | 18 |
| Hood River | Upper Dog River | 45.20°N, 121.31°W | 24 |
| Lwr Columbia River Gorge | Oneonta Creek | 45.59°N, 122.07°W | 34 |
| Lwr Columbia River Gorge | Lower Multnomah Creek | 45.58°N, 122.12°W | 30 |
| Lwr Columbia River Gorge | Upper Multnomah Creek | 45.58°N, 122.12°W | 29 |
| Lwr Columbia River Gorge | Bridal Veil Falls | 45.56°N, 122.18°W | 15 |

trout by Oregon Department of Fish and Wildlife personnel before tissue sampling and transport to the WTSGL.

Samples from the Hood River basin and the lower Columbia River Gorge would most likely consist of coastal rainbow trout and coastal cutthroat trout, while samples from the John Day River basin were suspected to be westslope cutthroat trout. Therefore, these populations were first screened with the *Hpa* I 3'/*Hpa* I 5' primer combination which efficiently distinguishes between rainbow and cutthroat trout species. Subsequent analyses were dictated by the results from this first primer combination, thus the total number of markers used in each analysis varied on a per population basis.

Species identity and hybrid status for each sample was determined by comparing fragments from PINE-PCR to the previously identified diagnostic fragments for rainbow and cutthroat trout subspecies. For example, a fish from the Hood River basin was classified as a rainbow trout if it had all of the rainbow trout markers and none of the cutthroat trout markers that were screened.

Hybrid fish were characterized as follows: $F_1$, backcross to rainbow trout ($F_1$ x RBT), backcross to cutthroat trout ($F_1$ x CTT), $F_2$, and post-$F_2$ hybrids. Since most PINE fragments are dominant (Spruell et al. 1999b) and inherited in a Mendelian manner (Greene and Seeb 1997, Spruell et al. 1999b), $F_1$ hybrids should possess every DNA fragment from both parental species. Backcrossed fish are expected to have all fragments of the species backcrossed to and approximately one half of the other species' fragments. It is expected that an $F_2$ hybrid would have approximately 75% of the fragments from each parental species. Due to the propensity of trout to form hybrid swarms, any fish with

proportions of fragments different from those described above was classified as a post $F_2$ hybrid.

## 2.3. RESULTS

### 2.3.1. Diagnostic Markers

As a result of the random distribution of interspersed nuclear elements throughout the salmonid genome, the likelihood of producing anonymous DNA fragments via PINE-PCR should increase if primers complementary to two different interspersed elements are used. Using a single primer for PINE-PCR resulted in smears and high molecular weight fragments that were unable to be accurately scored. However, using pairs of primers complimentary to the ends of interspersed elements resulted in clear, scoreable, anonymous DNA fragments. At least 30 anonymous DNA fragments were produced from each primer pair; two to eleven of which were diagnostic for a particular species. In sum, there were 42 species-specific fragments ranging in size from 66bp to 395bp (Table 4). The following nomenclature is used to identify diagnostic fragments, #X#Y ###. In this style, #X and #Y is the end of the element the primer was designed from, 5′ or 3′, and the first letter of the primer name. The two primers are listed alphabetically and ### is the size of the fragment. For example, the fragment labeled 5H5S 138 is 138 basepairs in size and derived from the *Hpa* I 5′/*Sma* I 5′ primer combination. However, note that 5F53 134 is a 134 basepair fragment from the *Fok* I 5′/*33.6* 5′ primer combination.

Coastal and inland rainbow trout have eleven diagnostic markers in common, while coastal rainbow trout have one diagnostic marker and inland rainbow trout have three diagnostic markers. Coastal, westslope, and Yellowstone cutthroat trout have three

**Table 4. Species-specific PINE fragments for two subspecies of rainbow trout and three subspecies of cutthroat trout.**

| Primer Combination | Fragment Size (bp) | *O. mykiss irideus* | *O. mykiss gairdneri* | *O. clarki clarki* | *O. clarki lewisi* | *O. clarki bouvieri* |
|---|---|---|---|---|---|---|
| *Hpa* I 3' /*Hpa* I 5' | 153 | --- | --- | XXX | XXX | --- |
| *Hpa* I 3' /*Hpa* I 5' | 70 | XXX | XXX | --- | --- | --- |
| *Hpa* I 3' /*Hpa* I 5' | 69 | --- | --- | XXX | XXX | XXX |
| *Hpa* I 3' /*Hpa* I 5' | 66 | XXX | XXX | --- | --- | --- |
| *Fok* I 5'/*33.6* | 170 | --- | --- | --- | --- | XXX |
| *Fok* I 5'/*33.6* | 168 | --- | --- | XXX | XXX | --- |
| *Fok* I 5'/*33.6* | 142 | --- | --- | --- | --- | XXX |
| *Fok* I 5'/*33.6* | 134 | XXX | XXX | --- | --- | --- |
| *Fok* I 5'/*33.6* | 127 | --- | --- | XXX | XXX | XXX |
| *Hpa* I 5'/*Sma* I 5' | 144 | --- | --- | --- | XXX | --- |
| *Hpa* I 5'/*Sma* I 5' | 138 | --- | XXX | --- | --- | --- |
| *Hpa* I 5'/*Sma* I 5' | 95 | XXX | XXX | --- | --- | --- |
| *Hpa* I 5'/*33.6* | 395 | XXX | XXX | --- | --- | --- |
| *Hpa* I 5'/*33.6* | 330 | XXX | XXX | --- | --- | --- |
| *Hpa* I 5'/*33.6* | 280 | XXX | XXX | --- | --- | --- |
| *Hpa* I 5'/*33.6* | 266 | XXX | XXX | --- | --- | --- |
| *Hpa* I 5'/*33.6* | 248 | --- | --- | --- | --- | XXX |
| *Hpa* I 5'/*33.6* | 170 | --- | XXX | --- | --- | --- |
| *Hpa* I 5'/*33.6* | 155 | --- | --- | --- | --- | XXX |
| *Hpa* I 5'/*33.6* | 148 | --- | --- | XXX | XXX | XXX |
| *Hpa* I 5'/*33.6* | 130 | --- | --- | --- | --- | XXX |
| *Hpa* I 5'/*33.6* | 126 | --- | --- | --- | --- | XXX |
| *Hpa* I 5'/*33.6* | 110 | --- | --- | --- | XXX | --- |
| *Fok* I 5' /*Sma* I 5' | 249 | --- | --- | --- | XXX | --- |
| *Fok* I 5' /*Sma* I 5' | 247 | --- | --- | XXX | --- | XXX |
| *Fok* I 5' /*Sma* I 5' | 110 | --- | --- | --- | --- | XXX |
| *Fok* I 5' /*Hpa* I 3' | 242 | --- | --- | --- | XXX | --- |
| *Fok* I 5' /*Hpa* I 3' | 173 | --- | --- | --- | --- | XXX |
| *Fok* I 5' /*Hpa* I 3' | 170 | --- | --- | --- | --- | XXX |
| *Fok* I 5' /*Hpa* I 3' | 162 | --- | --- | XXX | XXX | --- |
| *Fok* I 5' /*Tc* I 5' | 369 | XXX | XXX | --- | --- | --- |
| *Fok* I 5' /*Tc* I 5' | 170 | --- | XXX | --- | --- | --- |
| *Fok* I 5' /*Tc* I 5' | 159 | --- | --- | --- | --- | XXX |
| *Fok* I 5' /*Tc* I 5' | 138 | --- | --- | --- | --- | XXX |
| *Fok* I 5' /*Tc* I 5' | 132 | --- | --- | --- | XXX | --- |
| *Fok* I 5' /*Tc* I 5' | 122 | XXX | XXX | --- | --- | --- |
| *Hpa* I 3' /*Tc* I 5' | 207 | --- | --- | XXX | --- | --- |
| *Hpa* I 3' /*Tc* I 5' | 152 | --- | --- | --- | XXX | XXX |
| *Fok* I 5' /*RSg*-1 5' | 389 | XXX | XXX | --- | --- | --- |
| *Fok* I 5' /*RSg*-1 5' | 299 | --- | --- | XXX | --- | XXX |
| *Fok* I 5' /*RSg*-1 5' | 284 | --- | --- | --- | XXX | --- |
| *Fok* I 5' /*RSg*-1 5' | 278 | XXX | --- | --- | --- | --- |

diagnostic markers in common. There is one diagnostic marker for coastal cutthroat trout, six diagnostic markers for westslope cutthroat trout, and eleven diagnostic markers for Yellowstone cutthroat trout. The six remaining cutthroat trout markers were specific for two of the three cutthroat trout subspecies analyzed (Table 4). These 42 species-specific markers can be categorized by which subspecies they distinguish (Table 5).

### 2.3.2. PINE-PCR for Wild Populations

The John Day River basin is important as it is thought to contain the westernmost populations of westslope cutthroat trout (Behnke 1992). Putative westslope cutthroat trout were present in all three populations sampled from the drainage. Unfortunately, these were all hybrid swarms as DNA fragments that are specific to westslope cutthroat trout and inland rainbow trout were observed in all three populations. Also observed were DNA fragments present in both inland and coastal rainbow trout, DNA fragments shared by coastal, westslope, and Yellowstone cutthroat trout, and DNA fragments shared by coastal and westslope cutthroat trout only. Species-specific markers for Yellowstone cutthroat trout were not expected, and not found, in these wild populations since the lower Columbia River system is beyond the natural range of this subspecies. However, this lack of sympatry does not preclude the effects of stocking, and fragments characteristic of Yellowstone cutthroat trout would have been observed if present in the samples analyzed.

In lower Dixie Creek (LDXC), 24 fish were characterized as inland rainbow trout, four as westslope cutthroat trout, two as $F_1$ x RBT, and three as post-$F_2$ hybrids (Table 6). The inland rainbow trout were characterized by the presence of a fragment diagnostic for this subspecies, 5H5S 138, and the absence of any fragments diagnostic for

**Table 5. Matrix summarizing diagnostic PINE fragments derived from nine different primer pairs. The number of markers present in the column subspecies, but absent in the row subspecies, are shown.**

| Species | *O. mykiss irideus* | *O. mykiss gairdneri* | *O. clarki clarki* | *O. clarki lewisi* | *O. clarki bouvieri* |
|---|---|---|---|---|---|
| *O. mykiss irideus* | --- | 3 | 9 | 13 | 17 |
| *O. mykiss gairdneri* | 1 | --- | 9 | 13 | 17 |
| *O. clarki clarki* | 12 | 14 | --- | 7 | 12 |
| *O. clarki lewisi* | 12 | 14 | 3 | --- | 13 |
| *O. clarki bouvieri* | 12 | 14 | 4 | 9 | --- |

**Table 6. Species composition of sampled populations from the John Day River basin, Hood River basin, and lower Columbia River Gorge.**

| Drainage | Creek | N | Diagnostic Fragments Analyzed | Rainbow Trout | Cutthroat Trout | Hybrids | Population Status |
|---|---|---|---|---|---|---|---|
| John Day | Lower Dixie Creek | 33 | 12 | 24 | 4 | 5 | Hybrid |
| John Day | Upper Dixie Creek | 29 | 12 | 7 | 19 | 3 | Hybrid |
| John Day | Roberts Creek | 30 | 12 | - | 25 | 5 | Hybrid |
| Hood | Green Point Creek, N. Fork | 30 | 12 | 29 | - | 1 | Hybrid |
| Hood | Hood River, W. Fork | 9 | 12 | 9 | - | - | RBT |
| Hood | Rimrock Creek | 20 | 20 | - | 20 | - | CTT |
| Hood | Lower Dog River | 18 | 9 | 8 | 6 | 4 | Hybrid |
| Hood | Upper Dog River | 24 | 12 | - | 21 | 3 | Hybrid |
| C. Gorge | Bridal Veil Falls | 15 | 4 | - | 15 | - | CTT |
| C. Gorge | Lower Multnomah Creek | 30 | 7 | 8 | 4 | 18 | Hybrid |
| C. Gorge | Upper Multnomah Creek | 29 | 4 | - | 23 | 6 | Hybrid |
| C. Gorge | Oneonta Creek | 24 | 10 | 24 | - | - | RBT |

†Total diagnostic fragments analyzed

cutthroat trout. The westslope cutthroat trout were identified by the presence of a fragment specific for this subspecies, 5H5S 144, and the absence of any fragments diagnostic for rainbow trout. The two $F_1$ x RBT fish were characterized by the presence of all rainbow trout fragments, plus one (LDXC-17) or two (LDXC-04) cutthroat trout fragments. In the three post-$F_2$ hybrids, all cutthroat trout fragments were absent, and only a subset of rainbow trout fragments was present (Appendix A)

In the upper reaches of Dixie Creek (UDXC), one fish was identified as an $F_1$ x RBT, two as $F_1$ x CTT, seven as rainbow trout, and 19 as westslope cutthroat trout. The $F_1$ x RBT fish had all three rainbow trout fragments and one fragment common to coastal and westslope cutthroat trout. The two $F_1$ x CTT fish had all five fragments present for westslope cutthroat trout and one fragment common to both coastal and inland rainbow trout. The westslope cutthroat trout in this population were characterized by an absence of any fragments specific to rainbow trout and the presence of all fragments common to both coastal and westslope cutthroat trout, as well as a fragment unique to westslope cutthroat trout, 5F5S 249. The seven rainbow trout in this population lacked any fragments specific to cutthroat trout but exhibited fragments common to coastal and inland rainbow trout (Appendix B).

