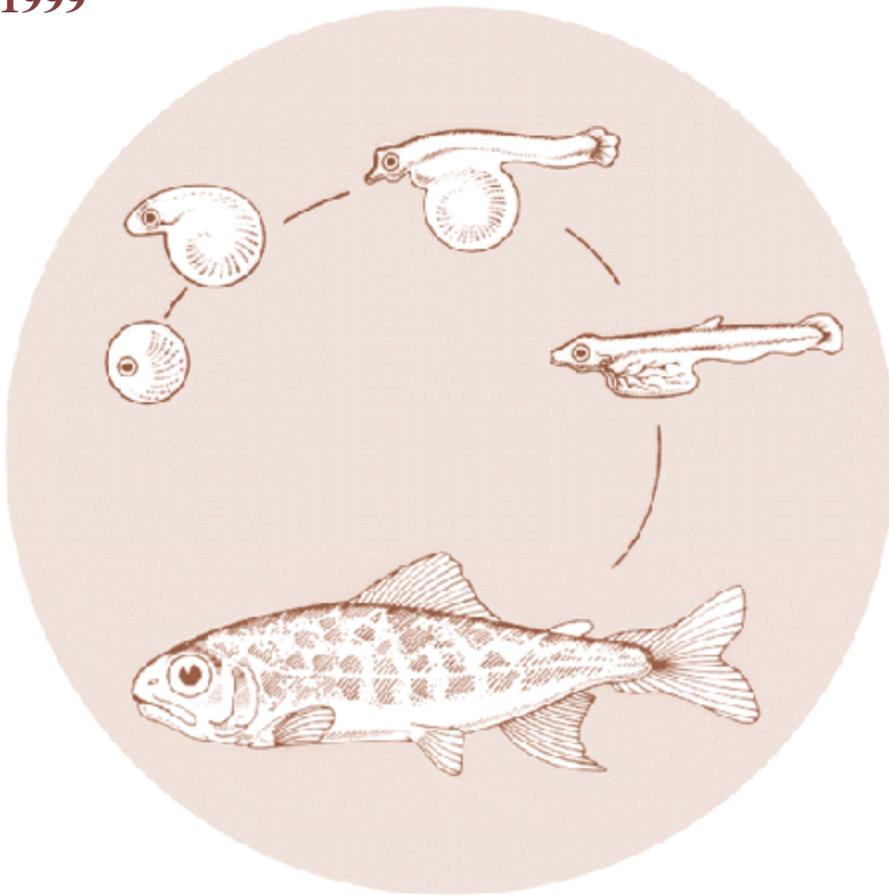


# Salmonid Gamete Preservation in the Snake River Basin

Annual Report  
1999



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**Salmonid Gamete Preservation in the Snake River Basin**

1999 Annual Report

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## ABSTRACT

Steelhead (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) populations in the Northwest are decreasing. Detrimental conditions causing these decreases can be improved in some cases, but time is required. The Nez Perce Tribe (Tribe) strives to ensure availability of a representative genetic sample of the male salmonid population by establishing and maintaining a germplasm repository. Our approach is to sample and cryopreserve male salmon and steelhead gametes from major river subbasins in the Snake River basin, assuming a metapopulation structure existed historically. Gamete cryopreservation permits the creation of a genetic repository, but is not a cure for decreasing fish stocks. The Tribe was funded in 1999 by the Bonneville Power Administration to coordinate gene banking of male gametes from Endangered Species Act listed steelhead and spring and summer chinook salmon in the Snake River basin. In 1999, a total of 335 viable chinook salmon semen samples from the Lostine River, Johnson Creek, Lake Creek, the South Fork Salmon River weir, and Sawtooth Hatchery (upper Salmon River stock), Rapid River Hatchery, Pahsimeroi Hatchery and Lookingglass Hatchery (Imnaha River stock) were cryopreserved. Also, 208 samples of male steelhead gametes from Dworshak Hatchery, Oxbow Hatchery, Johnson Creek, Pahsimeroi Hatchery and Little Sheep Creek were also cryopreserved. A total of 1,171 cryopreserved samples from Snake River basin steelhead and spring and summer chinook salmon, from 1992 through 1999, are stored in two independent locations at the University of Idaho and Washington State University. Two large freezer tanks are located at each university, each of which holds 25% of the cryopreserved sperm. One freezer tank at each university is considered long-term storage, while the other is short-term.

Fertility trials were conducted at each university to test the viability of the cryopreserved chinook salmon sperm. The experiments on the 1999 frozen and thawed sperm found a fertility of 40% relative to the fresh sperm control.

This document also summarizes chinook salmon and steelhead genetic analysis reports which were completed to date. The results of mitochondrial, nuclear DNA and microsatellite analysis found differences and shared haplotypes between the stocks of fish sampled for cryopreservation.

Recommendations for future gene banking efforts include a greater emphasis on wild fish, continued fertility trials, exploring field cryopreservation, initiate bull trout collections, and the establishment of a Northwest regional germplasm repository.

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## INTRODUCTION

Snake River steelhead (*Oncorhynchus mykiss*) and spring and summer chinook salmon (*Oncorhynchus tshawytscha*) spawning aggregates have experienced significant decline in numbers over the past five decades. These species are now listed as threatened under the ESA. These declines are due to many different factors. Most are the result of human activities.

Genetic conservation through population protection and monitoring has not been successful. With the constant threat of losing genetic diversity in specific native fish stocks, the establishment of a program for the long-term storage of fish germplasm serves as insurance against population collapse and extirpation. The Tribe has ensured the preservation of genetic diversity through cryopreservation of male gametes and development of a germplasm repository. At present, cryopreservation of semen is the best means of storing fish germplasm for extended periods of time. Cryopreserved salmonid semen will remain viable for long periods and can be easily shipped. Ashwood-Smith (1980), Whittingham (1980), and Stoss (1983) have estimated the storage time for fish semen held in liquid nitrogen to be between 200 and 32,000 years. This storage period is more than adequate for a germplasm repository. Although preservation of the maternal nuclear DNA component has been accomplished with some mammals (Rall and Fahy 1985, Fahning and Garcia 1992, Dobrinsky et al. 1991, Ali and Shelton 1993, Kono et al. 1988, Trounson and Mohr 1983, Hayashi et al. 1989), no similar techniques exist for fish species. Successful research and development to preserve germplasm components from female salmonids would increase future management options.

There are two important factors to be considered when developing a germplasm repository. First, this is a genetic repository and will not solve population problems of a fish stock that is at low levels of abundance and high risk of extirpation. Second, fertility of the stored semen currently is not as great as the fresh semen. The quality of the stored semen is usually a direct reflection of the quality of the sperm that was cryopreserved, and 50% motility of fresh sperm is considered good. There is a risk of lower fertilization rates and potential loss of eggs using cryopreserved semen. The Tribe, University of Idaho (UI) and Washington State University (WSU) conducted a small-scale fertilization trial using non-listed chinook salmon 1999 cryopreserved sperm and fresh eggs and sperm from Aberdeen Fish Hatchery.

The Nez Perce Tribe initiated chinook salmon cryopreservation activities in 1992 (Kucera 1999). The Lower Snake River Compensation Plan hatchery evaluation program funded through the U.S. Fish and Wildlife Service has provided a valuable, though limited, amount of financial support for this effort from 1992 through 1999. Bonneville Power Administration funded the Nez Perce Tribe in 1997 to coordinate and initiate a more comprehensive gene banking effort. More extensive male steelhead gamete cryopreservation was initiated in 1999.

Goals of the cryopreservation project are: 1) preserve the genetic diversity of listed salmonid populations at high risk of extirpation through application of cryogenic techniques, 2) maintain gene bank locations at independent sites for the short-term, and 3) establish and maintain long-term germplasm repositories.

## DESCRIPTION OF PROJECT AREA

The cryopreservation project currently seeks to preserve male spring and summer chinook salmon and steelhead gametes. The project area is the Snake River basin (Figure 1). In 1999, the sampling locations included: Lostine River, Johnson Creek, Lake Creek, Little Sheep Creek, South Fork Salmon River (SFSR), Dworshak Hatchery, Oxbow Hatchery, Pahsimeroi Hatchery, Sawtooth Hatchery (upper Salmon River spawning aggregate), Rapid River Hatchery and Lookingglass Hatchery (Imnaha River spawning aggregate). The fisheries personnel from the McCall and Enterprise field offices as well as the Lapwai office cover this geographically large collection area.

## METHODS

Fish handling protocol training was provided to all personnel prior to collection of adult male salmonids to minimize stress on the fish. Each team member was assigned a specific duty to improve the efficiency of sample collection. Semen from male kelts was collected from fish on the spawning grounds or from hatchery holding ponds. Fish were captured either by hand or dip net in the streams and sampled for sperm.

Chinook salmon spawning ground surveys are usually conducted on pre-determined stream reaches before handling any fish. Redd counts also determine where in each stream the collection of adult males occurs. Several team members locate the adults, being careful not to disturb the fish. Observations are made to visually identify male salmon. Males are identified by secondary sexual characteristics, which include a kype (greatly extended, narrowed snout, turned down at tip, also an enlarged lower jaw), large teeth, and a slim caudal peduncle that is not as worn as the female salmon. Females can be identified by a rounder head, thicker caudal peduncle, and a tattered, discolored (white) caudal fin from digging the redds. No harassment of actively spawning salmon occurs.

No one enters the water near any existing or active redds (i.e. where salmon are on the nests). A snorkeler enters the water to find solitary males, looking under cut banks, in logjams, in backwater habitats, etc. From the vantage point underwater, this person identifies fish for others to collect. It is easiest to collect the males in a constricted portion of the stream. Any females caught are returned to the water immediately, unharmed, and the capture is recorded.

All adult male salmon sampled are collected by hand, dip net, or seine in that order of priority:

Hand. Walk up to the identified fish and grasp the fish at caudal peduncle, put the fish into a dip net immediately. Always keep the fish in the water, pointing upstream, until ready to place in the tank.

Dip net. Stay away from active redds. Several dip netters get into position below the fish, with several people in the water upstream of the fish. The upstream people slowly herd fish towards the netters, moving slowly and quietly. Keep the large dip nets in the water in a line and let fish swim into the net. Net holders should be absolutely still as fish approach the nets.