In Roberts Creek (RBTC), one fish was identified as an $F_1$ x RBT, two as $F_1$ x CTT, two fish as post-$F_2$ hybrids and 25 fish as westslope cutthroat trout (Table 6 and Appendix A) The westslope cutthroat trout were identified by the presence of a fragment diagnostic for this subspecies, 5F5S 249, and the absence of any fragments specific to rainbow trout. The $F_1$ x RBT fish (RBTC-05) contained all fragments specific to rainbow trout and a subset of fragments specific to cutthroat trout. The two $F_1$ x CTT fish

amplified all cutthroat trout fragments as well as an additional rainbow trout fragment (RBTC-28) or two additional rainbow trout fragments (RBTC-18). Both post-$F_2$ hybrids (RBTC-14 and RBTC-23) were characterized by the presence of a subset of fragments specific to rainbow trout and subset of fragments specific to cutthroat trout (Appendix A).

Four populations of *Oncorhynchus* were sampled within the lower Columbia River Gorge. The 15 fish sampled from above Bridal Veil Falls (BRVE) were identified as cutthroat trout due to the presence of cutthroat trout fragments and the absence of any rainbow trout fragments. These samples were not categorized to the subspecies level, as only fragments common in coastal, westslope, and Yellowstone cutthroat trout were amplified. The 24 fish sampled from Oneonta Creek (ONEO) were identified as coastal rainbow trout due to the presence of fragments common to both inland and costal rainbow trout, the absence of a fragment diagnostic for inland rainbow trout, 5H5S 138, and the absence of any fragments specific to cutthroat trout subspecies. The 30 fish sampled from the lower section of Multnomah Creek (LMLT) were indicative of a hybrid swarm as evidenced by the following mixture of phenotypes: one $F_1$, 14 $F_1$ x RBT, three $F_1$ x CTT, eight rainbow trout, and four cutthroat trout. The eight rainbow trout could only be characterized to the species level, as the fragments amplified were common in both coastal and inland rainbow trout. The four cutthroat trout could only be characterized to the species level, as the fragments amplified were common in coastal, westslope, and Yellowstone cutthroat trout. For all fish identified as backcrosses to a parental species, all fragments specific to the parental species were amplified while a mixture of fragments specific to other species were also amplified. Evidence of hybridization was also found in the population above Multnomah Falls, in the upper

section of Multnomah Creek, where two $F_1$ x CTT, five post-$F_2$ hybrids, and 22 cutthroat trout were identified (Table 6 and Appendix B). The 22 cutthroat trout could not be categorized to the subspecies level, as the two cutthroat fragments amplified were common in coastal, westslope, and Yellowstone cutthroat trout. Two fish were classified as $F_1$ x CTT due the presence of both cutthroat trout markers and the presence of one rainbow trout marker. Five fish were identified as post-$F_2$ hybrids, for only one of two cutthroat trout markers were present and they lacked both rainbow trout markers.

Five populations were sampled from the Hood River basin. The 20 fish sampled from Rimrock Creek (RMRK) were identified as coastal cutthroat trout due to the absence of diagnostic fragments for westslope and Yellowstone cutthroat trout and the presence of fragments diagnostic for all three subspecies of cutthroat trout (Appendix B). Four fish sampled from the West Fork of the Hood River (WFHD) were identified as coastal rainbow trout. The remaining five fish from this population were classified as inland rainbow trout due to presence of a fragment diagnostic for inland rainbow trout, 5H5S 138 (Appendix B). Twenty-nine fish sampled from the North Fork of Green Point Creek (NFGC) were characterized as inland rainbow trout because of the presence of the 5H5S 138 fragment, as well as four other fragments found in both inland and coastal rainbow trout, and the absence of any species-specific cutthroat trout fragments. The other fish from this population was characterized as a post-$F_2$ hybrid, due to a disproportionate absence of fragments specific to each species. The fish sampled from the lower Dog River (LDOG) were representative of a hybrid swarm. There were six cutthroat trout, eight rainbow trout, three $F_1$ x CTT, and one $F_2$. The rainbow trout could not be classified to the subspecies level as only fragments common in both coastal and

inland rainbow trout were amplified. Likewise, the cutthroat trout could not be classified to the subspecies level as fragments that were common in both coastal and westslope cutthroat trout, as well as fragments common for coastal, westslope, and Yellowstone cutthroat trout, were amplified. The upper Dog River (UDOG) was also hybridized, though not as severely as the lower Dog River. There were 21 coastal cutthroat trout and three $F_1$ x CTT hybrids identified. These 21 cutthroat trout were identified as coastal cutthroat trout due to the presence of a fragment characteristic of both coastal and Yellowstone cutthroat trout, 5F5S 247, and the absence of a fragment diagnostic for Yellowstone cutthroat trout, 5F5S 110 (Appendix B).

### 2.3.3. Concordance Between PINEs and Allozymes

A survey for coastal cutthroat trout by the National Marine Fisheries Service in the lower Columbia River Gorge found that 83% of the fish in Multnomah Creek were hybrids (Johnson et al. 1999). Though they did not sample the same fish as in the present study, their results are in agreement with our findings.

The samples from the John Day basin were screened with five diagnostic allozyme loci (Robb Leary, unpublished data) to compare the results of a dominant marker based system of hybrid detection with a co-dominant marker based system of hybrid detection. On the population scale, the two methods were concordant, as all three populations were characterized as hybridized (Tables 7-9). However, though not surprisingly, there were identification discrepancies between PINE-PCR and allozyme electrophoresis at the level of individual samples (Tables 7-9 and Appendix A).

**Table 7. Comparison of PINE-PCR and allozyme classifications for 33 fish from lower Dixie Creek in the John Day basin, OR.**

| | Allozymes | | | | | | |
|---|---|---|---|---|---|---|---|
| **PINEs** | RBT | WCT | $F_1$ | $F_1$ x RBT | $F_1$ x CTT | $F_2$ | POST- $F_2$ |
| RBT | 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| WCT | 0 | 2 | 0 | 1 | 1 | 0 | 0 |
| $F_1$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $F_1$ x RBT | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| $F_1$ x CTT | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $F_2$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| POST- $F_2$ | 3 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 8. Comparison of PINE-PCR and allozyme classifications for 29 fish from upper Dixie Creek in the John Day basin, OR.**

| | Allozymes | | | | | | |
|---|---|---|---|---|---|---|---|
| **PINEs** | RBT | WCT | $F_1$ | $F_1$ x RBT | $F_1$ x CTT | $F_2$ | POST-$F_2$ |
| RBT | 5 | 0 | 0 | 2 | 0 | 0 | 0 |
| WCT | 0 | 19 | 0 | 0 | 0 | 0 | 0 |
| $F_1$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $F_1$ x RBT | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| $F_1$ x CTT | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| $F_2$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| POST- $F_2$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 9. Comparison of PINE-PCR and allozyme classifications for 30 fish from Roberts Creek in the John Day basin, OR.**

| | Allozymes | | | | | | |
|---|---|---|---|---|---|---|---|
| **PINEs** | RBT | WCT | $F_1$ | $F_1$ x RBT | $F_1$ x CTT | $F_2$ | POST- $F_2$ |
| RBT | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| WCT | 0 | 23 | 0 | 0 | 1 | 0 | 1 |
| $F_1$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $F_1$ x RBT | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| $F_1$ x CTT | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| $F_2$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| POST- $F_2$ | 0 | 2 | 0 | 0 | 0 | 0 | 0 |

## 2.4. DISCUSSION

The high-resolution, multi-fragment patterns produced by different PINE-PCR primer combinations are easily repeatable due to stringent PCR conditions. Furthermore, these patterns facilitate identification of cutthroat trout and rainbow trout and allow detection of hybridization between the various rainbow and cutthroat subspecies. Each PINE-PCR generates from two to 11 diagnostic fragments out of a total of 42 diagnostic fragments. Other nuclear DNA markers available for hybrid detection, such as RAPDs, are not as effective due to the limited number of markers available and decreased repeatability resulting from less stringent PCR conditions. Though there are many microsatellite markers available in trout, the inherent variability within the loci complicates the detection of hybridization. Diagnostic loci in one geographic area may not be useful in another part of the species' geographic range because allele frequencies are not fixed across the range.

### 2.4.1. Hybrid Detection

Though there were fish identified in all of the sampled populations as a subspecies of rainbow or cutthroat trout, considering the distribution of fragments within the populations, only four populations can be characterized as having non-hybridized individuals: the west fork of the Hood River (WFHD), Rimrock Creek (RMRK), Bridal Veil Falls (BRVE), and Oneonta Creek (ONEO). All of the remaining populations are hybridized populations.

The population classification results for PINE-PCR were consistent with those of allozymes. Roughly the same proportion of hybrids was detected in each population from the John Day River basin with either of the two methods. Previous comparisons of

the efficacy of PINE-PCR versus allozymes to detect hybrids between bull trout (*Salvelinus confluentus*), brook trout (*S. fontinalis*), and Dolly Varden (*S. malma*) have given similar results between the two methods (Spruell et al. 1999a).

The variation in assigning individual identity between the two data sets can be attributed to the random distribution of the different classes of markers throughout the genome of each species, combined with the fact that only a subset of each class of marker is analyzed. Therefore, we do not expect to detect hybridization in exactly the same individuals. It is expected that analyzing more diagnostic fragments and diagnostic allozyme loci would likely have decreased the discrepancies. Boecklen and Howard (1997) used statistical models to examine the power of molecular markers in discriminating between non-hybrid individuals, F1s, and backcrossed individuals. Their results indicate as few as four markers are needed to grossly distinguish individuals in hybrid zones. However, finer levels of distinction, such as between pure species and advanced backcrosses, require upwards of 70 diagnostic markers (Boecklen and Howard 1997).

Unfortunately, low-level introgression events, coupled with fertile hybrid progeny, help dilute the pool of effective diagnostic markers. The number of diagnostic markers between two species would be maximized if no genetic introgression occurred, thus allowing complete divergence to occur. However, even one migrant per generation is enough to keep two lineages from attaining complete divergence (Wright 1978).

The probability of misclassifying an $F_2$ or later hybrid as a pure species with the five allozyme loci used is 0.031 (probability = $0.5^N$ where N is the number of fixed loci), while the probability of doing the same using just five PINE-PCR markers is 0.0004

(probability = $[(0.75)^N(0.25)^N]*2$ where N is the number of PINE-PCR markers). If we increase the number of markers to 10, our probability of misidentification drops to $1.0x10^{-7}$. We have characterized 42 species-specific PINE markers.

### 2.4.2. Species-Specific Differences

The nature behind the species specificity of PINE markers is unclear. Numerous explanations could account for the differences that allow us to distinguish rainbow from cutthroat trout. Point mutations within the priming sites or deletions of entire interspersed nuclear elements from the genome of one species and not the other could account for some of the species-specific differences. Likewise, if the same two elements were found in the same genomic position within each species, but one species had an insertion or deletion event that changed the size of the anonymous fragment relative to the other species, another difference would arise. Another possibility arises when the origin of interspersed elements is considered. SINEs are thought to have arisen periodically throughout the evolution of salmonids (Kido et al. 1994, Takasaki et al. 1994). If a new round of SINE amplification were to have occurred after the divergence of rainbow and cutthroat trout in one species, e.g. rainbow trout but not cutthroat trout, then the presence of the new elements in rainbow trout could be used as a diagnostic marker to distinguish the two species. This data set precludes the discovery of precisely which aforementioned event is responsible for the species-specific differences we see at the nuclear DNA level. Nevertheless, the 42 diagnostic markers we have identified have proven reliable in comparisons against allozymes for the detection of hybridization.

### 2.4.3. Applications of PINE-PCR

PINE-PCR is constrained by the dominant nature of the amplified, species-specific fragments. The fragments are scored as present or absent and it is not possible to distinguish between heterozygous individuals and homozygous dominant individuals. Due to this limitation, PINE-PCR is best suited to straightforward documentation of hybridization events and species identification. The monetary cost difference between protein electrophoresis and PINE-PCR is negligible. However, the difference in cost for the population viability of a threatened trout population is appreciable to the lethal sampling required by protein electrophoresis, which could be devastating to small populations.

The effectiveness of PINE-PCR is restricted by the baseline data from which the diagnostic markers are derived. Though geographically distinct populations from across the Pacific Northwest were included in this study for each subspecies, extrapolating the results obtained with these markers to other cutthroat trout and rainbow trout populations outside of this region is not suggested. Further validation of these markers through testing in more geographically diverse populations is recommended. PINE-PCR is a powerful technique that, when used properly, can identify samples of cutthroat and rainbow trout and detect hybridization between these species. Similar application to other species complexes is limited only by the available knowledge to design appropriate primers for PINE-PCR.

## 2.5. CONCLUSSION

This study illustrates the value of PINE-PCR in identifying samples of cutthroat trout and rainbow trout and detecting hybridization between the two species in an

efficient and cost-effective manner. The method yields reproducible results that are as accurate, if not more so, than other methods of species identification such as meristic character counts and protein electrophoresis. Furthermore, PINE-PCR analysis is conducted via a non-lethal sampling protocol, which is an important consideration when addressing threatened or endangered populations.

# ANALYSIS OF THE PHYLOGENETIC RELATIONSHIP BETWEEN CUTTHROAT TROUT (*Oncorhynchus clarki*) AND RAINBOW TROUT (*Oncorhynchus mykiss*)

**Abstract:** Six combinations of primers complementary to the ends of interspersed nuclear elements were used, in conjunction with PINE-PCR, to analyze the phylogenetic relationships between five subspecies of cutthroat trout (*O. clarki* sp) and two subspecies of rainbow trout (*O. mykiss* sp). The six primer combinations amplified 440 anonymous DNA fragments. Similarity index values, based upon fragment sharing between samples, were compiled into 1000 pseudoreplicate distance matrices. UPGMA cluster analysis and principal coordinate analysis (PCoA) established phylogenetic relationships. Bootstrap values were greater than 90% at all major nodes in the UPGMA cluster analysis. The first four axes of the PCoA clearly separated the rainbow trout from the cutthroat trout and accounted for 80% of the total variance. The results strongly support the separation of rainbow trout and cutthroat trout subspecies into separate, monophyletic evolutionary lineages.

## 3.1. INTRODUCTION

Approximately 30-40 million years ago, the subfamily Salmoninae split into two lineages giving rise to five genera of fish. One branch of this division gave rise to the genera *Hucho*, *Brachymystax*, and *Salvelinus* while the other branch gave rise to the genera *Oncorhynchus* and *Salmo* (Behnke 1992). Behnke (1992) approximates that 15 million years later, *Salmo* and *Oncorhynchus* diverged into an Atlantic Ocean group (*Salmo*) and a Pacific Ocean group (*Oncorhynchus*). *Oncorhynchus* is thought to have further divided 5 million years ago into two lineages, one that gave rise to the Pacific salmon, and the other to the Pacific trout. The recent reclassification of the western North American trout species into the genus *Oncorhynchus* has allied these species closer to the Pacific salmon species than the Atlantic salmon and trout of the genus *Salmo* (Stearley and Smith 1993). Based on morphological characters, there are two major phylogenetic lines of Pacific trout within *Oncorhynchus*: the rainbow trouts (*O. mykiss* sp) and the cutthroat trouts (*O. clarki* sp) (reviewed in Behnke 1992). Furthermore, there

are 6 recognized subspecies of *O. mykiss* and 14 recognized subspecies of *O. clarki* (Behnke 1992).

Beyond these initial hypotheses, the phylogenetic relationships among the Pacific trout are uncertain. Leary et al. (1987) used protein electrophoresis to measure the genetic divergence between inland rainbow trout (*O. m. gairdneri*) and seven subspecies of cutthroat trout including Lahontan (*O. c. henshawi*), westslope (*O. c. lewisi*), Yellowstone (*O. c. bouvieri*), finespotted (*O. c.* sp), greenback (*O. c. stomias*), Colorado River (*O. c. pleuriticus*), and coastal cutthroat trout (*O. c. clarki*). Three of the cutthroat trout subspecies, Lahontan, westslope, and coastal cutthroat trout, were as similar to rainbow trout, if not more so, than they were to the other four subspecies of cutthroat trout analyzed. Reinitz (1974) found a similar relationship; westslope cutthroat trout were genetically more similar to rainbow trout than to Yellowstone cutthroat trout.

In contrast, Gyllensten and Wilson (1987) analyzed mitochondrial DNA (mtDNA) and showed that all subspecies of cutthroat trout form a distinct group from the rainbow trout subspecies. Loudenslager et al. (1986) used protein electrophoresis to show that three of the cutthroat trout subspecies, Lahontan, Yellowstone, and Colorado River cutthroat trout, cluster together indicating a distinct lineage from other western trout species, including rainbow trout. This separation between rainbow and cutthroat trout subspecies is additionally supported by the results of several karyotypic studies (Gold et al. 1977, Loudenslager and Thorgaard 1979, Thorgaard 1983) and classifications based upon the number of chromosomes for each subspecies. Coastal cutthroat trout have 68 chromosomes, westslope cutthroat trout have 66 chromosomes, and Yellowstone, Colorado River, and Lahontan cutthroat trout have 64 chromosomes. Inland rainbow

trout have 58 chromosomes, while coastal rainbow trout have from 58 to 64 chromosomes (Behnke 1992). Furthermore, subspecies of cutthroat trout have been shown to be morphologically more similar to each other than to rainbow trout (Leary et al. 1984, Leary et al. 1987).

Leary et al. (1987) present two explanations for the discordant hypotheses arising from the different data sets. Given the overlapping geographical distribution of several of the subspecies involved and the fact that trout hybrids are fertile, limited historical introgression could have slowed the rate of protein divergence between the subspecies without appreciably altering their morphological, karyological and mitochondrial evolution. This explanation is predicated upon Wright's (1978) "one migrant per generation" rule to maintain a low level of gene flow, thus preventing complete divergence. Without the retarding effect of minimal introgression, protein divergence would most likely have progressed at a rate conducive to further speciation.

Alternatively, the various rainbow and cutthroat trout subspecies might represent separate phylogenetic divergences from a common ancestor (Leary et al. 1987). In this scenario, the rainbow and cutthroat trout assemblages arose in separate speciation events and developed further into the subspecies recognized today under the influence of various environmental selective pressures.

There is no reason to discount either of the two previous explanations in favor of the other. Rather, considering the two previous explanations in tandem could account for the apparent discordance among the different phylogenetic data sets. If the rainbow and cutthroat trout species did arise from separate phylogenetic divergences, then there is no reason not to expect some low-level introgression events among the various subspecies,

as many of them are historically sympatric. These low-level introgression events would generate the present discordance evidenced by protein electrophoresis and mtDNA analysis. Unfortunately, it is this reticulate evolution that has hampered researchers from developing a clear picture of the phylogeny of rainbow and cutthroat trout subspecies.

Numerous biochemical techniques have been used to analyze the phylogenetic relationship between rainbow and cutthroat trout. Restriction fragment length polymorphisms (RFLPs) of both the nuclear and mitochondrial genomes have been used to infer the phylogenetic history of salmonids (Apostolidis et al. 1996, Hansen and Loeschcke 1996, Phillips et al. 1992, Thomas et al. 1986, Wilson et al. 1985). Likewise, sequence analysis of specific genes and genomic regions has been successful as well (Devlin 1993, McKay et al. 1996, Phillips et al. 1994, Pleyte et al. 1992, Shedlock et al. 1992). Though lethal in its sampling protocol, protein electrophoresis has been heavily utilized in phylogenetic analyses of the Salmonids (for a summary, see Utter and Allendorf 1994).

Randomly amplified polymorphic DNA (RAPD or RAPD-PCR) (Williams et al. 1990) markers have proven useful in species identification and hybrid detection (Bardacki and Skibinski 1994, Naish et al. 1995, Riesberg and Gerber 1995, Smith et al. 1996, Elo et al. 1997). They are especially appealing because they do not require lethal sampling of specimens and are relatively inexpensive. However, RAPD-PCR requires low stringency PCR conditions to facilitate annealing. Additionally, the small size of a RAPD primer, usually a decamer, coupled with lowered annealing temperatures, allows for potentially non-specific annealing throughout the genome being amplified. Furthermore, sequence heteroduplexes, generated by the annealing of differentially sized

allelic RAPD products, have been reported to form during RAPD-PCR (Riedy et al. 1992, Ayliffe et al. 1994).

Short interspersed elements (SINEs) have been utilized to characterize numerous species, including salmonids (Kido et al.1991, Murata et al. 1993, Takasaki et al. 1994, Murata et al. 1996, Greene and Seeb 1997, Hamada et al. 1997, Takasaki et al. 1997). During episodic bouts of amplification, multiple copies of a particular SINE element are generated and inserted at random into the genome via RNA intermediates (Deininger 1989, Tachida et al. 1996). Once inserted, the only means of removal of a SINE element is thought to be a recombination event (Kordis and Gubensek 1995, Cook and Tristem 1997). To date no one has been able to illustrate a case in which independent SINE insertions have occurred at the same location within distinct genomes (Takasaki et al.1997, Eyre-Walker 1999). Therefore, SINEs constitute a class of DNA markers that are very useful for phylogenetic analysis. However, due to the likely episodic nature of SINE insertion events, phylogenetic trees based upon SINEs should primarily be valued for their topology, with secondary emphasis given to their estimation of branch length and time of divergence (Cook and Tristem 1997).

### 3.1.1. Objectives

The discordance between the data sets of Leary et al. (1987) and Gyllensten and Wilson (1987) leaves the phylogenetic relationship of westslope, coastal, and Lahontan cutthroat trout to other rainbow and cutthroat trout subspecies unresolved. Therefore, the objective of this study was to use nuclear DNA markers to test if westslope, coastal, and Lahontan cutthroat trout are genetically more similar to rainbow trout versus other cutthroat trout subspecies.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Sample Collections

Eight species and subspecies of pacific trout, along with one species of pacific salmon, were analyzed in this study (Table 10). Sampled taxa included the following: coastal rainbow trout (*O. m. irideus*), inland rainbow trout (*O. m. gairdneri*), coastal cutthroat trout (*O. c. clarki*), westslope cutthroat trout (*O. c. lewisi*), Yellowstone cutthroat trout (*O. c. bouvieri*), Lahontan cutthroat trout (*O. c. henshawi*), Colorado River cutthroat trout (*O. c. pleuriticus*), Apache trout (*O. apache*), and chinook salmon (*O. tshawytscha*). Two fish were randomly selected for analysis from each of the populations sampled for each subspecies. The 32 samples used in this study represent multiple trout populations from across each subspecies' range in the western United States. Only one population of Yellowstone cutthroat trout was sampled due to the low level of genetic variation among its populations (Leary et al. 1987, Allendorf and Leary 1988). Additionally, only one population of Lahontan cutthroat trout, Colorado River, and Apache trout was analyzed due to the limited distribution of these subspecies and the small sizes of existing populations in the western United States (Carmichael et. al. 1993). Chinook salmon (*O. tshawytscha*) were included in the analysis as a representative of the pacific salmon species complex.