Seine. Two 5' x 30-40' seine nets are set up perpendicular to the flow of water, blocking a segment of stream. The upstream net is slowly moved downstream, trapping the fish in

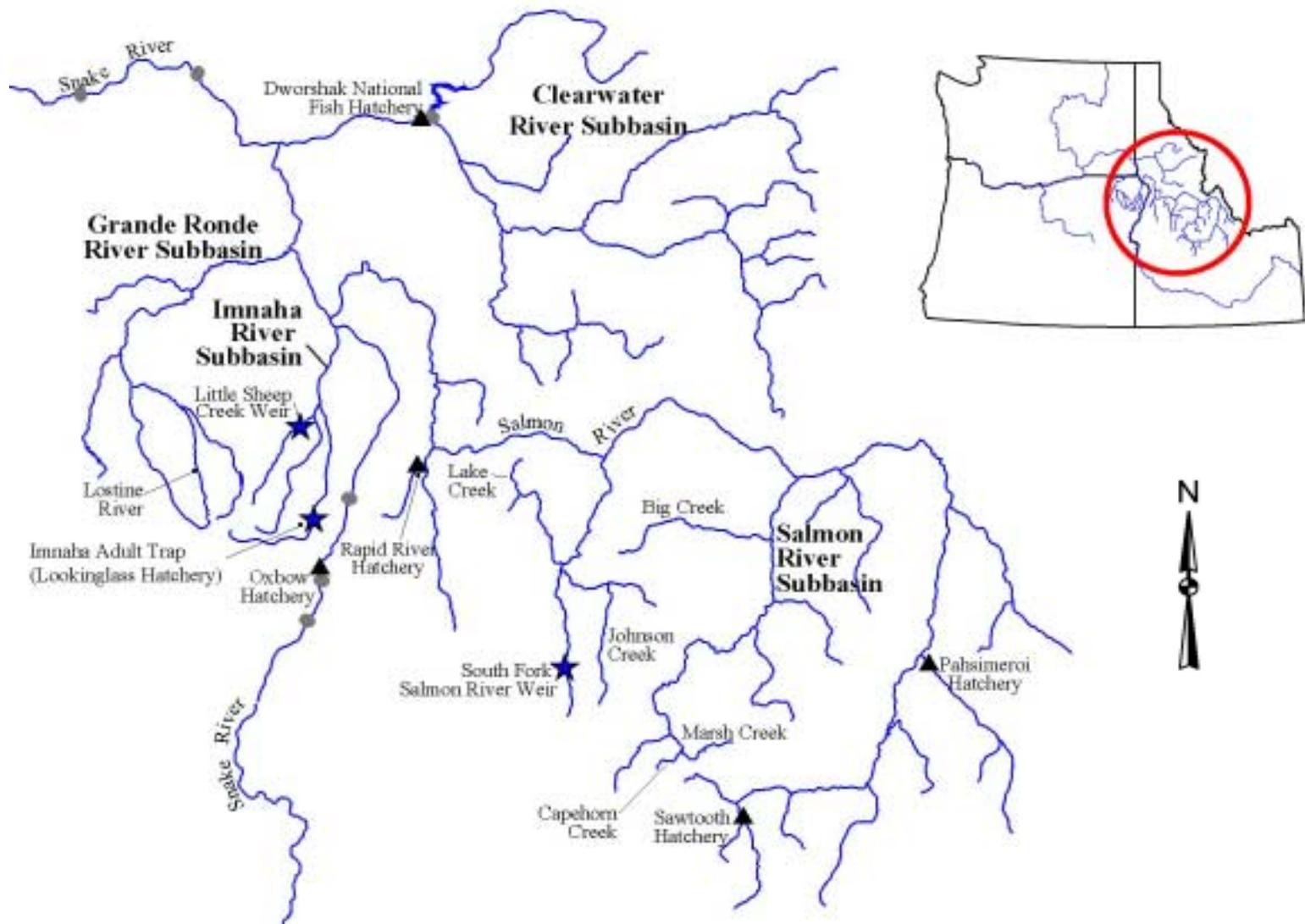


Figure 1. Snake River basin chinook salmon and steelhead cryopreservation locations in 1999.

a corral of decreasing area. Fish are collected with dip nets. If more than one fish is captured, determine which fish are to be sampled and release the others immediately. Upon capture of the male, the anesthetic bath tank is set up and filled. Captured fish are held in the stream before transfer to the anesthetic bath tank. General anesthetics first calm the fish, then cause it successively to lose mobility, equilibrium, consciousness, and finally reflex action (Summerfelt and Smith 1990). We wish to immobilize adult male salmon so they can be handled faster and less stressfully. A portable 35 gallon tank is set up along the stream to anaesthetize male chinook. Two people set up and fill the portable tank with seven 5-gallon buckets. Pre-measured Finquel<sup>TM</sup>, tricane methanesulfonate (MS-222), is used to anesthetize the adult male salmon, with the exception of hatchery fish. Sodium bicarbonate ( $\text{NaHCO}_3$ ) is used to buffer the acidic effect of the MS-222. It takes 3-5 minutes for the fish to be anesthetized. It is important to have one person time how long the fish is in the tank, and observe the fish all the time it is undergoing anesthesia. Fish handling/spawning protocols of Idaho Department of Fish and Game (IDFG) are used at the Idaho hatcheries, and thus the adults are not anesthetized before semen samples are taken. Imnaha River chinook salmon are anesthetized at Lookingglass Hatchery. Extra care is taken with semen collection to ensure the quality of preserved samples. The sedated fish is rinsed in the fresh water of the stream. The abdomen of the anesthetized male salmon is dried to reduce contamination of the semen samples and the milt is stripped (Figure 2).



Figure 2. Collecting chinook salmon milt from anaesthetized fish at Big Creek.

Semen samples were placed in two separately labeled Whirl Pak bags, aerated, and placed in an insulated cooler, on newspaper over wet ice. Some of the fish provide only enough semen for cryopreservation at one university. A few males are completely spawned out, so samples are not obtained. After the fish is released into the stream, the tank is emptied well away from the stream, so no chemicals are released into the stream proper. Fish biological information (fork length and mid-eye to hypural plate length, general condition, external marks) was recorded following semen collection (Figure 3). Caudal fin tissue was collected and preserved in ethyl alcohol for later genetic (DNA) analysis. Scales were taken for age assessment and scale pattern analysis. Following sampling and data collection, the anesthetized salmon were immediately returned to a slow water area and assisted until recovered.



Figure 3. Anaesthetized male chinook salmon on portable tank for measurements.

Semen samples are shipped to, cryopreserved and stored at each university as a safeguard to protect against catastrophic events that could destroy all germplasm samples if they were stored at one facility. Cryopreservation and storage occur independently at UI and WSU within a 12-hour period. Both universities started using nitrogen vapor freezing techniques in 1997. These samples were frozen in 20 0.5 ml straws if the quantity allowed. Any excess semen was cryopreserved in larger 5.0 ml straws. We continue to enlist the assistance of Dr. Joseph Cloud, professor of Zoology in the Department of Biological Sciences at the University of Idaho, Dr.

Gary Thorgaard and Paul Wheeler in the Thorgaard Lab at Washington State University, and Dr. Madison Powell, geneticist at the Fish Genetics Laboratory and Hagerman Experiment Station with the University of Idaho. These subcontractors are experts in the field of cryopreservation of reproductive physiology and/or fish genetics.

Sperm evaluation is an important component of the cryopreservation program in order to estimate the quality from the motility of the stored sperm. All semen from listed fish is stored regardless of motility. Fertility is evaluated by sperm motility (Figure 4), which is the percentage of motile sperm following the addition of a sperm activating solution (Mounib 1978). Motility correlates to post-thaw fertility.



Figure 4. Conducting pre-freeze motility estimates on fresh chinook salmon sperm.

There are four stages in the cooling sequence of cryopreservation of cells (Cloud and Osborne 1997): 1) Cooling cells to the point of ice formation - this does not appear to be a critical factor in the cryopreservation of salmonid sperm; 2) The formation of ice - the goal at this stage is to have ice form near the freezing point of the extracellular solution; 3) Cooling through the critical period - there is a net movement of water out of the cells as the temperature is constantly being reduced 4) Reduction to liquid nitrogen temperature - the frozen milt is then plunged into liquid nitrogen at -196°C.

The amount of sperm cryopreserved varied greatly by individual fish and by species. Chinook salmon produce greater volumes of milt (averaging ~15 ml), whereas steelhead produce less (average 2-4 ml). A sample of 5 ml of semen is sufficient to fill 20 0.5 ml straws, due to the dilution of semen with three parts freezing solution. Depending on the motility of the thawed

sperm, one 0.5 ml straw can fertilize up to 450 eggs, and a 5.0 ml straw can fertilize approximately 2,000 eggs. There is not a linear relationship of straw volume to fertilization capacity due to the heat of fusion and the surface area involved. Two additional large liquid nitrogen tanks, one for each university, were purchased in late 1998 to serve as emergency backups and long-term repositories. Each university now has two large nitrogen storage facilities, which became operational in 1999 (Figure 5).



Figure 5. Liquid nitrogen tank used to store cryopreserved samples at each university.

Fertilization experiments have been conducted every year on chinook salmon cryopreserved versus fresh sperm in order to assess our procedures. In 1999, Washington State University did not pool the eggs in the fertilization trial and each cross involved only one male and one female. Semen from one Aberdeen male was used to fertilize eggs from each of the three females as a control (three families). Five cryopreserved hatchery male gamete samples fertilized each of the three female's eggs for 15 different family groups. University of Idaho thawed four different

Rapid River hatchery male gamete samples and each sample fertilized two different groups of eggs. The eggs from the three Aberdeen females were pooled. Ten different family groups were created, two of which were controls.

## RESULTS

### Description of Spawning Aggregates

The background of the selected spawning aggregates for cryopreservation activities have a diverse history of transfers, stocking, and straying. Chinook salmon and steelhead hatcheries in the Snake River basin that are sampled for male gamete cryopreservation have developed broodstocks in a variety of ways. It is important to understand how broodstock development, stocking and straying occurred as it relates to the genetic profile of the existing stock of fish being propagated in these facilities. A genetic analysis was not conducted on any of the original hatchery or stream brood sources to the authors' knowledge. In that regard, we have compiled existing information of where the original chinook salmon and steelhead spawning aggregates brood sources were obtained.

#### Dworshak National Fish Hatchery (North Fork Clearwater River)

Dworshak National Fish Hatchery (DNFH) was built in 1969 by the U.S. Army Corps of Engineer as mitigation for Dworshak Dam, which blocks anadromous fish passage to the North Fork Clearwater River. The steelhead broodstock was started from native B-run steelhead returning to the North Fork to spawn (Howell et al. 1985b, IDFG et al. 1990). Based on allele frequencies, Milner (1977) reported that North Fork Clearwater steelhead were distinguishable from all other Columbia Basin steelhead by the high frequency of the allele for peptidase locus. To sample the run adequately, the hatchery operates the trap at different times throughout the run.

#### Lostine River

Straying of Grande Ronde chinook salmon hatchery fish from Lookingglass Hatchery occurs in the Lostine River. Based on the origin of carcasses recovered on spawning ground surveys, ODFW determined that a high proportion (20-75%) of natural spawners in the Lostine River were of Lookingglass Hatchery origin from 1986-1994 (Carmichael et al. 1998). The broodstock history at Lookingglass is varied and complex (Table 1).

Table 1. Broodstock history of Lookingglass Hatchery Grande Ronde spring chinook salmon (Carmichael et al. 1998).

| Brood Year | Source   |
|------------|--|
| 1978       | Rapid River  |
| 1980-1984  | Carson / Wilamette Hatchery                            |
| 1985-1987  | Carson / Lookingglass Hatchery<br>Rapid River          |
| 1988       | Rapid River  |
| 1989       | Carson / Lookingglass Hatchery<br>Rapid River          |
| 1990-1997  | Rapid River / Lookingglass Hatchery                    |
| 1995-2000  | native Lostine, upper Grande Ronde,<br>Catherine Creek |

#### Lookingglass Hatchery (Imnaha River)

Lookingglass Hatchery is an LSRCF facility constructed in 1982 with adult trapping and smolt acclimation facilities on the Imnaha River. The LSRCF hatchery program provides mitigation for salmon and steelhead losses incurred by the construction and operation of the four lower Snake River dams. Native Imnaha River chinook salmon were collected in 1982 for the supplementation program (Howell et al. 1985a, Carmichael et al. 1998) and only this stock has been used.

#### Irrigon Hatchery (Little Sheep Creek)

The summer steelhead LSRCF supplementation program in the Imnaha River began with the trapping of native steelhead adults in Little Sheep Creek in 1982 (Howell et al. 1985b). In 1983, hatchery-reared smolts were released into Little Sheep Creek and the Imnaha River and adults began to return to the basin in 1985 (Whitesel et al. 1998). The collection, holding and spawning of natural and hatchery adults occurs at a permanent facility on Little Sheep Creek.

#### Rapid River Hatchery (Rapid River)

Rapid River Hatchery was constructed in 1964 through the Idaho Power Company (IPC) as part of the mitigation for spring chinook salmon losses due to the construction and operation of Brownlee Dam, Oxbow Dam, and Hells Canyon Dam on the Snake River (IDFG et al. 1990, Howell et al. 1985). Broodstock was obtained from adult collection at Hells Canyon Dam from

1964 through 1968. These fish originated from the mid-Snake River tributaries such as Eagle Creek, Powder River and the Weiser River (Howell et al. 1985a, IDFG et al. 1990). As such this hatchery brood source represents the genetic diversity contained from these chinook salmon subpopulations. In addition, the hatchery broodstock probably contains natural spring and summer chinook salmon from Rapid River.

#### Lake Creek

Straying of hatchery fish (missing an adipose fin), presumably from McCall Fish Hatchery (MFH) South Fork Salmon River program has been noted in Lake Creek and the Secesh River.

#### Johnson Creek

The Johnson Creek summer chinook salmon population has received supplementation outplants of SFSR summer chinook salmon reared at MFH in 1984 through 1988 (Table 2). These outplants primarily occurred as fingerling releases and were distributed from the headwater reaches to the Ice Hole in Johnson Creek. However, no evaluations of these releases were conducted. It is not known how these supplementation efforts have affected the Johnson Creek summer chinook salmon population in terms of stock mixing, population interactions, spawning distribution, or genetic effects. Minimal straying has occurred into Johnson Creek, on the order of one fish with a missing adipose, probably from the MFH SFSR program, per year.

Table 2. South Fork Salmon River Summer Chinook Salmon Releases in Johnson Creek (MFH 1984-1988).

| Brood Year          | Release Date | Fish Released | Fish Size      |
|---------------------|--------------|---------------|----------------|
| BY 1984             | 8/02/85      | 50,000        | Fry            |
| BY 1985             | 5/09/86      | 177,606       | Fry            |
| BY 1986             | 5/05/87      | 90,000        | Fry            |
| BY 1986             | 6/12/87      | 28,400        | Fry            |
| BY 1987             | 5/09/88      | 194,600       | Fry            |
| BY 1987             | 5/31/88      | 259,200       | Fry            |
| BY 1988             | 5/08/89      | 200,500       | Fry            |
| BY 1988             | 8/8-10/89    | 290,000       | Fingerling     |
| Total Fish Released |              | 1,290,306     | Fry/Fingerling |

### McCall Fish Hatchery (South Fork Salmon River)

McCall Fish Hatchery was constructed in 1980 through the LSRCP program to supplement summer chinook salmon in the SFSR. The LSRCP hatchery program provides mitigation for salmon and steelhead losses incurred by the construction and operation of the four lower Snake River hydroelectric dams. The broodstock was initially collected at Little Goose Dam in 1978, at Lower Granite Dam in 1979, and at Lower Granite Dam and the SFSR in 1980 (Howell et al. 1985a, Kucera 1987, IDFG et al. 1990). After 1980, adult returns to the SFSR were used for broodstock purposes. As such, the broodstock is thought to be a mixture mainly of Snake River summer chinook, but may also contain genetic diversity from spring chinook salmon subpopulations as well.

### Capehorn Creek

In 1975, 22,000 spring chinook salmon fry from Rapid River Hatchery were outplanted in Capehorn Creek. This was the only nonindigenous fish outplanting in the Middle Fork Salmon River (Matthews and Waples 1991).

### Pahsimeroi Hatchery Chinook Program (Pahsimeroi River)

Pahsimeroi Hatchery was constructed in the mid 1960's by the Idaho Power Company (IPC) as part of the mitigation for chinook salmon and steelhead losses due to the construction and operation of Brownlee Dam, Oxbow Dam, and Hells Canyon Dam on the Snake River (IDFG et al. 1990). Broodstock for the Pahsimeroi Hatchery chinook program was developed from native summer chinook in the Pahsimeroi River in 1968 (Howell et al. 1985a). When the hatchery was expanded in 1980, a spring chinook salmon program was started and the summer chinook program continued. In 1982-1985, over 700 females from the Hayden Creek hatchery in the Lemhi River subbasin were spawned and added to the Pahsimeroi broodstock. In 1987 the hatchery program converted to a summer chinook salmon program and eggs were obtained from the South Fork Salmon River (McCall Hatchery). Transplants from Rapid River spring chinook occurred in 1982 and 1987 to 1989. The genetic diversity contained within the current stock includes at least spring and summer chinook from the Pahsimeroi River, Rapid River (see Rapid River above), Lemhi River and SFSR chinook salmon (see MFH above). It is unclear what other program modifications may have occurred. The broodstock makeup has been stable since 1990, meaning only the fish returning to the hatchery have been used for spawning purposes.

### Pahsimeroi Hatchery Steelhead Program (Pahsimeroi River)

Idaho's hatchery steelhead program began in 1965 after the Federal Power Commission ordered IPC to transplant Snake River steelhead trapped at Hells Canyon Dam to the Salmon River for mitigation due to the Hells Canyon dam complex construction on the Snake River. This Snake River stock has been the basis for all hatchery A-strain programs in the Salmon River (Ball 1998). B-run steelhead from Dworshak National Fish Hatchery were released in the Pahsimeroi River in 1973 - 1974 (Ball 1998, IDFG et al. 1990).

### Sawtooth Hatchery (Upper Salmon River)

Sawtooth Hatchery was constructed and became operational in 1985 (Hassemer 1998). It is an LSRCF facility designed to compensate spring chinook salmon in the upper Salmon River. These spring chinook must migrate 900 plus miles from the ocean and spawn and rear at an elevation over 6,000 feet. Broodstock development occurred from adult collections on the East Fork Salmon River and the upper Salmon River (Hassemer 1998, IDFG et al. 1990). During the early and late 1970's Rapid River hatchery chinook salmon were released into the East Fork Salmon River and upper Salmon River in response to severe declines in adult escapement (Howell et al. 1985a, Kucera 1987, Hassemer 1998). A total of 985,400 Rapid River hatchery smolts were released in 1978 and 1,012,300 smolts were released in 1979. Adult broodstock collection has occurred in the upper Salmon River since 1981 (Howell et al. 1985a). The current hatchery broodstock may reflect the genetic diversity contained in both the upper Salmon River chinook and the Rapid River hatchery chinook.

#### 1999 Chinook Salmon Cryopreservation Sample Collections

Gametes from male chinook salmon were sampled from Lostine River, Johnson Creek, Lake Creek, the SFSR weir, Pahsimeroi Hatchery, Rapid River Hatchery, Sawtooth Fish Hatchery and Lookingglass Hatchery (Table 3). Sampling at hatchery facilities has been designed to collect gametes from the spectrum of the run.

Table 3. Cryopreserved samples taken from listed Snake River basin spring and summer chinook in 1999; dates collected, unmarked (wild/natural) and marked (hatchery) fish numbers, fork length and percent sperm motility.

| Spawning Aggregate      | Collection Dates                  | Unmarked Fish | Marked fish | Fork Length (cm) | % Sperm Motility |
|-------------------------|-----------------------------------|---------------|-------------|------------------|------------------|
| Lostine River           | Aug. 27                           | 2             | -           | 77-79            | 0-80             |
| Imnaha River            | Aug.19, 25 & Sept. 1, 9, 16       | 9             | 86          | 50-84            | 30-90            |
| Rapid River             | Aug. 16, 26, 30 & Sept. 7         | -             | 68          | not measured     | 40-90            |
| South Fork Salmon River | Aug. 18, 27, 31, & Sept. 3, 7, 10 | 10            | 78          | not measured     | 30-90            |
| Lake Creek              | Aug. 17, 19                       | 6*            | -           | 49-82*           | 50-90            |
| Johnson Creek           | Aug. 31                           | 4             | 1           | 49-94            | 40-90            |
| Pahsimeroi River        | Sept. 8, 23                       | -             | 31          | 44-86            | 50-90            |
| Up. Salmon River        | Aug. 25 & Sept. 1                 | 30            | 10          | 50-108           | 20-90            |

\* 12 additional unmarked precocial males were sampled, fork lengths ranged from 10-12 cm. Lostine River

The Lostine River is a tributary to the Wallowa River, which empties into the Grande Ronde River. Two wild male chinook salmon were sampled on August 27. One of the two fish collected in 1999, sample # NPT-002LR had three opercle punches, indicating it had been passed over the adult fish weir between July 11-24, 1999. Field efforts were again hampered by the refusal of a landowner in the prime spawning area to allow project personnel access to his property. This was the sixth consecutive year of cryopreservation sampling in the Lostine River. A total of 15 cryopreserved semen samples taken from 1994 to 1999 are now in the germplasm repository.

#### Imnaha River

Sperm was cryopreserved from 95 Imnaha River chinook salmon out of the 101 fish that were spawned at Lookingglass Fish Hatchery. Samples from nine natural (unmarked) and 86 hatchery (adipose clipped) fish were frozen. More than half (58%) of the males sampled were jacks. They are all ESA-listed fish. These samples were collected in coordination with the ODFW during hatchery spawning operations. The chinook salmon males ranged in fork length from 50

to 84 cm. Two additional collection years, 1994 and 1995, with 64 samples were added to our repository this year from ODFW. Including these samples, we have six years of cryopreservation sampling with a total of 312 cryopreserved semen samples taken from 1994 to 1999.

### Rapid River Hatchery

Milt was collected and cryopreserved from 68 Rapid River spring chinook salmon considered excess to production needs. These salmon are Snake River origin fish but are not listed under the ESA. A total of 160 semen samples from Rapid River are cryopreserved. Most of the hatchery fish are returning adults to Rapid River, though three of the 1999 males were transported from the Snake River trap at Hells Canyon Dam.

### South Fork Salmon River

The upper SFSR program at the MFH is the only summer chinook salmon program operated within the Lower Snake River Compensation Plan (LSRCP) (Hassmer 1998). Eighty-eight fish were sampled over a three-week period at the SFSR weir with IDFG hatchery personnel. The breakdown of the origin of these fish is as follows: 78 were adipose fin-clipped (hatchery reared), and 10 were natural fish. Twelve out of the 88 or 13% of the chinook males sampled were jacks. This was the third year of cryopreservation sampling at the SFSR weir. A total of 197 cryopreserved semen samples taken from 1996 to 1999 are now in storage.

### Lake Creek

Five wild fish were sampled in Lake Creek on August 17<sup>th</sup> and the 19<sup>th</sup>. A number of juvenile precocial males were observed both in the stream near actively spawning females and in the Tribe's outmigrant screw traps. We counted over 30 smaller males hovering around an actively spawning pair of adult chinook. The juveniles would dart into the redd area until chased out by the adult male. These little males probably are successful in fertilizing some of the eggs. So we cryopreserved the pooled milt from 12 precocial males due to the low volume. These fish ranged in size from 102 mm to 123 mm. Two female chinook salmon were netted and immediately released upon identification. This was the fourth year of cryopreservation sampling in Lake Creek, a tributary of the Secesh River in the SFSR watershed. A total of 16 cryopreserved semen samples taken from 1996 through 1999 are now in storage.

### Johnson Creek

Five chinook salmon semen samples were cryopreserved from Johnson Creek in 1999. One female chinook was netted and immediately released upon identification. The Nez Perce Tribe initiated a supplementation program in 1998 on this stream, although the adult weir was not operational in 1999. Ninety six percent (22 redds were counted by August 30 of the total 23 redds counted on September 13) of the redds constructed in the index area were completed when cryopreservation collection took place. One of the two jacks sampled and milt cryopreserved from on August 31 was retrieved as a carcass on September 8. The other jack sampled had a left ventral fin clip indicating a hatchery stray probably from the SFSR MFH. Ventral fin-clipped fish are supplementation fish meaning they are the progeny of a wild and a hatchery (probably from the SFSR fish). Johnson Creek is a tributary of the East Fork SFSR. This was the third year

of cryopreservation sampling in Johnson Creek, for a total of 29 fish cryopreserved.

### Big Creek

We have attempted to collect semen from chinook salmon in Big Creek, a tributary to the Middle Fork Salmon River since 1992. Big Creek experienced three consecutive years (1994-1996) of cohort collapse when samples were not obtained. No salmon were netted in the stream this year either. Several females, four redds, and carcasses were observed. A total of 24 fish were taken in 1992, 1993, 1997 and 1998 are now in storage.