### 3.2.2. DNA Isolation

DNA was obtained either from tissue subsamples of caudal fin clips, approximately 1.0 $cm^2$, or tissue muscle cores from whole frozen fish. Caudal fin clips

**Table 10. Species of western North American *Oncorhynchus* analyzed for phylogenetic analysis, locations of populations sampled, and individual sample designations.**

| Species | Strain | Source (Sponsor) | Location | ID |
|---|---|---|---|---|
| *O. mykiss irideus* | Arlee | Jocko River Trout Hatchery (MTFWP) | Arlee, MT | MIJK |
| | Wild | Little Sandy River, OR | 45.426°N, 122.206°W | MILS |
| *O. mykiss gairdneri* | Shasta | Dworshak National Fish Hatchery (USFWS) | Ahsahka, ID | MGDW |
| | Wild | Wolf Creek, MT | 48.229°N, 115.292°W | MGWC |
| | Kamloops | Ennis National Fish Hatchery (USFWS) | Ennis, MT | MGEN |
| *O. clarki clarki* | Wild | Gierin Creek, WA | 48.115°N, 123.058°W | CCGC |
| | Wild | Emil Creek, OR | 45.556°N, 121.586°W | CCEC |
| | Wild | Lady Creek, OR | 45.314°N, 121.838°W | CCLC |
| *O. clarki lewisi* | Fish Lake | McCall Fish Hatchery (IDFG) | McCall, ID | CLMC |
| | Wild | Marshall Creek, MT | 46.888°N, 113.924°W | CLMR |
| | Hungry Horse | Washoe Park Trout Hatchery (MTFWP) | Anaconda, MT | CLWP |
| *O. clarki bouvieri* | McBride | Yellowstone River Trout Hatchery (MTFWP) | Big Timber, MT | CBMB |
| *O. clarki pleuriticus* | Trappers Lake | Bozeman Fish Technology Center (USFWS) | Bozeman, MT | CPBZ |
| *O. clarki henshawi* | Pyramid Lake | Captain Dave Numana Hatchery (PLPT) | Nixon, NV | CHPL |
| *O. apache* | Wild | Williams Creek National Fish Hatchery (USFWS) | White River, AZ | OAWC |
| *O. tshawytscha* | Green River | Issaquah State Salmon Hatchery (WDFW) | Issaquah, WA | OTGR |

were stored at room temperature in 95% ethanol, while whole fish were stored at -40°C until sampled. DNA was extracted from subsamples of caudal fin clips or muscle cores of frozen fish using the Puregene® kit (Gentra Systems Inc.) as per the manufacturer's instructions. The DNA concentration for all samples was estimated through visualization on agarose gels stained with ethidium bromide (Maniatis et al. 1982).

### 3.2.3. Paired Interspersed Nuclear Element PCR

In this study, the phylogenetic relationship among species of pacific trout was established by analyzing numerous anonymous DNA fragments amplified from Paired Interspersed Nuclear Element-PCR or PINE-PCR. This technique uses florescently labeled primers, complementary to the ends of short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs), to amplify the intergenic region between two nuclear elements. Thus far, five SINEs have been identified within the salmonids. Kido et al. (1991) described *Fok* I, an element specific to the genus *Salvelinus*. Pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*) share the *Sma* I element (Kido et al. 1991). Furthermore, Spruell and Thorgaard (1996) discovered that a portion of the 5′ end of *Fok* I and *Sma* I was found throughout the salmonids. *Hpa* I is found within all members of the family Salmonidae (Kido et al. 1991) as is the *Hpa* I-like *Ava* III element (Kido et al. 1994). Ginatulin and Ginatulin (1996) described another SINE element, *Pvu* II, specific to pink salmon. Additionally, Winkfein et al. (1988) identified *RSg*-1; a long interspersed nuclear element (LINE) found within rainbow trout.

Primers for this study were synthesized from the *Fok* I, *Hpa* I, and *Sma* I SINE families (Kido et al. 1991), and Jeffreys 33.6 core fragment (Jeffreys et al. 1985) (Table

11). Jeffreys' core fragment was selected for it had been shown previously to be an effective probe in fingerprinting studies of rainbow trout (Spruell et al. 1994) and sockeye salmon (Thorgaard et al. 1995). Six different primer combinations were used to amplify anonymous, nuclear DNA fragments via PINE-PCR from approximately 25.0 ng of genomic DNA in a 10 μL PCR reaction containing 1X Stoffel buffer (100 mM KCL, 100 mM Tris-HCL, pH 8.3), 5.0 mM $MgCl_2$, 0.20 mM dNTP, 0.25 μM of each primer, and 1.0 U AmpliTaq® DNA Polymerase Stoffel Fragment (Perkin-Elmer). Amplifications occurred in MJ Research PTC-100 thermal cyclers using the following profile: 1 cycle of 95°C/1.5 min, 30 cycles of 91°C/1 min, 60°C/1 min, 72°C/1.5min, 1 cycle of 72°C/1.5 min, and 12°C until termination. The resulting DNA fragments were electrophoretically separated in 4.5% denaturing polyacrylamide gels and visualized using a Hitachi FMBIO-100 fluorescent imager. Fragments were scored relative to MapMarker ladder (BioVentures) using version 6.0 of the FMBIO-100 data analysis software (Hitachi Software). The MapMarker ladder has a range of 400bp to 70bp and all identifiable fragments within that range were scored, based upon size, as unique. Each sample was scored for the presence or absence of each unique fragment. All fragments for each sample were then pooled across all primer combinations to generate a single data matrix.

### 3.2.4. Statistical Analysis

For each primer combination, the products of PINE-PCR for all 32 fish were electrophoresed on the same gel so that fragment sizes could be accurately assessed. Due to the dominant nature of PINE-PCR fragments, heterozygosity is not discernible. Therefore, the presence of a fragment on a gel is scored as a "1", while the absence is scored as a "0". With binary data such as this, the similarity index (*S*) of Nei and Li

**Table 11. Primer sequences used in PINE-PCR for phylogenetic analysis.**

| Primer | Sequence (5′ → 3′) | Reference |
|---|---|---|
| *Fok* I 5′ | CCAACTGAGCCACACGGGAC | Kido et al. 1991 |
| *Hpa* I 3′ | TGAGCTGACAAGGTACAAATC | Kido et al. 1991 |
| *Hpa* I 5′ | AACCACTAGGCTACCCTGCC | Kido et al. 1991 |
| *Sma* I 5′ | AACTGAGCTACAGAAGGACC | Kido et al. 1991 |
| 33.6 | TGGAGGAGGGCTGGAGGAGGGC | Jeffreys et al. 1985 |

(1985) reports the proportion of shared fragments between two samples A and B as $S = 2N_{AB}/(N_A + N_B)$. In this equation, $N_{AB}$ is the number of bands that sample A and sample B have in common, while $N_A$ and $N_B$ are the number of bands found in each sample respectively. The FORTRAN program RAPDPLOT (Black 1995) calculated genetic distance values ($1 - S$), based on the proportion of shared fragments, for all pairwise sample combinations and generated 1000 pseudoreplicate distance matrices. Cluster analysis was performed using the unweighted paired-group method of averaging (UPGMA; Sneath and Sokal 1973) in the NEIGHBOR program from PHYLIP 3.57c (Felsenstein 1993). A consensus dendrogram, with bootstrap support at each node, was generated from the 1000 pseudoreplicate distance matrices using the CONSENSE program from PHYLIP 3.57c (Felsenstein 1993).

A potential source of error in the estimation of phylogenetic relationships from binary data such as PINEs is the inclusion of co-migrating, yet non-homologous fragments in the data set, otherwise know as homoplasy. The ordination technique of principal coordinate analysis (PCoA) has been suggested as an alternative method of phylogenetic estimations since it is insensitive to errors of this type (Adams 1975, Adams and Demeke 1993). Therefore, the binary data matrix was also subjected to principal coordinate analysis (PCoA), following Gower (1996), using the software package MVSP (Kovach 1999). A major advantage of PCoA is that PCoA searches for similarities between cases while Principal Components Analysis (PCA) searches for patterns between variables. PCoA analyses a matrix of distances between cases while PCA reduces variable dimensionality by an eigenanalysis of a correlation or covariance matrix.

Furthermore, any distance metric can be used, in this case $1 - S$, based upon the similarity index of Nei and Li (1985).

## 3.3. RESULTS

The six combinations of primers used in PINE-PCR amplified from 49 to 103 anonymous DNA fragments per reaction, for a total of 440 fragments (Table 12). Four hundred and thirty three of the 440 fragments were polymorphic (98.4%).

Table 13 shows the averaged genetic distance ($1 - S$) between all sampled species on the lower left-hand matrix, while the upper right-hand matrix shows the number of pairwise differences, i.e. total number of fragment presences and absences, between two subspecies. The greatest distance is between inland rainbow trout and chinook salmon, while the smallest genetic distance is between Yellowstone cutthroat trout and Colorado River cutthroat trout. See Appendix C for all pairwise genetic distances based upon the proportion of shared fragments.

### 3.2.1 Cluster Analysis

Using cluster analysis, both rainbow and cutthroat trout are delineated from chinook salmon (Figure 2). A clear distinction between the rainbow and cutthroat trout lineages is apparent and has 100% bootstrap support (Figure 2). The Apache trout are more closely related to rainbow trout as indicated by their position as a sister group to the rainbow trout lineage. Furthermore, within the rainbow trout lineage, inland rainbow trout and coastal rainbow trout cluster separately as two distinct groups with 100% bootstrap support (Figure 2). Bootstrap support of interior rainbow trout was

**Table 12. Primer combinations and total number of fragments amplified across sampled species.**

| Primer Combination | Number of Fragments |
|---|---|
| *Hpa* I 5'/*Sma* I 5' | 66 |
| *Fok* I 5'/*33.6* | 48 |
| *Fok* I 5'/*Sma* I 5' | 54 |
| *Fok* I 5'/*Hpa* I 3' | 74 |
| *Hpa* I 5'/*33.6* | 95 |
| *Hpa* I 3'/*Hpa* I 5' | 103 |
| **Total** | **440** |

**Table 13. Averaged genetic distances (1 – *S*) across nine species of the genus *Oncorhynchus* in lower left-hand matrix and total number of pairwise differences between two subspecies in upper right-hand matrix.**

| Species | *Omi* | *Omg* | *Occ* | *Ocl* | *Ocb* | *Ocp* | *Och* | *Oa* | *Ot* |
|---|---|---|---|---|---|---|---|---|---|
| *Omi* | - | 97 | 148 | 143 | 148 | 136 | 148 | 112 | 179 |
| *Omg* | 0.315 | - | 149 | 162 | 165 | 153 | 165 | 127 | 196 |
| *Occ* | 0.564 | 0.556 | - | 127 | 118 | 116 | 132 | 138 | 173 |
| *Ocl* | 0.580 | 0.611 | 0.477 | - | 117 | 111 | 127 | 125 | 160 |
| *Ocb* | 0.656 | 0.678 | 0.498 | 0.557 | - | 52 | 100 | 122 | 155 |
| *Ocp* | 0.604 | 0.634 | 0.513 | 0.528 | 0.307 | - | 84 | 108 | 145 |
| *Och* | 0.609 | 0.637 | 0.537 | 0.549 | 0.498 | 0.447 | - | 112 | 165 |
| *Oa* | 0.467 | 0.516 | 0.547 | 0.567 | 0.642 | 0.603 | 0.533 | - | 145 |
| *Ot* | 0.791 | 0.817 | 0.737 | 0.746 | 0.813 | 0.805 | 0.790 | 0.753 | - |