### Capehorn Creek and Marsh Creek

No salmon were found in either of these headwater streams of the Middle Fork Salmon River. Idaho Department of Fish and Game had their redd count training on these streams and found no fish. Additional passes were made over a month's time. Semen samples from 14 salmon from 1997 and 1998 are cryopreserved in the gene bank.

### Pahsimeroi River

This is the first year this hatchery was sampled. Fifty-three salmon were sampled for semen and 31 of the samples were cryopreserved on two different days in September. Eleven of the salmon were unmarked, presumed natural fish; the remaining 42 were hatchery-reared fish. Their fork lengths ranged from 44 to 86 cm.

### Upper Salmon River

Forty salmon held in the Sawtooth Fish Hatchery from the upper Salmon River were sampled in 1999. Male chinook collected ranged in fork length from 50 to 108 cm. (Figure 7). Most of the fish sampled from Sawtooth hatchery were four year old fish. Seventy five percent of the fish cryopreserved in 1999 and eighty eight percent of the fish sampled in 1998 were natural, the remainder was hatchery-reared fish. Whereas 75% of the fish sampled in 1997 were from the hatchery. There are 132 samples from the upper Salmon River spawning aggregate (1997-1999) represented in the repository.

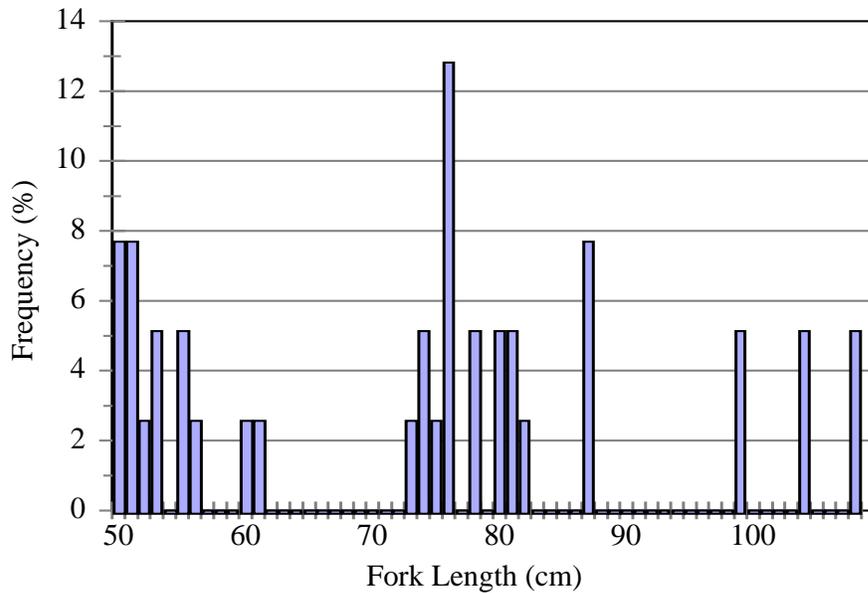


Figure 6. Length frequency distribution of male chinook salmon sampled for cryopreservation in the upper Salmon River in 1999.

#### 1999 Steelhead Cryopreservation Sample Collections

Male steelhead gametes were collected at Dworshak Hatchery, Oxbow Hatchery, Pahsimeroi Hatchery, Johnson Creek and at the ODFW weir on Little Sheep Creek, a tributary to the Imnaha River (Table 4).

Table 4. Cryopreserved samples taken from Snake River basin steelhead in 1999; dates collected, unmarked and marked fish numbers, fork length and percent sperm motility.

| Spawning Aggregate          | Collection Dates                      | Unmarked Fish | Marked fish | Fork Length (cm) | % Sperm Motility |
|-----------------------------|---------------------------------------|---------------|-------------|------------------|------------------|
| North Fork Clearwater River | Feb. 9, 23, March 16, April 13, May 4 | -             | 58          | not measured     | 20-90            |
| Little Sheep Creek          | April 6, 13, May 4                    | 1             | 24          | 57-69            | 50-90            |
| Johnson Creek               | April 27, May 14                      | 2             | -           | 21-22            | 0-80             |
| Pahsimeroi River            | May 6, 7                              | -             | 47          | 52-73            | 40-90            |
| Snake River                 | March 10, 25<br>April 1, 5, 19, 26    | 1             | 57          | not measured     | 0-90             |

### North Fork Clearwater River

Semen was cryopreserved from 58 hatchery steelhead that were held for spawning at the DNFH. This hatchery propagates the anadromous B-run steelhead run from the North Fork Clearwater River. All of these fish were hatchery origin steelhead, which means they are not ESA listed fish. These samples were collected in coordination with the U.S. Fish and Wildlife Service during hatchery spawning operations. This was the first year of steelhead cryopreservation sampling at DNFH.

### Little Sheep Creek

Steelhead semen was collected from 38 fish at the adult weir on Little Sheep Creek, a tributary of the Imnaha River, which is operated by the ODFW. This hatchery stock originated from native Little Sheep Creek steelhead. Only 25 steelhead samples, ranging in size from 57-69 cm in size were cryopreserved either due to low volume or low motility. All 25 fish had an adipose or an adipose plus left ventral fin clip, except one unmarked fish.

### Johnson Creek

Three precocial steelhead or rainbow trout were caught in the Tribe's emigrant screw trap on Johnson Creek in April and May. A small quantity of milt was hand stripped from each fish and two of the samples were cryopreserved. The cryopreserved fish were 211 and 224 mm in length, and had not migrated to the ocean. It is unclear whether these fish were resident rainbow trout or precocial steelhead. The definition of steelhead, however, is any rainbow/steelhead found in anadromous waters of the Columbia River Basin. One of the samples had zero motility but was frozen for its DNA. There is no steelhead hatchery supplementation program in the South Fork Salmon River drainage.

### Pahsimeroi River

At the end of the Pahsimeroi Hatchery steelhead spawning season, we were able to collect 47 hatchery fish semen samples on two consecutive days when surplus males were being killed after spawning was completed. We attempted to transport whole testes from wild kelts caught coming downstream in the hatchery weir, but the testes arrived at the university with no motility. This hatchery was started using A-run Snake River steelhead from Hells Canyon. These fish are ESA-listed.

### Snake River

Snake River steelhead milt was sampled at Oxbow Hatchery for the first time in 1999. These fish are not listed under the ESA. In 1999, hatchery steelhead milt (91 samples) considered excess to the production needs was collected, and 76 of the samples collected from the spectrum of the run were preserved. These fish are trapped below Hells Canyon Dam in the fall and again starting in February. Wild, unmarked fish are released to spawn. Semen from one wild steelhead was cryopreserved even though the sperm showed zero motility.

## Fertility Trials

Fertilization experiments were conducted in 1999 using ESA non-listed fall chinook salmon gametes acquired from Aberdeen Hatchery, Washington. Eggs from three females and semen from three males were stripped on November 18. The gametes were shipped overnight to WSU and UI and the fertility trial was conducted on November 19. Gametes from nine cryopreserved hatchery ESA non-listed chinook males were thawed and used to fertilize approximately 100 eggs each (Appendix 1 Table 4). The control groups using fresh semen showed a low fertilization rate (mean of 45%) perhaps due to the next day fertilization method. WSU found a relative fertility (% eyed / %eyed in control) of 41% and UI showed 39%. The relative fertilization rate of 40% from the 1999 cryopreserved semen is what can be expected if 1999 frozen chinook salmon sperm is used for production. Lower fertilization rates using cryopreserved sperm may be acceptable where genetic concerns warrant them.

## Salmonid Genetic Analysis

The University of Idaho Center for Salmonid and Freshwater Species at Risk analyzed and reported on the results of the chinook salmon collected in 1998. A summary of the preliminary findings follows. These results are being refined further, since new markers and analyses are continually being developed. A preliminary report is attached (Appendix 3) and will be updated annually.

Spring and summer chinook salmon collected from ten locations within the Snake River basin were examined using three molecular genetic methods (mitochondrial DNA, nuclear DNA and microsatellites). Analysis of mitochondrial DNA (mtDNA) variation among the sample locations revealed five composite haplotypes with one haplotype (designated Ot-01) observed in all locations. Nuclear DNA was examined for restriction fragment length polymorphisms within the p53 gene locus and revealed two alleles among sample locations. Intron D of the chinook salmon growth hormone 2 gene was also screened for restriction site polymorphisms but no variation was observed. Two microsatellite loci designated Pu-RBT and 77-RBT (approximating PuPuPy and Omy-77 in published literature) were examined and found to have a total of 11 and 9 alleles, respectively, among all samples. Estimates of nucleotide diversity and divergence were calculated for all locations. Diagrammatic representations of inferred evolutionary relationships among observed mitochondrial haplotypes and sample locations (dendrograms) were constructed based upon genetic distances. Statistical evaluation of geographic heterogeneity among haplotype distributions (population structure) was also performed. These tests and estimates were done to demonstrate the utility of these methods, and do not necessarily reflect statistically robust conclusions for all sample locations. Sample sizes from 6 of the 10 locations consisted of  $n \leq 15$  and any conclusions drawn from such data should be considered preliminary.

Washington State University analyzed and reported results of genetic analysis of selected 1999 steelhead. The mitochondrial DNA haplotypes and microsatellite allelic distribution appear to agree on the similarities and relationships among Snake River basin steelhead trout collected by the Tribe. The most striking preliminary result is the relationship among the Pahsimeroi River natural and hatchery fish and the Oxbow hatchery steelhead. The microsatellite allele frequency

data suggests that Pahsimeroi River natural fish may be more similar to Oxbow fish than they are to Pahsimeroi hatchery steelhead. Likewise, the fact that hatchery and natural Pahsimeroi fish each have a mitochondrial type not shared by the other and the striking similarities in mitochondrial haplotypes for Oxbow and Pahsimeroi natural fish further support the microsatellite data. These relationships certainly reflect the historical makeup of the hatchery stocks, but are not conclusive for the wild stocks without larger sample sizes of wild fish and more information from other steelhead stocks in the Salmon River system. Any conclusions that are drawn from this particular relationship should be cautiously done since the sample sizes were so small for these wild fish.

It is also important to remember that both of the above genetic analyses were conducted on putatively neutral markers that are not subject to natural selection, as compared to adaptive markers. It is difficult to analyze adaptive markers, so this is rarely done. Some adaptive traits are: distance migrated, size, fecundity, age at return, run timing, egg emergence, emigration time, and habitat used.

## DISCUSSION

Semen samples from 543 wild/natural and hatchery steelhead and spring and summer chinook salmon were collected for cryopreservation in 1999. Chinook salmon spawning aggregates to sample were chosen based upon geographic distribution. More semen samples for cryopreservation were collected this year than any other year. All of the five stream sites sampled for chinook salmon produced minimal sample numbers, totaling 15 samples. Adult salmon spawner escapement in 1999 was very low, so a large sample size was not obtained.

A total of 335 spring and summer chinook were sampled with 73 of those fish, or 22%, being of natural origin. Adult chinook salmon migrating upstream past Bonneville Dam from March through May, and June through July are categorized as spring- and summer-run fish respectively (Burner 1951). Some streams in the Snake River are considered to have only spring chinook, some mainly summer-run fish (e.g., those in the SFSR), and some both forms (e.g., Middle Fork Salmon River and upper Salmon River). In most cases where the two forms coexist, spring-run fish spawn earlier and in the headwaters of the tributaries, whereas summer chinook spawn later and farther downstream (Matthews and Waples 1991). We are sampling chinook in the major river subbasins in the Snake River drainage, thereby preserving gametes from both runs.

A total of 208 steelhead male gametes were cryopreserved out of 258 gametes collected (249 were hatchery fish, 9 were unmarked wild fish). Both of the major extant groups (A and B-run) of steelhead in the Snake River basin were sampled. The A-group passes Bonneville Dam (Columbia River kilometer 235) before August 25 and the B-group pass Bonneville after August 25 (CBFWA 1990, IDFG 1994), but both reach the lower Snake River dams at about the same time. A-run steelhead are defined as predominately one ocean fish, while B-run steelhead are defined as two ocean (IDFG 1994). A-run steelhead are believed to occur throughout the steelhead-bearing streams of the Snake River basin (Busby et al. 1996). B-run steelhead are thought to be produced only in the Clearwater, Middle Fork Salmon River and South Fork Salmon Rivers (IDFG 1994). Steelhead of the B-group are larger, averaging 11-15 pounds (or 5-7 kilograms) with maximum size up to 35 pounds (or 16 kilograms).

Sustained productivity of salmonids in the Pacific Northwest is possible only if the genetic resources that are the basis of such productivity are maintained (National Research Council 1996). Much of the genetic diversity that historically existed probably has already been lost. The germplasm repository is an effort to conserve the genetic diversity that remains in existing salmon runs and steelhead runs for future management options. The spawning aggregates sampled represent only a small portion of the stocks in the Snake River basin. We have attempted to sample and preserve salmonid genetic diversity within the major river subbasins in the Snake River basin. An adequate number of individuals should be sampled from each genetically unique conservation unit to ensure conserving the genetic diversity contained in the runs of chinook salmon and steelhead.

Sampling of male chinook salmon in the streams is restricted to the later part of the spawning period to avoid affecting reproductive success. By limiting the sampling period, the genetic diversity contained in early spawning fish may not be preserved in the germplasm repository. Although it would be very time-consuming and labor-intensive to observe and sample early spawning fish while avoiding harassment of fish that were not yet ready for spawning, it should be investigated.

The urgency to create a germplasm repository becomes apparent when reviewing the status of the runs (Kucera 1998) and the number of samples being preserved. The number of samples collected in the streams in 1999 was minimal. Fish were not found in some streams, like Big Creek, Marsh Creek and Capehorn Creek for sampling.

The use of cryopreserved sperm to fertilize eggs in a hatchery setting enables hatchery managers to produce threatened salmonids via captive broodstock programs. Captive breeding programs require consideration of small population vulnerabilities to preserve high levels of genetic diversity (Ryder et al. 2000). It is estimated that 200 unrelated individual samples are needed to establish a breeding program (Cloud personal communication).

Many precocial male juvenile chinook salmon were observed in the streams that had actively spawning females in 1999. Because cryopreservation is intended to capture the spectrum of genetic diversity contained within target populations, it is essential that all sexually mature individuals be represented. It is possible that precocial maturation is under some genetic control and represents an important evolutionary strategy that might be lost if not included in gene banking efforts (Moran 1999). The Tribe's research at Lake Creek and the Secesh River has found that a few of the outmigrating fish detected by PIT tags spend an extra year in freshwater (NPT unpublished data). This potentially adds another year class to the spawning population.

The preliminary genetic analysis reveals population-level identities and concerns. Chinook salmon returning to Sawtooth Hatchery (n=36) initially showed one composite haplotype. Based on this information, managers of the hatchery initiated a genetically based spawning matrix in 1999. This dissimilarity matrix using DNA analysis identified directly related individuals and recommended spawning unique genotypic fish to maximize genetic diversity in the offspring. Further genetic analysis has since shown more differences in that spawning aggregate than formerly thought.

Johnson Creek (n=15), Lake Creek (n=8), and Marsh Creek (n=3) also had genotypic and allelic frequencies for the p53 gene locus which indicates a more inbred population or non-random breeding. Further analysis with a larger sample size is needed to draw conclusions about the genetic diversity in the fish chosen for cryopreservation. The preliminary report on chinook salmon genetic diversity can be found in Appendix 6. The report will be updated annually with each year's salmon tissue samples.

The "ownership" of the genetic resources and responsibility for the timing and circumstances of attempts at restoration are complicated issues to be resolved. Many of the controversial practical and ethical issues related to captive breeding populations and their reintroduction into restored and changing natural habitats apply to restoration via genome banking (Corley-Smith and Brandhorst 1999). In the future, we believe that more requests will be made to use cryopreserved semen in hatchery production programs and in research. We recommend and support only the ethical use of cryopreserved genetic material from the germplasm repository. The judicious use of this vital genetic material is imperative. To that end, we provide criteria for accessing and using cryopreserved semen samples from the germplasm repository that will assist in rational use and inventory management. A database of the straw inventory has been established. Appendix 2 proposes a request form that will allow inventory management of the germplasm repository.

#### Other Gene Banking Efforts in the Snake River Basin

Idaho Department of Fish and Game have been cryopreserving Redfish Lake sockeye salmon as part of their captive sockeye program. They also freeze male gametes from chinook salmon from the Lemhi River, Yankee Fork and the East Fork Salmon River from their captive rearing project. IDFG were involved in cryopreserving the same chinook salmon gametes as the NPT at Sawtooth and Pahsimeroi hatcheries in 1999. The Tribe's captive broodstock evaluation project cryopreserves male gametes from the captive Lostine River, upper Grande Ronde River and Catherine Creek chinook salmon broodstocks at Bonneville Hatchery. Washington Department of Fish and Wildlife Snake River Lab freezes fall chinook salmon gametes from Lyons Ferry Hatchery and the Tucannon River.

#### RECOMMENDATIONS

The genetic diversity within existing chinook salmon and steelhead spawning aggregates is not replaceable and should be conserved to protect present and future options (NRC 1996). The recommendation of the gamete preservation project is to cryopreserve gametes from as many genetically diverse conservation units as possible. Coordination with other Tribal, state and federal agencies will continue to help identify unique spawning aggregates. Further genetic analysis may help separate different salmon and steelhead subpopulations.

Snake River chinook salmon and steelhead are in a downward spiral toward extinction. This is the case with the wild and hatchery fish. The gene bank has an inventory of both wild and hatchery salmonids, though many more hatchery fish, due to the ease of collections at a known time and location. Future collections should emphasize semen collection from wild/natural fish. A new, interesting opportunity for sampling steelhead gametes is collecting and stripping kelts (spawned-out fish) as they are handled for downstream passage at the lower Snake River dams.

The cryopreservation project would benefit from input from Tribal, state, and federal agencies to identify steelhead conservation units for gamete cryopreservation. We plan to formally request this information from the appropriate management agencies.

It would be more efficient to freeze the few wild fish gametes collected at streamside. This option of cryopreserving the gametes on dry ice, then nitrogen, will be explored in 2000. We will store the samples in a 32-liter dewar, then transfer the straws to the main repository at the end of the spawning period for that species.

Fertility trials are conducted after each species' spawning season as a standard practice. The trials involve fertilizing eggs with cryopreserved versus fresh sperm. Our field versus laboratory techniques will be evaluated in the same manner.

The goal of the gene bank is to preserve genetic diversity. Diversity can be quantified with analysis of the DNA from sampled fish. We recommend that the genetic analysis and report of results keep current with the cryopreservation of the gametes to make project evaluation possible. The means to preserving species genetic diversity is collection of at least 100 samples per year from each spawning aggregate, covering at least five collection years for chinook and at least four years for steelhead.

It is expected with the ESA listing of bull trout as a threatened species, that more requests for cryopreserving male gametes from this species will occur. It is wise to move proactively to cryopreserve genetic diversity while the spawning aggregates of these species are relatively healthy instead of reacting to threatened population levels. We recommend cryopreserving male gametes from bull trout in 2000. Delayed coordination with the U.S. Fish and Wildlife Service precluded bull trout cryopreservation efforts in 1999. We propose to collect gametes from some of the bull trout populations in 2000 after the fish spawn in the fall.

Currently a limited and relatively uncoordinated effort exists to preserve tissues, gametes and frozen viable cells (Ryder et al. 2000). We are being insightful in proposing a regional aquatic germplasm repository based in Moscow, Idaho. Sagacious managers of gene banks should coordinate at a national and worldwide level.

## SUMMARY

The cryopreservation project currently has a total of 930 chinook salmon and 241 steelhead semen samples in the germplasm repositories at the University of Idaho and Washington State University. Each university has backup freezer tanks, which act as a safeguard mechanism in case of primary tank failure. They also serve as long-term archival storage, which is not disturbed.

Semen collection should continue until sufficient genetic diversity from as many salmonid subpopulations as possible is represented in the germplasm repository. Fish in some streams such as Big Creek are low in abundance and may require a longer sampling period. Genetic analysis needs to continue annually as semen samples are collected for cryopreservation. Assessment of genetic resources can serve a valuable planning purpose in the overall

conservation effort, as well as in determination and management of the well being of species and the richness of ecosystems (Ryder et al. 2000).

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## APPENDIX

## APPENDIX 1

Table 1. Snake River Basin Chinook Salmon Samples Cryopreserved from 1992-1999.

| Spawning<br>Aggregate   | Number of Samples Cryopreserved, by Year |      |      |      |      |      |      |      | Total Number<br>of Samples<br>per Stream |
|-------------------------|--|------|------|------|------|------|------|------|--|
|                         | 1999                                     | 1998 | 1997 | 1996 | 1995 | 1994 | 1993 | 1992 |  |
| Lostine River           | 2  | 3    | 2    | 3    | 1    | 4    |      |      | 15                                       |
| Imnaha River            | 95                                       | 79   | 41   | 33   | 43*  | 21*  |      |      | 312                                      |
| Rapid River             | 68                                       | 92   |      |      |      |      |      |      | 160                                      |
| So. Fork Salmon River   | 88                                       | 45   | 45   | 19   |      |      |      |      | 197                                      |
| Lake Creek              | 6  | 3    | 4    | 3    |      |      |      |      | 16                                       |
| Johnson Creek           | 5  | 17   | 7    |      |      |      |      |      | 29                                       |
| Big Creek               | 0  | 1    | 6    | 0    | 0    | 0    | 10   | 7    | 24                                       |
| Capehorn Creek          | 0  | 6    | 2    |      |      |      |      |      | 8  |
| Marsh Creek             | 0  | 2    | 4    |      |      |      |      |      | 6  |
| Pahsimeroi River        | 31                                       |      |      |      |      |      |      |      | 31                                       |
| Upper Salmon River      | 40                                       | 41   | 51   |      |      |      |      |      | 132                                      |
| Total # samples by year | 335                                      | 289  | 162  | 58   | 44   | 25   | 10   | 7    | 930                                      |

\* denotes samples collected by ODFW and transferred to NPT repository in 1999.