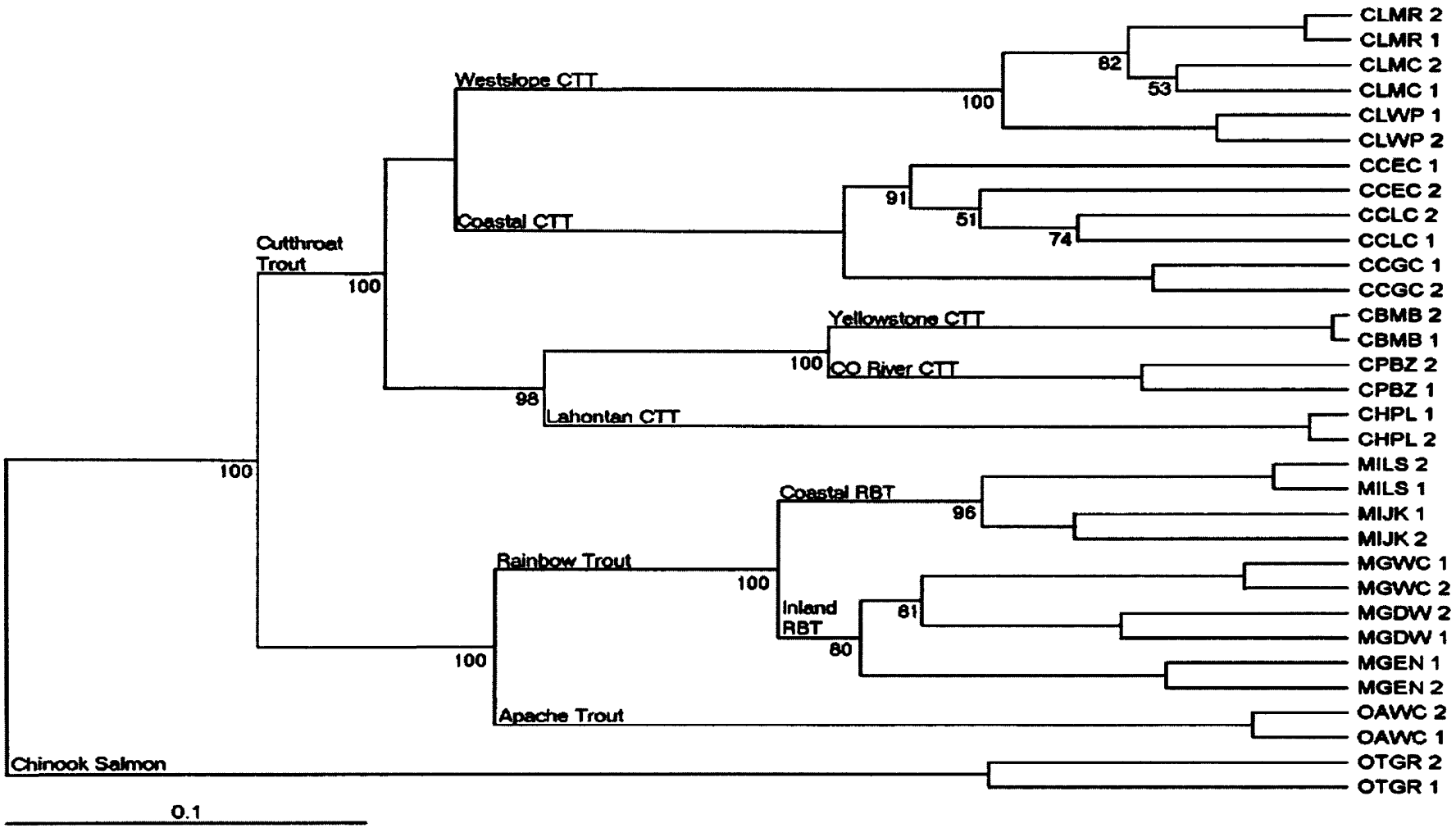


**Figure 2. UPGMA dendrogram of 32 fish derived from 440 PINE fragments. Branch lengths are proportional to 1 - *S*. Percent bootstrap support is indicated below each branch. Unless noted otherwise, bootstrap support at terminal nodes is 100%. See Table 10 to identify fish based upon terminal node designations.**

approximately 80%.

The cutthroat trout lineage resolves into two distinct groupings with 100% bootstrap support (Figure 2). The first cutthroat trout group is composed of a Yellowstone plus Colorado River cutthroat trout group and a Lahontan cutthroat trout sister group, segregated by 98% bootstrap support (Figure 2). The second cutthroat group further separates into distinct subgroups of westslope cutthroat trout and coastal cutthroat trout with 91% bootstrap support (Figure 2). Bootstrap support at nodes separating populations of coastal cutthroat trout and populations of westslope cutthroat trout ranged from 50% to 100% (Figure 2). Though the sample sizes are small, it is worth noting that greater differentiation of populations is apparent among the samples of westslope cutthroat trout than within the populations of coastal cutthroat trout. This is not surprising given the high genetic divergence among westslope cutthroat trout populations (Allendorf and Leary 1988).

### 3.2.2. Principal Coordinates Analysis

Principal coordinates analysis corroborates the results from cluster analysis by clearly separating the samples into species-based clusters. Within the first four axes, 80% of the variance was explained (Table 14). Axis 1 explained 39% of the variance and separated the samples into three separate species complexes: a rainbow trout group, an apache trout group, and a cutthroat trout group (Figure 3 and Table 15). Axis 2 explained 18% of the variance and delineated three different groups: a group composed of Yellowstone cutthroat, Lahontan cutthroat, and Colorado River cutthroat trout; a group composed of coastal cutthroat trout, Apache trout, and rainbow trout; and a group of westslope cutthroat trout (Figure 3 and Table 15).

**Table 14. The eigenvalue and percent variance explained by the first 15 axes.**

| | Eigenvalues | Percent Variance Explained | Cumulative Percent Variance |
|---|---|---|---|
| Axis 1 | 1.52 | 39 | 39 |
| Axis 2 | 0.70 | 18 | 57 |
| Axis 3 | 0.53 | 14 | 71 |
| Axis 4 | 0.37 | 9 | 80 |
| Axis 5 | 0.22 | 6 | 86 |
| Axis 6 | 0.15 | 4 | 90 |
| Axis 7 | 0.12 | 3 | 93 |
| Axis 8 | 0.10 | 2 | 95 |
| Axis 9 | 0.06 | 1 | 96 |
| Axis 10 | 0.05 | 1 | 97 |
| Axis 11 | 0.04 | 1 | 98 |
| Axis 12 | 0.03 | 1 | 99 |
| Axis 13 | 0.03 | 1 | 100 |

**Table 15. PCoA scores for the first four axes averaged across eight subspecies of Pacific trout.**

| Species Group | Axis 1 | Axis 2 | Axis 3 | Axis 4 |
|---|---|---|---|---|
| Coastal RBT | 0.28 | -0.02 | 0.06 | 0.02 |
| Inland RBT | 0.31 | 0.00 | -0.02 | 0.06 |
| Coastal CTT | -0.13 | 0.05 | -0.24 | -0.03 |
| Westslope CTT | -0.20 | -0.26 | 0.06 | 0.03 |
| Yellowstone CTT | -0.26 | 0.25 | 0.08 | 0.14 |
| Colorado CTT | -0.20 | 0.21 | 0.15 | 0.11 |
| Lahontan CTT | -0.15 | 0.17 | 0.18 | -0.17 |
| Apache Trout | 0.10 | 0.01 | 0.08 | -0.32 |

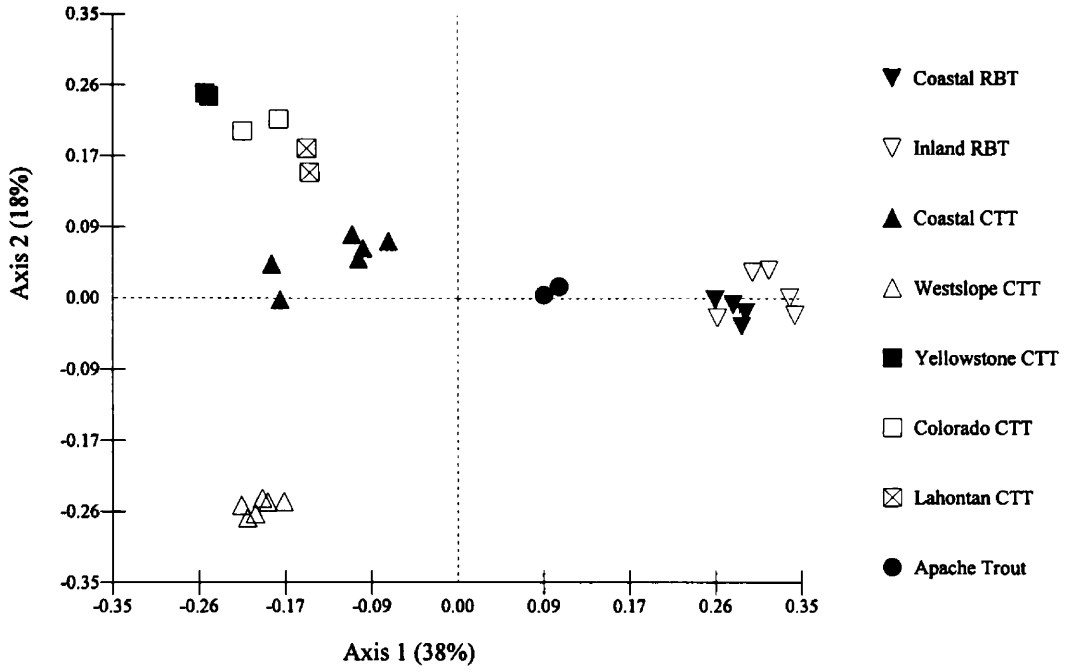


**Figure 3. Plot of axes 1 and 2 for the principal coordinate analysis (PCoA) of 8 Pacific trout species using 440 PINE fragments. Axis 1 explained 39% of the variance and delineated three groups: a rainbow trout group, an apache trout group, and a cutthroat trout group. Axis 2 explained 18% of the variance and separated the samples into three groups: a group composed of Yellowstone cutthroat, Lahontan cutthroat, and Colorado River cutthroat trout; a group composed of coastal cutthroat trout, Apache trout, and rainbow trout; and a group of westslope cutthroat trout.**

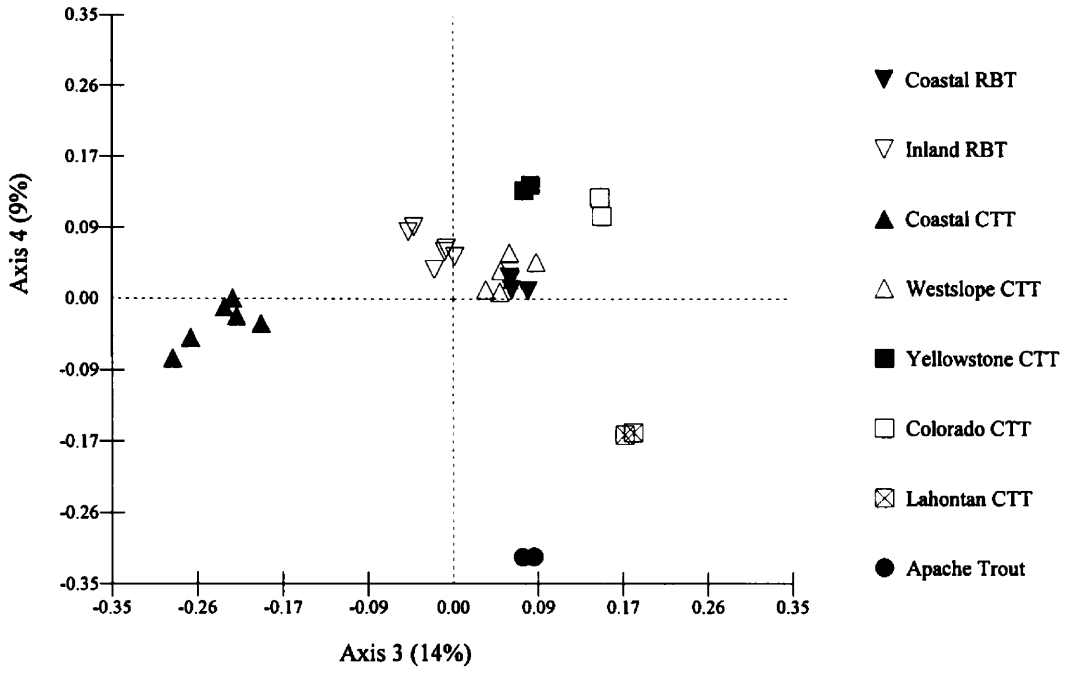


**Figure 4. Plot of axes 3 and 4 for the principal coordinate analysis (PCoA) of 8 Pacific trout species using 440 PINE fragments. Axis 3 explains 14% of the variation and separates coastal cutthroat trout from the other western trout subspecies. Axis 4 explains 9% of the variation and delineates both Apache trout and Lahontan cutthroat trout from the other 6 western trout subspecies.**

Axis 3 explained 14% of the variation and separated coastal cutthroat trout from the other western trout subspecies (Figure 4 and Table 15). Axis 4 explained 9% of the variation and delineated both Apache trout and Lahontan cutthroat trout from the other 6 western trout subspecies (Figure 4 and Table 15).