Table 2. Snake River Basin Steelhead Cryopreserved from 1997-1999.

| Spawning<br>Aggregate         | Number of Samples<br>Cryopreserved, by Year |      |      | Total Number<br>of Samples<br>per Stream |
|-------------------------------|---|------|------|--|
|                               | 1999  | 1998 | 1997 |  |
| Little Sheep Creek            | 25  | 25   | 5    | 55                                       |
| North Fork Clearwater River   | 58  |      |      | 61                                       |
| Snake River<br>Oxbow Hatchery | 76  |      |      | 76                                       |
| Johnson Creek                 | 2   |      |      | 2  |
| Pahsimeroi Hatchery/River     | 47  |      |      | 47                                       |
| Total # samples by year       | 208   | 25   | 5    | 241                                      |

Table 3. Total number of straws in storage from 1992-1999.

| Spawning Aggregate          | Total Number 0.5 ml Straws, 1999 | Total Number 5.0 ml Straws, 1999 | Total Number of Straws,1992-1999 |
|-----------------------------|----------------------------------|----------------------------------|----------------------------------|
| North Fork Clearwater River | 1,167                            | 4                                | 1,171                            |
| Lostine River               | 60                               | 8                                | 741                              |
| Little Sheep Creek          | 310                              | 2                                | 671                              |
| Imnaha River                | 1,879                            | 64                               | 6,287                            |
| Rapid River                 | 1,674                            | 67                               | 4,080                            |
| South Fork Salmon River     | 2,516                            | 355                              | 5,958                            |
| Lake Creek                  | 211                              | 13                               | 522                              |
| Johnson Creek               | 210                              | 42                               | 1,104                            |
| Big Creek                   | 0                                | 0                                | 787                              |
| Capehorn Creek              | 0                                | 0                                | 263                              |
| Marsh Creek                 | 0                                | 0                                | 179                              |
| Pahsimeroi River            | 1,110                            | 0                                | 1,110                            |
| Upper Salmon River          | 1,194                            | 180                              | 4,180                            |
| Snake River, Oxbow          | 1,253                            | 13                               | 1,266                            |
| Totals                      | 11,584                           | 748                              | 28,319                           |

Table 4. Summary of 1999 fertility trial of cryopreserved chinook salmon semen.

| Hatchery Female | Hatchery Male | Number Eye-up | Number of Eggs | Relative Fertility <sup>1</sup> | Date Male Cryopreserved | Pre-freeze Motility |
|-----------------|---------------|---------------|----------------|---------------------------------|-------------------------|---------------------|
| Non-pooled eggs |               |               |                |                                 |                         |                     |
| Aberdeen 1      | Aberdeen 1    | 27            | 135            | - <sup>2</sup>                  | -                       | -                   |
| Aberdeen 2      | Aberdeen 1    | 37            | 63             | - <sup>2</sup>                  | -                       | -                   |
| Aberdeen 3      | Aberdeen 1    | 45            | 81             | - <sup>2</sup>                  | -                       | -                   |
| Aberdeen 1      | SFSR-170      | 9             | 114            | 40                              | 9-7-99                  | 90                  |
| Aberdeen 2      | SFSR-170      | 19            | 91             | 36                              | 9-7-99                  | 90                  |
| Aberdeen 3      | SFSR-170      | 38            | 113            | 61                              | 9-7-99                  | 90                  |
| Aberdeen 1      | RR-372        | 5             | 129            | 20                              | 8-30-99                 | 90                  |
| Aberdeen 2      | RR-372        | 5             | 85             | 10                              | 8-30-99                 | 90                  |
| Aberdeen 3      | RR-372        | 21            | 112            | 34                              | 8-30-99                 | 90                  |
| Aberdeen 1      | PAH-509       | 4             | 121            | 15                              | 9-8-99                  | 90                  |
| Aberdeen 2      | PAH-509       | 11            | 87             | 22                              | 9-8-99                  | 90                  |
| Aberdeen 3      | PAH-509       | 37            | 105            | 63                              | 9-8-99                  | 90                  |
| Aberdeen 1      | RR-334        | 7             | 123            | 30                              | 8-23-99                 | 90                  |
| Aberdeen 2      | RR-334        | 33            | 116            | 49                              | 8-23-99                 | 90                  |
| Aberdeen 3      | RR-334        | 69            | 116            | 105                             | 8-23-99                 | 90                  |
| Aberdeen 1      | SFSR-143      | 6             | 105            | 30                              | 9-3-99                  | 90                  |
| Aberdeen 2      | SFSR-143      | 15            | 97             | 25                              | 9-3-99                  | 90                  |
| Aberdeen 3      | SFSR-143      | 53            | 122            | 77                              | 9-3-99                  | 90                  |
| Pooled eggs     |               |               |                |                                 |                         |                     |
| A               | Aberdeen 1    | 73            | 97             | - <sup>2</sup>                  | -                       | -                   |
| B               | Aberdeen 1    | 71            | 100            | - <sup>2</sup>                  | -                       | -                   |
| A               | RR-174        | 28            | 95             | 40                              | 8-16-99                 | 80                  |
| B               | RR-174        | 34            | 94             | 51                              | 8-16-99                 | 0                   |
| A               | RR-178        | 43            | 98             | 58                              | 8-16-99                 | 90                  |
| B               | RR-178        | 41            | 96             | 60                              | 8-16-99                 | 90                  |
| A               | RR-293        | 32            | 97             | 44                              | 8-23-99                 | 80                  |
| B               | RR-293        | 33            | 95             | 48                              | 8-23-99                 | 80                  |
| A               | RR-298        | 39            | 106            | 49                              | 8-23-99                 | 80                  |
| B               | RR-298        | 16            | 89             | 25                              | 8-23-99                 | 80                  |

<sup>1</sup> Relative fertility = percent eyed/percent eyed in control

<sup>2</sup> Control group, second day fertilization

APPENDIX 2. Snake River Germplasm Repository Cryopreserved Semen Request Form

Snake River Germplasm Repository Committee  
P.O. Box 1942, 125 South Mission St  
McCall, ID 83638  
Phone: (208) 634-5290  
Fax: (208) 634-4097

**Snake River Germplasm Repository Cryopreserved Semen Request Form**

Name: \_\_\_\_\_ Affiliation: \_\_\_\_\_  
Phone number: (\_\_\_\_\_) \_\_\_\_\_ Address: \_\_\_\_\_  
Date of request: \_\_\_\_\_ Date need by: \_\_\_\_\_  
Species/stock requested: \_\_\_\_\_ Hatchery or wild/natural: \_\_\_\_\_  
Number of individuals: \_\_\_\_\_ Number of straws needed: \_\_\_\_\_ 0.5ml \_\_\_\_\_ 5.0ml  
Reason for request (clearly demonstrate need or type of hatchery program): \_\_\_\_\_

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Fertilization experience using cryopreserved semen: \_\_\_\_\_

\_\_\_\_\_  
\_\_\_\_\_

Name, address, and phone number of person samples should be delivered to: \_\_\_\_\_

\_\_\_\_\_  
\_\_\_\_\_

Please use additional pages as necessary.

The salmon managers of the Snake River Basin are concerned with how cryopreserved samples are being used and retains the right to refuse samples for inappropriate use of the threatened salmonid species gametes. The Nez Perce Tribe can arrange to deliver and assist in the fertilization of eggs. Please call Robyn Armstrong at the McCall Field Office (address above) to coordinate transfer. The Nez Perce Tribe also may request data on the performance of the semen (percent of eggs fertilized, post-thaw sperm motility, etc.).

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

APPENDIX 3. Genetic Analysis of Threatened Chinook Salmon Populations from the Snake River Basin.

# Genetic Analysis of Threatened Chinook Salmon (*Oncorhynchus tshawytscha*) Populations from the Snake River Basin.

By M. S. Powell and J. C. Faler. Center for Salmonid and Freshwater Species at Risk, Hagerman Fish Culture Experiment Station / University of Idaho

## EXECUTIVE SUMMARY

Spring/summer chinook salmon (*Oncorhynchus tshawytscha*) collected from ten locations within the Snake River drainage were examined using three molecular genetic methods. Analysis of mitochondrial DNA variation among the sample locations revealed five composite haplotypes with one haplotype (designated Ot-01) observed in all locations. Nuclear DNA was examined for restriction fragment length polymorphisms within the p53 gene locus and revealed two alleles among sample locations. Intron D of the chinook salmon growth hormone 2 gene was also screened for restriction site polymorphisms but no variation was observed. Two microsatellite loci designated Pu-RBT and 77-RBT (approximating PuPuPy and Omy-77 in published literature) were examined and found to have a total of 11 and 9 alleles among all samples, respectively.

Estimates of nucleotide diversity and divergence were calculated for all locations. Diagrammatic representations of inferred evolutionary relationships among observed mitochondrial haplotypes and sample locations (dendrogram) were constructed based upon genetic distances. Statistical evaluation of geographic heterogeneity among haplotype distributions (population structure) was also performed. These tests and estimates were done to demonstrate the utility of this investigation and the methods used and do not necessarily reflect statistically robust conclusions for all sample locations. In brief, sample sizes from 6 of the 10 locations consisted of  $N \leq 15$  and any conclusions drawn from such data should be considered preliminary.

## INTRODUCTION

In a 1994 final ruling, remaining Snake River spring and summer chinook salmon runs in the Snake River Basin were listed as threatened under the Endangered Species Act (NMFS, 1995). Subsequently, conservation measures including captive propagation programs and the cryopreservation of sperm from returning males were instituted by state and federal agencies and Native American tribes for several subpopulations in low abundance. Additional subpopulations within the designated Snake River spring/summer ESU, summarized in the NMFS (1998) status review, will likely require the intervention of captive propagation in the near future to prevent extirpation.

Captively raised chinook subpopulations within the ESU are currently kept separated in all ongoing programs. Milt from captively reared males and from anadromous males returning to several spawning locations has been collected and cryopreserved. Genetic differentiation and reproductive isolation among many of these Snake River spring/summer ESU subpopulations has been previously examined using protein electrophoresis (see NMFS, 1998). Allele frequency data from those studies were used in part to describe genetic groups for future conservation management.

However, some captive propagation programs have used cryopreserved milt to augment

frequently skewed sex ratios of returning adults or to theoretically increase the effective population size ( $N_e$ ) without the benefit of current genetic information. This policy may compound the genetic and demographic risks associated with these small populations and thus effect the long-term persistence of the ESU overall. While the cryopreservation of milt from threatened chinook salmon populations should be considered an essential part of recovery efforts, the judicious use of such vital genetic material is also important. This preliminary study uses three different molecular methods to genetically identify tissue samples taken from chinook salmon where milt was cryopreserved and infer possible evolutionary relationships among them. This information is intended to facilitate the future use of cryopreserved sperm in Snake River chinook salmon recovery efforts.

## METHODS

### Collections

Employees of the Nez Perce Tribe collected tissue samples from 250 spring/summer chinook salmon from 10 locations within the Snake River Basin. Samples were stored in ethanol until they were transferred to the University of Idaho and DNA extractions were performed.

### DNA Extraction

Extraction of DNA was accomplished using methods modified from Sambrook et al., (1989) and Dowling et al. (1990). Approximately 100 mg of tissue was excised from each sample, transferred to a 1.7 µl tube, homogenized in 650 µl of digestion buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 50 mM EDTA, 1% sodium dodecyl sulfate, 0.2% dithiothreitol, 0.5% Proteinase K), and incubated overnight at 55°C. Following overnight digestion, the samples were extracted twice with equal volumes of chloroform/isoamyl alcohol, the aqueous phase was removed, and DNA precipitated with 1/10 volume of 3 M ammonium acetate and 2 volumes of cold 100% ethanol. DNA was centrifuged at 13,000 rpm in a microfuge for 10 min and the resulting pellet was washed twice in 500 µl of 70% ethanol, dried at 37°C and resuspended in 100 µl of 1X TE.