## 3.4. DISCUSSION

The high-resolution, multi-fragment patterns produced by different primer combinations in PINE-PCR are easily repeatable due to stringent PCR conditions. Additionally, the primer sites for PINE-PCR are known segments of DNA that have shown strong resolving power in phylogenetic applications (Kido et al. 1991, Murata et al. 1993, Murata et al. 1996). The patterns produced by PINE-PCR were analyzed to infer the phylogenetic relationship between the rainbow and cutthroat trout subspecies. The relationships among the various subspecies were clearly delineated by ordination through principal coordinates analysis, and by clustering, with significant bootstrap support at all major nodes. These high bootstrap values increase our confidence in the accuracy of the results (Hillis & Bull 1993).

### 3.4.1. Cutthroat and Rainbow Trout Phylogenetics

My results negate the suggestion that westslope, coastal, and Lahontan cutthroat trout are as similar to rainbow trout, if not more so, than they are to other cutthroat trout subspecies (Leary et al. 1987). Rather, the theory that the rainbow trout and cutthroat trout species complexes represent separate, monophyletic origins is supported (Behnke 1992, Phillips et al. 1992).

The results present a phylogenetic relationship among rainbow and cutthroat trout subspecies that is in line with the overall consensus view of the phylogenetic relationship for all of *Oncorhynchus* put forth by Utter and Allendorf (1994). In this view, the Pacific salmon, including chinook salmon, are the next closest relatives, though in a separate lineage, to rainbow and cutthroat trout (see also Smith and Stearly 1989, Thomas et al. 1986, Philips and Pleyete 1991, Phillips et al. 1992, McKay et al 1996, Domanico et al. 1997, Takasaki et al. 1997, Oakley et al. 1999). This differs from Stearly and Smith (1993) who suggest that rainbow trout are the sister group of Pacific salmon, not cutthroat trout, based upon parsimony analysis of 119 morphological characters.

The relationship between Lahontan, Colorado River, and Yellowstone cutthroat trout is consistent with the study by Loudenslager et al. (1986). However, the placement of the Lahontan cutthroat trout differs from Utter and Allendorf (1994). In their study, Lahontan cutthroat trout were a sister group to coastal cutthroat trout, the two of which together were a sister group to westslope cutthroat trout.

We find no evidence that coastal cutthroat trout are a sister group to chinook salmon, which in turn, are a sister group to rainbow and Apache trout as suggested by Nielsen et al. (1998). However, these results do support the relationship between Apache and rainbow trout subspecies, i.e. they are sister groups, congruent with Nielsen et al. (1998).

### 3.4.2 Homoplasy

The PINE-PCR fragments used in this analysis are assumed homologous. However, a confounding factor in a study such as this is homoplasy, or the presence of co-migrating, yet non-homologous bands. Even a minimal level of homoplasy in the data

set may significantly affect the resultant phylogeny (Swofford and Olsen 1990). A limited number of studies have addressed the issue of homology in RAPD fragments, which are very similar to PINEs, and found the assumption to be valid (Halward et al. 1992, Wilkie et al. 1993). However others studies have found the assumption to be false (Smith et al. 1994, Thormann et al. 1994, and Riesberg 1996). Testing all 440 fragments for homology via sequencing or restriction digestion is prohibited by time and expense constraints. Additionally, verifying sequence homology does not guarantee positional homology. It is possible for co-migrating fragments to have arisen from multiple loci dispersed throughout the genome (Williams and St. Clair 1993), especially in a tetraploid organism such as a salmonid. Nonetheless, the sheer number of informative markers (433), coupled with the random distribution of SINE elements throughout the genome of *Oncorhynchus*, effectively drowns out any errors of homology as noise. This noise in the data set is subsequently accounted for when analyzed by principal coordinates analysis, which is robust to errors from homplasy (Adams 1975, Adams and Demeke 1993).

### 3.4.3. Species-Specificity of PINEs

Numerous mechanisms could affect the reproducibility of the PINE markers analyzed in this study. Insertion and deletion events could alter the size of a fragment to the point that it is no longer recognizable when co-migrating with a homologous fragment. Massive insertion events could prevent PCR from completing amplification between two interspersed elements. Furthermore, deletions of entire interspersed elements or point mutations within the priming sites could also disrupt amplification. Likewise, inversion events at a specific locus of either interspersed element involved with amplification would prevent fragment amplification. Subsequent rounds of SINE

amplification proceeding from an initial divergence from a common ancestor would also yield detectable differences. Over evolutionary time, mechanisms such as those described above could have led to the phylogenetic differences we see today. However, we are prevented from knowing the exact mechanism due to the dominant nature of the DNA fragments amplified from PINE-PCR. Regardless, PINE-PCR is a powerful for analyzing the relationships of closely related species.

## 3.5. CONCLUSION

Six PINE-PCR reactions amplified 440 anonymous, DNA fragments, 98.4% of which were informative. This method yields reproducible results that are, for the most part, concordant with other published views of phylogenetic relationship of the genus *Oncorhynchus*, principally that the rainbow trout and cutthroat trout complexes have separate monophyletic origins. Furthermore, PINE-PCR analysis is conducted via a non-lethal sampling protocol, which is an important consideration when analyzing threatened, endangered, or extinct taxa, for data can still be acquired from museum specimens.

## NOTES REGARDING APPENDICES A AND B

Species classifications are as follows: *Om* - rainbow trout; *Omi* - coastal rainbow trout; *Omg* - inland rainbow trout; *Oc* - cutthroat trout; *Occ* - coastal cutthroat trout; *Ocl* - westslope cutthroat trout; BCC - backcrossed to cutthroat trout BCR - backcrossed to rainbow trout; $F_1$ - $F_1$ hybrid; $F_2$ - $F_2$ hybrid; and $PF_2$ - post-$F_2$ hybrid.

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**Appendix A. Data from 5 allozyme loci and 15 PINE-PCR markers for 92 fish sampled in John Day River basin, OR. Sampled populations were lower Dixie Creek (LDXC), upper Dixie Creek (UDXC), and Roberts Creek (RBTC). The following were omitted from the analysis due to poor DNA extractions: LDXC-11, LDXC-24, and RBTC-21. For each of the five allozyme loci scored, alleles 100 and/or 76 are indicative of rainbow trout. All other allelic values are indicative of cutthroat trout. See Table 4 to determine species specificity for each fragment. For each PINE-PCR marker, fragments are scored with a '1' indicating presence, and a '0' indicating absence. Discrepancies in classification between allozymes and PINE-PCR in are indicated by BOLD text for the sample, i.e. LDXC-04.**

| Sample | sAAT-1 | CK-A2 | GPI-B2 | SDH-2 | LDH-B2 | Species | 5H5S 138 | 5H5S 95 | 5F53 134 | 3H5H 70 | 3H5H 66 | 5F5S 247 | 5F53 170 | 5F53 142 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 3H5H 69 | 5F53 127 | 5F53 168 | 3H5H 153 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LDXC-01 | 100 | 100 | 100 | 100 | 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-02 | 100 | 100 | 100 | 100 | 100 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-03 | 100 | 100 | 100 | 100 | 100 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| **LDXC-04** | **100** | **100** | **100** | **100** | **76** | ***Om*** | **1** | **1** | **1** | **1** | **1** | **-** | **0** | **0** | **-** | **-** | **0** | **0** | **0** | **1** | **1** | **BCR** |
| **LDXC-05** | **200 100** | **100** | **100** | **100 40** | **100** | **BCR** | **0** | **0** | **0** | **0** | **0** | **-** | **0** | **0** | **-** | **-** | **1** | **1** | **1** | **1** | **1** | ***Ocl*** |
| LDXC-06 | 100 | 100 | 100 | 100 | 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-07 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-08 | 100 | 100 | 100 | 100 | 100 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-09 | 100 | 100 | 100 | 100 | 100 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-10 | 100 | 100 | 100 | 100 | 100 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-12 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-13 | 100 | 100 | 100 | 100 | 100 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-14 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-15 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| **LDXC-16** | **200** | **84** | **100 92** | **40** | **100** | **BCC** | **0** | **0** | **0** | **0** | **0** | **-** | **0** | **0** | **-** | **-** | **1** | **1** | **1** | **1** | **1** | ***Ocl*** |
| **LDXC-17** | **100** | **100** | **100** | **100** | **76** | ***Om*** | **1** | **1** | **1** | **1** | **1** | **-** | **0** | **0** | **-** | **-** | **0** | **1** | **0** | **0** | **0** | **BCR** |

**Appendix A. Continued**

| Sample | sAAT-1 | CK-A2 | GPI-B2 | SDH-2 | LDH-B2 | Species | 5H5S 138 | 5H5S 95 | 5F53 134 | 3H5H 70 | 3H5H 66 | 5F5S 247 | 5F53 170 | 5F53 142 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 3H5H 69 | 5F53 127 | 5F53 168 | 3H5H 153 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LDXC-18 | 100 | 100 | 100 | 100 | 76 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| **LDXC-19** | **100** | **100** | **100** | **100** | **100** | ***Om*** | **0** | **0** | **1** | **1** | **1** | **-** | **0** | **0** | **-** | **-** | **0** | **0** | **0** | **0** | **0** | **PF2** |
| LDXC-20 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| LDXC-21 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| **LDXC-22** | **100** | **100** | **100** | **100** | **100** | ***Om*** | **0** | **1** | **1** | **1** | **1** | **-** | **0** | **0** | **-** | **-** | **0** | **0** | **0** | **0** | **0** | **PF2** |
| LDXC-23 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-25 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-26 | 200 | 84 | 92 | 40 | 100 | *Ocl* | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | - | - | 1 | 1 | 1 | 1 | 1 | *Ocl* |
| LDXC-27 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| LDXC-28 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| LDXC-29 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| LDXC-30 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| LDXC-31 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-32 | 200 | 84 | 92 | 40 | 100 | *Ocl* | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | - | - | 1 | 1 | 1 | 1 | 1 | *Ocl* |
| LDXC-33 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| LDXC-34 | 100 | 100 | 100 | 100 | 100 | *Om* | 1 | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 | *Omg* |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| **LDXC-35** | **100** | **100** | **100** | **100** | **100** | ***Om*** | **1** | **1** | **0** | **1** | **1** | **-** | **0** | **0** | **-** | **-** | **0** | **0** | **0** | **0** | **0** | **PF2** |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| UDXC-01 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-02 | 100 | 100 | 100 | 100 | 76 | *Om* | - | - | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | *Om* |
| UDXC-03 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-04 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-05 | 100 | 100 | 100 | 100 | 76 | *Om* | - | - | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | *Om* |
| **UDXC-06** | **200** | **100** | **100** | **100** | **100** | **PF2** | **-** | **-** | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **-** | **0** | **0** | **0** | **1** | **BCR** |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| UDXC-07 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-08 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-09 | - | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-10 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-11 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-12 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **UDXC-13** | **200** | **84** | **92** | **40** | **100** | ***Ocl*** | **-** | **-** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **-** | **1** | **1** | **1** | **1** | **BCC** |
| UDXC-14 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-15 | 100 | 100 | 100 | 100 | 100 | *Om* | - | - | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | *Om* |
| | | | | | 76 | | | | | | | | | | | | | | | | | |
| UDXC-16 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |

**Appendix A. Continued**

| Sample | sAAT-1 | CK-A2 | GPI-B2 | SDH-2 | LDH-B2 | Species | 5H5S 138 | 5H5S 95 | 5F53 134 | 3H5H 70 | 3H5H 66 | 5F5S 247 | 5F53 170 | 5F53 142 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 3H5H 69 | 5F53 127 | 5F53 168 | 3H5H 153 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UDXC-17 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-18 | 100 | 100 | 100 | - | 76 | *Om* | - | - | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | *Om* |
| **UDXC-19** | **100** | **100** | **100 92** | **100 40** | **100 76** | **BCR** | - | - | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | - | **0** | **0** | **0** | **0** | ***Om*** |
| UDXC-20 | 200 | 84 | 100 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-21 | - | 84 | 100 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **UDXC-22** | - | **100** | **100 92** | **100** | **76** | **BCR** | - | - | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | - | **0** | **0** | **0** | **0** | ***Om*** |
| UDXC-23 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-24 | 100 | 100 | 100 | 100 | 100 76 | *Om* | - | - | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | *Om* |
| UDXC-25 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **UDXC-26** | **200** | **84** | **92** | **40** | **100** | ***Ocl*** | - | - | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | - | **1** | **1** | **1** | **1** | **BCC** |
| UDXC-27 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-28 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| UDXC-29 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-01 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-02 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-03 | 200 | 84 | 100 92 | 40 | 100 76 | **BCC** | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | ***Ocl*** |
| RBTC-04 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **RBTC-05** | **200** | **84** | **92** | **40** | **100** | ***Ocl*** | - | - | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **1** | - | **1** | **0** | **0** | **1** | **BCR** |
| RBTC-06 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-07 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **RBTC-08** | **100** | **100** | **92** | - | **100** | **PF2** | - | - | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | - | **1** | **1** | **1** | **1** | ***Ocl*** |
| RBTC-09 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-10 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-11 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-12 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-13 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **RBTC-14** | **200** | **84** | **92** | **40** | **100** | ***Ocl*** | - | - | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **1** | - | **1** | **0** | **1** | **1** | **PF2** |
| RBTC-15 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-16 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-17 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **RBTC-18** | **200** | **84** | **92** | **40** | **100** | ***Ocl*** | - | - | **0** | **1** | **1** | **0** | **0** | **0** | **0** | **1** | - | **1** | **1** | **1** | **1** | **BCC** |
| RBTC-19 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-20 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-22 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **RBTC-23** | **200** | **84** | **92** | **40** | **100** | ***Ocl*** | - | - | **1** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | - | **1** | **0** | **0** | **1** | **PF2** |
| RBTC-24 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-25 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |

**Appendix A. Continued**

| Sample | sAAT-1 | CK-A2 | GPI-B2 | SDH-2 | LDH-B2 | Species | 5H5S 138 | 5H5S 95 | 5F53 134 | 3H5H 70 | 3H5H 66 | 5F5S 247 | 5F53 170 | 5F53 142 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 3H5H 69 | 5F53 127 | 5F53 168 | 3H5H 153 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RBTC-26 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-27 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| **RBTC-28** | **200** | **84** | **92** | **40** | **100** | ***Ocl*** | - | - | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | - | **1** | **1** | **1** | **1** | **BCC** |
| RBTC-29 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-30 | 200 | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |
| RBTC-31 | - | 84 | 92 | 40 | 100 | *Ocl* | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 1 | 1 | 1 | *Ocl* |

**Appendix B. PINE-PCR data for 291 fish from five creeks in the Hood River basin and four creeks in the Lower Columbia River Gorge. Creeks are as follows: NFGC – North Fork of Green Point Creek; RMRK – Rimrock Creek; WFHD- West Fork of Hood River; LDOG – lower Dog River; UDOG – upper Dog River; ONEO – Oneota Creek, LMLT – lower Multnomah Creek, UMLT – upper Multnomah Creek, and BRVE – Bridal Vail Falls. See Table 4 to determine species specificity for each diagnostic fragment. For each PINE-PCR marker, fragments are scored with a '1' indicating presence, a '0' indicating absence, or a '-' indicating that fragment was not scored for that sample.**

| Sample | 5H53 170 | 5H5S 138 | 5H53 395 | 5H53 330 | 5H53 280 | 5F53 134 | 5H5S 95 | 3H5H 70 | 3H5H 66 | 5H53 248 | 5F53 170 | 5F53 142 | 5H53 130 | 5H53 126 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 5H53 110 | 5F5S 247 | 5F53 168 | 3H5H 153 | 5H53 148 | 3H5H 69 | 5F53 127 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NFGC-01 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-02 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-03 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-04 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-05 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-06 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-07 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-08 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-09 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-10 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-11 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-12 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-13 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-14 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-15 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-16 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-17 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-18 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-19 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-20 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-21 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-22 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-23 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-24 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-25 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-26 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-27 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-28 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| NFGC-29 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 1 | 0 | - | 0 | 1 | $PF_2$ |
| NFGC-30 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omg* |
| RMRK-01 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | - | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-02 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-03 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-04 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-05 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-06 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |

**Appendix B. Continued**

| Sample | 5H53 170 | 5H5S 138 | 5H53 395 | 5H53 330 | 5H53 280 | 5F53 134 | 5H5S 95 | 3H5H 70 | 3H5H 66 | 5H53 248 | 5F53 170 | 5F53 142 | 5H53 130 | 5H53 126 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 5H53 110 | 5F5S 247 | 5F53 168 | 3H5H 153 | 5H53 148 | 3H5H 69 | 5F53 127 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RMRK-07 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-08 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | - | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-09 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | - | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-10 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-11 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-12 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-13 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-14 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-15 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-16 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-17 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-18 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-19 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| RMRK-20 | 0 | - | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | - | 0 | 1 | 1 | 1 | 1 | 1 | 1 | *Occ* |
| WFHD-01 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omi* |
| WFHD-02 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| WFHD-03 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| WFHD-04 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| WFHD-05 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omi* |
| WFHD-06 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omi* |
| WFHD-07 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| WFHD-08 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Omi* |
| WFHD-09 | - | 1 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| LDOG-01 | - | - | - | - | - | 1 | - | 1 | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| LDOG-02 | - | - | - | - | - | 0 | - | 0 | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 1 | 1 | - | 1 | 1 | BCC |
| LDOG-03 | - | - | - | - | - | 1 | - | 1 | 1 | - | 0 | - | - | - | - | - | - | - | - | - | 0 | - | 0 | 0 | *Om* |
| LDOG-04 | - | - | - | - | - | 0 | - | 0 | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 1 | 1 | - | 1 | 1 | BCC |
| LDOG-05 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | - | - | - | - | - | 1 | 1 | - | 1 | 1 | *Oc* |
| LDOG-06 | - | - | - | - | - | 1 | - | 1 | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| LDOG-07 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | - | - | - | - | - | 1 | 1 | - | 1 | 1 | *Oc* |
| LDOG-08 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| LDOG-09 | - | - | - | - | - | - | - | 1 | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| LDOG-10 | - | - | - | - | - | 1 | - | 0 | 0 | - | 0 | 0 | - | - | - | - | - | - | - | 1 | 1 | - | 1 | 1 | BCC |
| LDOG-11 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| LDOG-12 | - | - | - | - | - | 1 | - | 1 | 0 | - | 0 | 0 | - | - | - | - | - | - | - | 1 | 1 | - | 0 | 1 | $F_2$ |
| LDOG-13 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | - | - | - | - | - | 1 | 1 | - | 1 | 1 | *Oc* |
| LDOG-14 | - | - | - | - | - | 1 | - | 1 | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| LDOG-15 | - | - | - | - | - | 1 | - | 1 | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| LDOG-16 | - | - | - | - | - | - | - | 1 | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| LDOG-17 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | - | - | - | - | - | 1 | 1 | - | 1 | 1 | *Oc* |
| LDOG-18 | - | - | - | - | - | 1 | - | - | 1 | - | 0 | 0 | - | - | - | - | - | - | - | 0 | 0 | - | 0 | 0 | *Om* |
| UDOG-01 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-02 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-03 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-04 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |

**Appendix B. Continued**

| Sample | 5H53 170 | 5H5S 138 | 5H53 395 | 5H53 330 | 5H53 280 | 5F53 134 | 5H5S 95 | 3H5H 70 | 3H5H 66 | 5H53 248 | 5F53 170 | 5F53 142 | 5H53 130 | 5H53 126 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 5H53 110 | 5F5S 247 | 5F53 168 | 3H5H 153 | 5H53 148 | 3H5H 69 | 5F53 127 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UDOG-05 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-06 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-07 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-08 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-09 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-10 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-11 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-12 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-13 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-14 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-15 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-16 | - | - | - | - | - | - | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-17 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-18 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-19 | - | - | - | - | - | 0 | - | 1 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | BCC |
| UDOG-20 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-21 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-22 | - | - | - | - | - | 0 | - | 0 | 0 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | *Occ* |
| UDOG-23 | - | - | - | - | - | 0 | - | 0 | 1 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | BCC |
| UDOG-24 | - | - | - | - | - | 0 | - | 0 | 1 | - | 0 | 0 | - | - | 0 | 0 | - | - | 1 | 1 | 1 | - | 1 | 1 | BCC |
| ONEO-01 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-02 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-03 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-04 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-05 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-06 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-07 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-08 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-09 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-10 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-11 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-12 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-13 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-14 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-15 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-16 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-17 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-18 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-19 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-20 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-21 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-22 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-23 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| ONEO-24 | - | 0 | - | - | - | 1 | 1 | 1 | 1 | - | 0 | 0 | - | - | - | - | 0 | - | - | - | 0 | - | 0 | - | *Omi* |
| LMLT-01 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 1 | - | *Oc* |

**Appendix B. Continued**

| Sample | 5H53 170 | 5H5S 138 | 5H53 395 | 5H53 330 | 5H53 280 | 5F53 134 | 5H5S 95 | 3H5H 70 | 3H5H 66 | 5H53 248 | 5F53 170 | 5F53 142 | 5H53 130 | 5H53 126 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 5H53 110 | 5F5S 247 | 5F53 168 | 3H5H 153 | 5H53 148 | 3H5H 69 | 5F53 127 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LMLT-02 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 0 | - | *Om* |
| LMLT-03 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 1 | - | BCR |
| LMLT-04 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 1 | - | $F_1$ |
| LMLT-05 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 1 | - | *Oc* |
| LMLT-06 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 0 | - | BCR |
| LMLT-07 | - | - | - | - | - | - | - | 0 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 1 | - | BCC |
| LMLT-08 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 1 | - | *Oc* |
| LMLT-09 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 1 | - | BCR |
| LMLT-10 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 0 | - | 1 | - | BCR |
| LMLT-11 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 0 | - | *Om* |
| LMLT-12 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 1 | - | BCR |
| LMLT-13 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 0 | - | *Om* |
| LMLT-14 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 0 | - | *Om* |
| LMLT-15 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 0 | - | *Om* |
| LMLT-16 | - | - | - | - | - | - | - | 0 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 1 | - | BCC |
| LMLT-17 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 0 | - | BCR |
| LMLT-18 | - | - | - | - | - | - | - | 1 | 0 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 1 | - | BCC |
| LMLT-19 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 1 | - | 0 | - | BCR |
| LMLT-20 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 0 | - | BCR |
| LMLT-21 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 0 | - | *Om* |
| LMLT-22 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 0 | - | *Om* |
| LMLT-23 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 0 | - | BCR |
| LMLT-24 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 1 | - | 0 | - | BCR |
| LMLT-25 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 0 | - | BCR |
| LMLT-26 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 0 | - | 0 | - | *Om* |
| LMLT-27 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 0 | - | 0 | - | BCR |
| LMLT-28 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 0 | - | 1 | - | 0 | - | BCR |
| LMLT-29 | - | - | - | - | - | - | - | 1 | 1 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 0 | - | BCR |
| LMLT-30 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | 0 | 0 | - | - | 1 | - | 1 | - | 1 | - | *Oc* |
| UMLT-01 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-02 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-03 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 0 | - | 1 | - | $PF_2$ |
| UMLT-04 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-05 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-06 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 0 | - | 1 | - | $PF_2$ |
| UMLT-07 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-08 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-09 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-10 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-11 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 0 | - | 1 | - | $PF_2$ |
| UMLT-12 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-13 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-14 | - | - | - | - | - | - | - | 1 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | BCC |
| UMLT-15 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-16 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |

**Appendix B. Continued**

| Sample | 5H53 170 | 5H5S 138 | 5H53 395 | 5H53 330 | 5H53 280 | 5F53 134 | 5H5S 95 | 3H5H 70 | 3H5H 66 | 5H53 248 | 5F53 170 | 5F53 142 | 5H53 130 | 5H53 126 | 5F5S 110 | 5F5S 249 | 5H5S 144 | 5H53 110 | 5F5S 247 | 5F53 168 | 3H5H 153 | 5H53 148 | 3H5H 69 | 5F53 127 | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UMLT-17 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-18 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-19 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-20 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-21 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-22 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-23 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-24 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-25 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 0 | - | 1 | - | PF2 |
| UMLT-26 | - | - | - | - | - | - | - | 1 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | BCC |
| UMLT-27 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| UMLT-28 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 0 | - | 1 | - | $PF_2$ |
| UMLT-29 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-01 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-02 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-03 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-04 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-05 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-06 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-07 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-08 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-09 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-10 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-11 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-12 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-13 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-14 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |
| BRVE-15 | - | - | - | - | - | - | - | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | *Oc* |

**Appendix C. Summary data matrix of genetic distance values (1 - *S*) calculated for 32 fish. Values were derived using RAPDPLOT (Black 1995) which calculates 1 - *S* assuming $S = 2N_{AB}/[N_A + N_B]$, therefore, the smaller the value in the matrix, the greater the similarity between two fish. See Table 10 for sample species designations.**

| SAMPLE | MIJR1 | MIJR2 | MILS1 | MILS2 | MGDW1 | MGDW2 | MGWC1 | MGWC2 | MGEN1 | MGEN2 | CCGC1 | CCGC2 | CCEC1 | CCEC2 | CCLC1 | CCLC2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MIJK1 | 0.000 | | | | | | | | | | | | | | | |
| MIJk2 | 0.140 | 0.000 | | | | | | | | | | | | | | |
| MILS1 | 0.233 | 0.219 | 0.000 | | | | | | | | | | | | | |
| MILS2 | 0.215 | 0.211 | 0.045 | 0.000 | | | | | | | | | | | | |
| MGDW1 | 0.301 | 0.307 | 0.277 | 0.259 | 0.000 | | | | | | | | | | | |
| MGDW2 | 0.297 | 0.294 | 0.251 | 0.243 | 0.140 | 0.000 | | | | | | | | | | |
| MGWC1 | 0.354 | 0.330 | 0.311 | 0.326 | 0.224 | 0.229 | 0.000 | | | | | | | | | |
| MGWC2 | 0.371 | 0.368 | 0.352 | 0.345 | 0.240 | 0.245 | 0.043 | 0.000 | | | | | | | | |
| MGEN1 | 0.330 | 0.347 | 0.286 | 0.279 | 0.273 | 0.237 | 0.295 | 0.280 | 0.000 | | | | | | | |
| MGEN2 | 0.360 | 0.357 | 0.308 | 0.300 | 0.303 | 0.267 | 0.283 | 0.279 | 0.079 | 0.000 | | | | | | |
| CCGC1 | 0.542 | 0.508 | 0.525 | 0.520 | 0.516 | 0.516 | 0.505 | 0.506 | 0.522 | 0.514 | 0.000 | | | | | |
| CCGC2 | 0.568 | 0.534 | 0.554 | 0.549 | 0.532 | 0.543 | 0.511 | 0.511 | 0.538 | 0.542 | 0.138 | 0.000 | | | | |
| CCEC1 | 0.587 | 0.573 | 0.561 | 0.568 | 0.593 | 0.584 | 0.562 | 0.552 | 0.544 | 0.560 | 0.276 | 0.292 | 0.000 | | | |
| CCEC2 | 0.567 | 0.542 | 0.576 | 0.558 | 0.573 | 0.575 | 0.553 | 0.542 | 0.547 | 0.550 | 0.244 | 0.259 | 0.205 | 0.000 | | |
| CCLC2 | 0.602 | 0.589 | 0.590 | 0.585 | 0.644 | 0.612 | 0.602 | 0.593 | 0.560 | 0.539 | 0.300 | 0.329 | 0.265 | 0.257 | 0.000 | |
| CCLC2 | 0.593 | 0.569 | 0.593 | 0.588 | 0.622 | 0.591 | 0.593 | 0.583 | 0.552 | 0.532 | 0.289 | 0.280 | 0.281 | 0.182 | 0.147 | 0.000 |
| CLMC1 | 0.577 | 0.564 | 0.586 | 0.570 | 0.594 | 0.607 | 0.598 | 0.589 | 0.569 | 0.551 | 0.491 | 0.474 | 0.525 | 0.516 | 0.439 | 0.399 |
| CLMC2 | 0.590 | 0.577 | 0.600 | 0.584 | 0.637 | 0.640 | 0.654 | 0.646 | 0.604 | 0.587 | 0.508 | 0.492 | 0.542 | 0.533 | 0.472 | 0.432 |
| CLMR1 | 0.596 | 0.572 | 0.584 | 0.567 | 0.624 | 0.626 | 0.640 | 0.632 | 0.589 | 0.571 | 0.488 | 0.471 | 0.509 | 0.500 | 0.436 | 0.407 |
| CLMR2 | 0.604 | 0.592 | 0.593 | 0.577 | 0.642 | 0.634 | 0.648 | 0.640 | 0.609 | 0.591 | 0.500 | 0.483 | 0.521 | 0.524 | 0.463 | 0.434 |
| CLWP1 | 0.580 | 0.567 | 0.566 | 0.561 | 0.620 | 0.611 | 0.626 | 0.617 | 0.595 | 0.576 | 0.491 | 0.460 | 0.526 | 0.516 | 0.450 | 0.432 |
| CLWP2 | 0.589 | 0.587 | 0.576 | 0.571 | 0.617 | 0.620 | 0.623 | 0.614 | 0.582 | 0.563 | 0.491 | 0.461 | 0.500 | 0.490 | 0.412 | 0.396 |
| CBMC1 | 0.648 | 0.635 | 0.663 | 0.670 | 0.707 | 0.701 | 0.672 | 0.665 | 0.665 | 0.648 | 0.480 | 0.497 | 0.512 | 0.515 | 0.466 | 0.473 |
| CBMC2 | 0.651 | 0.639 | 0.667 | 0.674 | 0.710 | 0.704 | 0.676 | 0.669 | 0.668 | 0.652 | 0.497 | 0.503 | 0.518 | 0.533 | 0.485 | 0.491 |
| CPBZ1 | 0.604 | 0.591 | 0.617 | 0.612 | 0.678 | 0.636 | 0.628 | 0.631 | 0.644 | 0.626 | 0.518 | 0.512 | 0.529 | 0.545 | 0.440 | 0.474 |
| CPBZ2 | 0.611 | 0.598 | 0.600 | 0.595 | 0.665 | 0.633 | 0.624 | 0.615 | 0.617 | 0.610 | 0.535 | 0.516 | 0.548 | 0.551 | 0.483 | 0.503 |
| CHPL1 | 0.624 | 0.604 | 0.616 | 0.612 | 0.640 | 0.633 | 0.655 | 0.648 | 0.639 | 0.624 | 0.533 | 0.528 | 0.554 | 0.568 | 0.514 | 0.551 |
| CHPL2 | 0.615 | 0.594 | 0.606 | 0.602 | 0.640 | 0.633 | 0.645 | 0.638 | 0.629 | 0.614 | 0.513 | 0.508 | 0.554 | 0.568 | 0.514 | 0.540 |
| OAWC1 | 0.455 | 0.463 | 0.477 | 0.471 | 0.545 | 0.514 | 0.536 | 0.525 | 0.486 | 0.500 | 0.543 | 0.561 | 0.556 | 0.521 | 0.547 | 0.539 |
| OAWC2 | 0.460 | 0.457 | 0.470 | 0.486 | 0.535 | 0.515 | 0.526 | 0.527 | 0.479 | 0.503 | 0.543 | 0.560 | 0.556 | 0.523 | 0.560 | 0.552 |
| OTGR1 | 0.781 | 0.801 | 0.797 | 0.794 | 0.811 | 0.817 | 0.846 | 0.843 | 0.815 | 0.790 | 0.750 | 0.770 | 0.742 | 0.756 | 0.738 | 0.747 |
| OTGR2 | 0.775 | 0.798 | 0.792 | 0.789 | 0.808 | 0.804 | 0.824 | 0.819 | 0.826 | 0.797 | 0.739 | 0.748 | 0.714 | 0.745 | 0.693 | 0.706 |

## Appendix C. Continued

| SAMPLE | CLMC1 | CLMC2 | CLMR1 | CLMR2 | CLWP1 | CLWP2 | CBMC1 | CBMC2 | CPBZ1 | CPBZ2 | CHPL1 | CHPL2 | OAWC1 | OAWC2 | OTGR1 | OTGR2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MIJK1 | | | | | | | | | | | | | | | | |
| MIJk2 | | | | | | | | | | | | | | | | |
| MILS1 | | | | | | | | | | | | | | | | |
| MILS2 | | | | | | | | | | | | | | | | |
| MGDW1 | | | | | | | | | | | | | | | | |
| MGDW2 | | | | | | | | | | | | | | | | |
| MGWC1 | | | | | | | | | | | | | | | | |
| MGWC2 | | | | | | | | | | | | | | | | |
| MGEN1 | | | | | | | | | | | | | | | | |
| MGEN2 | | | | | | | | | | | | | | | | |
| CCGC1 | | | | | | | | | | | | | | | | |
| CCGC2 | | | | | | | | | | | | | | | | |
| CCEC1 | | | | | | | | | | | | | | | | |
| CCEC2 | | | | | | | | | | | | | | | | |
| CCLC2 | | | | | | | | | | | | | | | | |
| CCLC2 | | | | | | | | | | | | | | | | |
| CLMC1 | 0.000 | | | | | | | | | | | | | | | |
| CLMC2 | 0.102 | 0.000 | | | | | | | | | | | | | | |
| CLMR1 | 0.136 | 0.109 | 0.000 | | | | | | | | | | | | | |
| CLMR2 | 0.156 | 0.117 | 0.047 | 0.000 | | | | | | | | | | | | |
| CLWP1 | 0.222 | 0.179 | 0.143 | 0.164 | 0.000 | | | | | | | | | | | |
| CLWP2 | 0.217 | 0.174 | 0.152 | 0.172 | 0.076 | 0.000 | | | | | | | | | | |
| CBMC1 | 0.552 | 0.533 | 0.561 | 0.571 | 0.554 | 0.553 | 0.000 | | | | | | | | | |
| CBMC2 | 0.557 | 0.538 | 0.566 | 0.575 | 0.560 | 0.558 | 0.022 | 0.000 | | | | | | | | |
| CPBZ1 | 0.521 | 0.515 | 0.519 | 0.518 | 0.497 | 0.509 | 0.281 | 0.302 | 0.000 | | | | | | | |
| CPBZ2 | 0.551 | 0.543 | 0.548 | 0.547 | 0.527 | 0.539 | 0.313 | 0.333 | 0.154 | 0.000 | | | | | | |
| CHPL1 | 0.557 | 0.550 | 0.554 | 0.563 | 0.570 | 0.558 | 0.485 | 0.500 | 0.422 | 0.456 | 0.000 | | | | | |
| CHPL2 | 0.536 | 0.540 | 0.534 | 0.543 | 0.548 | 0.537 | 0.495 | 0.510 | 0.433 | 0.478 | 0.028 | 0.000 | | | | |
| OAWC1 | 0.547 | 0.573 | 0.544 | 0.566 | 0.573 | 0.548 | 0.636 | 0.640 | 0.588 | 0.608 | 0.531 | 0.520 | 0.000 | | | |
| OAWC2 | 0.569 | 0.594 | 0.567 | 0.587 | 0.584 | 0.548 | 0.643 | 0.647 | 0.609 | 0.605 | 0.541 | 0.541 | 0.049 | 0.000 | | |
| OTGR1 | 0.757 | 0.765 | 0.744 | 0.739 | 0.745 | 0.728 | 0.819 | 0.821 | 0.783 | 0.824 | 0.797 | 0.797 | 0.760 | 0.750 | 0.000 | |
| OTGR2 | 0.747 | 0.769 | 0.745 | 0.739 | 0.746 | 0.726 | 0.805 | 0.808 | 0.790 | 0.824 | 0.782 | 0.782 | 0.750 | 0.752 | 0.242 | 0.000 |

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