### Mitochondrial DNA

The polymerase chain reaction (PCR) was used to amplify sequences using a thermocycler profile of 95°C for 45 sec, 48°C for 40 sec, 70°C for 2 min 30 sec, for 38 cycles followed by 3 min at 70°C. Nucleotide primers specific for the mitochondrial NADH dehydrogenase subunit 1 and 5/6 gene regions were used in a 40 µl reaction volume containing 1-3 µl sample DNA, 16 pmol of primers, 1 mM dNTPs, 0.5 U *Thermus aquaticus* (Taq) DNA polymerase, and 1X reaction buffer supplied by the manufacturer (Perkin-Elmer Corporation). Primers were generally designed for salmonids and amplified well for chinook samples (ND1, #381 5' ACC CCG CCT GTT TAC CAA AAA CAT<sup>3'</sup> and #563-B 5' GGT TCA TTA GTG AGG GAA GG<sup>3'</sup>; ND 5/6, #763 5' AAT AGC TCA TCC ATT GGT CTT AGG<sup>3'</sup> and #764 5' TAA CAA CGG TGG TTT TTC AAG TCA<sup>3'</sup>). Amplified mitochondrial DNA gene regions were digested using 5 restriction endonucleases *Ase* I, *Dde* I, *Hae* III, *Rsa* I, and *Bst*U I. The resulting mtDNA fragments were separated by electrophoresis using 3% agarose/TAE gels. Vertical 6% polyacrylamide/TBE gels were used to separate small fragments and questionable co-migrating fragments. Gels were stained with ethidium bromide and restriction fragment patterns visualized using UV light then photographed. Restriction fragment length polymorphisms (RFLPs) observed among samples were given alphabetical designations as simple haplotypes. The size of

each DNA fragment from each mtDNA gene region was estimated by comparison to a size standard, pUC-19 marker (Bio-Synthesis). Alphabetical designations for RFLPs of each mtDNA gene region were combined into composite mtDNA haplotypes. An estimate of the number of nucleotide substitutions per site ( $p$ ) for each RFLP was calculated via the Nei (1987) method using REAP ver. 4.0 (Restriction Enzyme Analysis Package) (McElroy et al., 1991) and was then used to generate a matrix comparing  $p$  values (distance) between all pairs of identified composite haplotypes. The KITSCH program in PHYLIP ver. 3.5 (Felsenstein, 1993) which assumes independence and equal rates of divergence was used to generate an distance dendrogram using the least-squares method of Fitch and Margoliash (1967) to illustrate the estimated evolutionary relationships and distance among the identified composite haplotypes. The extent of geographic heterogeneity among population frequency distributions was examined using a Monte Carlo simulation of a chi-square analysis with 1000 iterations (Roff and Bentzen, 1989; MONTE program in REAP ver. 4.0). Nucleotide diversity and divergence among and within populations was estimated using Nei (1987) equations 10.19, 10.7, 10.20, and 10.21 in the DA program of REAP ver. 4.0. The resulting matrix of nucleotide divergence among populations was used to construct a neighbor-joining tree using NTSYS-pc 1.80 (Roff, 1993).

#### Nuclear Gene Loci p53 and GH2

The polymerase chain reaction was used to amplify both nuclear sequences using thermocycler profiles listed in Park et al (1995, 1996). Nucleotide primers specific for the gene regions were used in a 40  $\mu$ l reaction volume again containing 1-3  $\mu$ l sample DNA, 16 pmol of primers, 1 mM dNTPs, 0.5 U *Thermus aquaticus* (Taq) DNA polymerase, and 1X reaction buffer supplied by the manufacturer (Perkin-Elmer Corporation). Primers were constructed for chinook salmon from published sources and the sequences are listed in Park et al. (1995) for intron D of growth hormone 2, (GH2) and Park et al. (1996) for the p53 locus. Amplified p53 and GH2 gene regions were digested using restriction endonucleases *Hha* I and *Dra* I, respectively. The resulting DNA fragments were separated by using gel electrophoresis as described above and compared to published fragment sizes.

Genotypic and Allelic frequencies were calculated for all sample locations and examined using  $X^2$  analyses to test for Hardy-Weinberg equilibrium (HWE). The program, Genetic Data Analysis ver. 12 (Lewis and Zaykin, 1999) was used to calculate descriptive statistics, F-statistics and genetic distance matrices for the nuclear gene alleles.

#### Microsatellite Loci

The polymerase chain reaction was used to amplify two microsatellite sequences using thermocycler profiles listed in Morris et al (1996). Nucleotide primers for the microsatellite regions were used in a 10  $\mu$ l reaction volume containing 10 ng sample DNA, 0.5  $\mu$ M of primers, 0.2 mM dNTPs, 0.5 U *Thermus aquaticus* (Taq) DNA polymerase, and 1X reaction buffer supplied by Perkin-Elmer Corporation. Primers were constructed for chinook salmon from sequences of rainbow trout DNA published in Morris (1996) and approximate the primers used for the microsatellite loci *Omy-77* and PuPuPy. Amplified microsatellite fragments were separated and analyzed using a Perkin-Elmer ABI 310 Genetic Analyzer and compared to published fragment sizes for *Omy-77* and PuPuPy.

Genotypic and Allelic frequencies were calculated for all sample locations. The program,

Genetic Data Analysis ver. 12 (Lewis and Zaykin, 1999) was used to calculate descriptive statistics for the microsatellite alleles.

## RESULTS

### Mitochondrial DNA

Table 1 shows the two mitochondrial gene regions that were PCR amplified and digested with restriction enzymes and how the polymorphisms observed were combined into composite haplotypes. A total of 5 composite mitochondrial restriction fragment length polymorphisms (composite haplotypes, henceforth referred to simply as haplotypes) were observed among the 250 samples examined. Table 2 lists the sample locations, the number of samples from each location and the distribution of observed haplotypes among them. The relative frequency of each haplotype at each location is shown diagrammatically in Figure 1. The most common haplotype, Ot-01, was observed in samples from all locations and ranged in frequency from 0.600 – 1.000. Three of the 10 sample locations were fixed for this haplotype, Lake Creek, Marsh Creek, and Salmon River/Sawtooth Hatchery. Haplotype Ot-02 resulting from a restriction site polymorphism in the ND 5/6 gene region was observed in samples from 4 locations, Big Creek, Cape Horn Creek, Looking Glass Hatchery and Rapid River Hatchery. This haplotype ranged in frequency from 0.125 in the Cape Horn Creek samples to 0.200 in samples from Big Creek. Haplotypes Ot-03 and Ot-04 were observed in samples from 3 locations, Looking Glass Hatchery, Rapid River Hatchery, and the South Fork of the Salmon River. These haplotypes were lowest in frequency and ranged from 0.014 for Ot-04 in Lookingglass Hatchery samples to 0.098 for Ot-03 in Rapid River Hatchery samples.

The percent sequence divergence calculated between identified composite haplotypes and standard errors for each distance value are reported in the matrix shown in Table 3. These values range from 0.63% between Ot-01 and Ot-04 to 7.13% between Ot-03 and Ot-05. Using the least-squares method of Fitch and Margoliash (1967), a dendrogram representing genetic distance among haplotypes is diagrammed in Figure 2. In this instance, Ot-02 and Ot-03 cluster together as well as Ot-01 and Ot-04. Haplotype Ot-05 clusters with other identified haplotypes most distantly using this method.

Nucleotide divergence calculated from REAP was used as input to diagram genetic relationships among sample locations (populations) using neighbor-joining (Roff, 1993). The resulting dendrogram in Figure 3, clusters samples from Big Creek, Lostine River, and Johnson Creek together. Haplotype Ot-05 was observed in samples from these locations. Samples from Lake Creek cluster with Marsh Creek and samples from the Salmon River/Sawtooth Hatchery. Samples from these three locations were fixed for haplotype Ot-01. Looking Glass Hatchery samples clustered together with samples from Rapid River Hatchery. These two locations share haplotypes Ot-01, Ot-02, Ot-03, and Ot-04. A test of geographic heterogeneity among haplotype frequency distributions using MONTE was significant at  $P < 0.05$  ( $X^2 = 54.76$ ).

### Nuclear Gene Loci p53 and GH2

Table 4. shows genotypic and allele frequencies for the p53 locus at each location. Two alleles (a restriction site difference) were observed for p53 and are the same as described in Park (1996). One location, Marsh Creek was fixed for the “A” allele. Both alleles were observed in the

remaining populations with “A” in higher frequency (66.7% to 100.0%) than “B” (0.0% to 33.3%). Chi square analysis of genotype frequencies among p53 alleles did not show any population with a significant over abundance of homozygous individuals (i.e. a null hypothesis of HWE was not rejected in any instance).

The GH2 gene region was screened for polymorphisms using the restriction enzyme *Dra* I as previously described in Park (1995) but no polymorphisms were detected among the samples thus far examined.

#### Microsatellite Loci

Numerous alleles were observed for both microsatellite loci examined. A total of 11 alleles were observed for the PuPuPy locus and 9 alleles were observed for the *Omy-77* locus among the samples. Most individuals from the sample locations were heterozygous for both PuPuPy and *Omy-77* (i.e. *H* approached 100%). In some locations, such as Marsh Creek, every sample was heterozygous. Accounting for minor size differences typically encountered between laboratories, the sizes of alleles and variance did correspond, in general, with previously published size ranges for each loci (Banks et al, 1996; Morris et al, 1996). However, though descriptive statistics were calculated for both microsatellite loci, they not reported because the abundance of heterozygous individuals adversely effect the outcome of the data and in most cases meaningful calculations were not possible.

#### DISCUSSION AND RECOMMEDATIONS

The concordance of multiple data sets provides the strongest evidence in the analysis of population structure and intraspecific hierarchy. In this preliminary study three molecular genetic methods were used, mitochondrial DNA variation, nuclear gene variation and microsatellite variation.

Mitochondrial haplotype diversity among the ten sample locations was relatively modest with only 5 composite haplotypes identified. This is most likely a result of both a small sample number from several locations and from the relatively few restriction enzymes (5) used to screen for restriction fragment length polymorphisms (i.e. there were a small number of nucleotides examined overall). However, the analysis does show patterns similar to those observed in other chinook populations and in other species (Powell and Faler, unpublished data). Typically, salmonid populations are dominated by one or two haplotypes that account for approximately 80% of the individuals examined. Minor haplotypes, those with a frequency less than 5% (typically around 1-2%) account for the remaining individuals surveyed. In the 4 locations with the largest number of samples, Lookingglass Hatchery (N=71), Rapid River Hatchery (N=51), Salmon River/Sawtooth Hatchery (N=36) and South Fork Salmon River (N=44) this pattern is evident. Haplotype Ot-01 accounts for 78.4 – 100.0% of the individuals in those populations. As expected, the Rapid River and Lookingglass Hatchery populations had the highest diversity of haplotypes observed, followed by samples from the South Fork Salmon River.

From Figure 2, the dendrogram showing the estimated relationships of the observed haplotypes, Ot-03 and Ot-02 are closely related. The difference between these two haplotypes arising from a polymorphism in the ND5/6 gene region. Haplotypes Ot-02 and Ot-03 share a polymorphism in

the ND 1 region (when digested with *Rsa* I) that is not present in the other haplotypes. Haplotype Ot-04 appears to be a minor haplotype closely related to Ot-01. The difference in these two haplotypes arising from a polymorphism in the ND 1 gene region when digested with *Hae* III. Haplotype Ot-05 is the most distant haplotype from Ot-02 and Ot-03. The percent sequence divergence of 5.04% and 7.13% respectively is higher than commonly observed. As a general rule, haplotypes don't vary more than 1-2% in sequence divergence intraspecifically among salmonids (Ferris and Berg, 1987 and references therein). Again, the small number of restriction sites surveyed may have had an influence and resulted in an over estimate of sequence divergence. Haplotype Ot-05 was observed in the sample locations, Big Creek, Lostine River and Johnson Creek. Not surprisingly these three locations were clustered together in the neighbor-joining tree produced from nucleotide divergence estimates (Figure 3). The Rapid River and Lookingglass hatcheries also clustered together in this dendrogram. Cape Horn Creek clusters with these hatchery samples more distantly. These examples point out that while the neighbor-joining tree did show some resolution (as with Rapid River and Lookingglass hatcheries) it also placed some geographically distant locations together based upon shared haplotypes. The Monte-Carlo chi-square analysis of haplotype frequency distributions though used especially for small class sizes may not be very robust in this instance because of the few samples from some locations. In summary, mitochondrial haplotype variation and diversity can help to resolve questions regarding the genetic relatedness of spring/summer chinook salmon in the Snake River. However, the distinctions at this point are weak. Larger sample sizes from most locations are needed. Samples from different year classes are needed, and a greater number of nucleotides need to be surveyed. The utility of genetically identifying cryopreserved milt for use in captive breeding programs will be best served when we also have the opportunity to survey the populations they come from with statistical power.

The nuclear genes surveyed (p53 and GH2) show promise in being able to provide F statistics and heterozygosity values without having to sacrifice animals as with allozyme analysis. This is especially important for some cryopreserved milt samples when there are no corresponding tissue samples remaining. In this study GH2 was invariant when it was screened for polymorphisms with *Dra* I. However, it may show variability when cut with other restriction enzymes. The p53 locus was fixed in only one location. Samples from Marsh Creek (N=3) were fixed for the "A" allele. This is likely due to the general abundance of the "A" allele among all sample locations (frequencies of 66.7-100%) coupled with the very small sample size. Chi-square analysis was used to test for departures from random mating. An over abundance of homozygous individuals can result from non-random mating and this phenomenon can be observed because Hardy-Weinberg expectations are violated (see Hartl and Clark, 1997 for a review). It is a relatively weak test but one location, the Salmon River/Sawtooth Hatchery, come relatively close to falsifying the null hypothesis of HWE at 3.44 ( $P < 0.05$ ). In summary, the nuclear genes used have great potential for genetically identifying chinook salmon. In this instance a "panel" of perhaps 8-12 of these nuclear gene markers would make a very robust analysis. As it is, heterozygosity can be calculated for the single variable marker p53 but, one variable marker would not be a good representative of the overall variation.

Our current analysis of microsatellite variation in these samples was disappointing from a population analysis standpoint. The level of polymorphism observed among both loci was higher than unexpected. A previous study (Banks et al., 1996) used these two loci successfully

to develop population level information on fall chinook salmon in the Sacramento River. The primers used in this study to PCR amplify these loci were constructed from sequences reported by Morris et al. (1996). The only differences in primers were minor modifications made to reflect chinook sequences rather than the original rainbow trout (*Oncorhynchus mykiss*) sequences. The size of microsatellite repeats obtained from the primers generally follow the size ranges previously reported Morris et al. (1996). We have begun to examine fall chinook populations with these two microsatellite loci and thus far they are showing roughly the same number of alleles (Powell and Faler, unpublished data). The utility of these two microsatellite loci for population level analysis may be increased with larger sample sizes from each location. Likewise, minor sequence modifications or other technical modifications may yield different results. It should be noted that the utility of these two microsatellite loci might be borne out in kinship and parentage analyses. Such is the level of variation needed to construct breeding matrices and pedigrees for captive animals. Presuming all the alleles observed at these two loci are Mendelian inherited and are not procedural or genetic anomalies (i.e. mispriming, stutter, or null alleles etc.), the number of all possible genotypes at the PuPuPy locus would be 177,147 and the number of possible genotypes for the *Omy-77* locus would be 19,683. In summary, these microsatellite markers are probably best employed in pedigree and kinship analyses. Additional microsatellite markers are becoming available for chinook salmon (see Banks et al., 1999) and will likely be very useful in future analysis of spring/summer chinook populations in the Snake River.

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APPENDIX 4. Genetic Diversity in *Oncorhynchus mykiss* from the Snake River Basin and Eastern Oregon.

# GENETIC DIVERSITY IN *Oncorhynchus mykiss* FROM THE SNAKE RIVER BASIN AND EASTERN OREGON

A report to the Nez Perce Tribe December 1999  
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## INTRODUCTION AND OVERVIEW

The following report discusses the genetic diversity of steelhead trout (*Oncorhynchus mykiss*) collected by the Nez Perce Tribe for cryopreservation of male gametes. We have utilized both mitochondrial DNA D-loop sequencing and four microsatellite loci to discern genetic diversity and the relationship among the populations of interest. The genetic markers chosen for this analysis coincide with those utilized in other population level analyses for *Oncorhynchus mykiss*, particularly for those aimed at differentiating steelhead and rainbow trout in California, Oregon, Washington, and Alaska (Nielsen et al., 1994a, 1994b, 1997; Wenburg et al., 1997; Nielsen and Fountain, 1999; Nielsen, 1999). These markers are putatively neutral, have higher rates of mutation than expressed genes, and thus can provide fine-level differentiation between closely related populations of organisms (Nielsen, 1999 and references therein).

Many of the *O. mykiss* sampled for this study originated from hatchery adult steelhead that returned to spawn and were captured at hatchery weirs. Included in this study are fish from Dworshak National Fish Hatchery, Oxbow Hatchery, Pahsimeroi Hatchery, and Johnson Creek in Idaho, and Little Sheep Creek (Imnaha River) in northeastern Oregon (Table 1). Additionally, redband trout populations were included in this study to evaluate the relationships of eastern Oregon *O. mykiss* with Snake River steelhead trout. Dworshak hatchery was established to mitigate losses of North Fork Clearwater River drainage steelhead following installation of Dworshak dam. Oxbow and Pahsimeroi are Idaho Power funded hatcheries run by Idaho Department of Fish and Game personnel to mitigate for salmon and steelhead losses due to the building of the Hells Canyon Complex of dams. Each of the hatcheries has its own unique history regarding stock establishment for hatchery populations. With this in mind, we have attempted to discuss the putative relationships of these hatchery stocks, as well as the relationship of natural Pahsimeroi River steelhead with all other populations.

**Table 1.** Population information for genetic study of *O. mykiss* in Idaho and Oregon.

| <b>Population</b>        | <b>Collection site</b>             | <b>Age</b>                      | <b>Historical makeup</b>                          | <b>Population information</b>                                  |
|--------------------------|------------------------------------|---------------------------------|---|--|
| Clearwater (CW)          | Dworshak Nat. Fish Hatchery        | Returning adults                | North Fork Clearwater River                       | B-run, no wild fish for hatchery spawning                      |
| Oxbow, Snake River (OX)  | Hells Canyon Dam                   | Returning adults                | Snake River                                       | Early returning A- and B-run, some wild fish                   |
| Pahsimeroi (PR)          | Pahsimeroi Hatchery and river weir | Returning adults and wild kelts | Snake River                                       | A-run, hatchery not listed, ESA-listed wilds released to spawn |
| Johnson Creek (JC)       | Johnson Creek screw trap (NPT)     | Juvenile                        | South Fork Salmon River Basin                     | Resident rainbows or steelhead?                                |
| Little Sheep Creek (LSC) | Little Sheep Creek weir            | Returning adults                | Little Sheep Creek, Innaha River                  | A-run, ESA-listed few wild fish                                |
| Home Creek (HC)          | Home Creek, SE Oregon              |                                 | Wild redband trout, plus Hatchery stocked rainbow |  |
| Blitzen River (BL)       | Blitzen River, SE Oregon           |                                 | Wild redband trout, plus Hatchery stocked rainbow |  |
| Three Mile Creek (TM)    | Three Mile Creek, SE Oregon        | Spawning adults                 | Wild redband trout                                | Protected  |

## MATERIALS AND METHODS

### *Tissue collection and DNA extraction*

Steelhead trout (*Oncorhynchus mykiss*) fin tissue samples were collected in Idaho and Oregon at collection weirs and traps during spring spawning by the Nez Perce Tribe. These samples were collected at hatcheries located on the Snake River, Pahsimeroi River, North Fork Clearwater River, Little Sheep Creek (Oregon), and Johnson Creek. A summary of the origin and makeup of these populations is provided in Table 1. Most fish collected were of hatchery origin with the exception of seven unclipped 'wild' fish from Pahsimeroi River and Johnson Creek. NPT samples were collected from February to May 1999. Redband trout samples were collected independently by hook and line sampling in southeastern Oregon. Blitzen River and Home Creek samples were collected in July of 1999. Three Mile Creek samples were collected in May of 1995.

Tissue samples were collected using either opercular punches or caudal fin clips. Tissue was immediately placed in 6 ml tubes containing 80% ethanol for storage and shipment. Total genomic DNA was extracted from 1 mm<sup>2</sup> tissue samples using the Puregene<sup>®</sup> DNA Isolation Kit D-5000A and solid tissue protocol (Gentra Systems). Extracted DNA was then quantified using the Hoefer DNA fluorometer Model TKO 100 using Hoechst dye. Samples were diluted in Tris-EDTA (TE) to 50ng/μl prior to PCR amplification.

### *Mitochondrial Sequencing*

PCR amplification of the highly variable 3' end of the mitochondrial control region was carried out using primers known to amplify this region in salmonids (Nielsen et. al., 1994, and references therein). The two primers used were S-phe (5'-GCTTTAGTTAAGCTACG-3') and P2 (5'-TGTTAAACCCCTAAACCAG-3'). The 193 bp amplified product includes the 3' end of the control region along with 5 bp of the adjacent phenylalanine tRNA gene. Double stranded PCR amplifications were carried out in 40  $\mu$ l reactions containing 8.0  $\mu$ l 5X Buffer C (300mM Tris-HCL, 75mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 12.5mM MgCl<sub>2</sub>), 3.2  $\mu$ l 10mM dNTP's (2.5mM dATP, 2.5mM dCTP, 2.5mM dGTP, 2.5mM dTTP), 0.8  $\mu$ l DMSO, 6.0  $\mu$ l of each primer (4 pM/ $\mu$ l), 13.3  $\mu$ l ddH<sub>2</sub>O, 0.2  $\mu$ l *Taq* DNA polymerase (5 U/ $\mu$ l, GibcoBRL). Amplifications were performed in an AMPLITRON<sup>®</sup> II (Thermolyne) for 40 cycles of denaturation at 95°C for 50 s, annealing at 55°C for 50 s and extension at 72°C for 2 min 30 s with a 4°C chill upon completion.

Amplified double stranded products (5  $\mu$ l) were then electrophoresed on 1% agarose gels. Gels were stained with an ethidium bromide solution and visualization of DNA was performed using UV trans-illumination. Successfully amplified products were purified using the GeneClean III (Bio 101, Inc.) for use in the second PCR reactions to produce single stranded DNA for sequencing. Single stranded DNA amplification was carried out using fluorescent dye-terminator biochemistry. The cycle sequencing reaction mixture contained 2.0  $\mu$ l terminator dye premix (Perkin Elmer/ABI), 1.0  $\mu$ l DMSO, 0.5  $\mu$ l primer (4 pM/ $\mu$ l), 3.5  $\mu$ l ddH<sub>2</sub>O, 2.0  $\mu$ l 2.5X Sequence Buffer (5 mM MgCl<sub>2</sub>, 200mM Tris-HCl), and 1.0  $\mu$ l purified DNA for a total volume of 10.0  $\mu$ l. Sequencing reactions were run for 25 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 15 s, and extension at 60°C for 4 min. with a 4°C chill upon completion. After cycle sequencing, excess unincorporated dye terminators were removed by running samples through 400  $\mu$ l of Sephadex G-50 in Centri-Sep spin columns (Princeton Separations, Inc.) and vacuum dried. Dried samples were resuspended in 1.0  $\mu$ l of loading buffer (five parts deionized formamide to one part 30 mg/ml Blue Dextran). DNA sequencing was performed on an ABI 377 automated sequencer using 6% acrylamide gel. DNA sequences were assembled and analyzed using the Sequencher<sup>™</sup> 3.1 computer program (Gene Codes Corporation).

### *Microsatellites*

Four microsatellite loci were chosen based upon their polymorphisms and use in studies of other *O. mykiss* populations. Omy77, One2, One6, and One8 were used and have been named previously according to the species in which they were isolated; Omy was isolated in *O. mykiss* and One were isolated in *Oncorhynchus nerka*. Microsatellite loci were amplified from fluorescent labeled forward and unlabeled reverse primers developed previously for Omy77 (Morris et al., 1996) and One2, One6, and One8 (Scribner et al., 1996). Omy77 and One2 were amplified in single reactions alone, while One6 and One8 were amplified in the same PCR reaction. Polymerase chain reaction (PCR) was performed in a total volume of 20  $\mu$ L containing 2.5 mM MgCl<sub>2</sub> for Omy77 and One6,8 duplex or 1.5 mM MgCl<sub>2</sub> for One2, 1X PCR buffer without MgCl<sub>2</sub> (Gibco BRL), 250  $\mu$ M each of dATP, dCTP, dGTP, dTTP, 0.25  $\mu$ L of *Taq* DNA polymerase (5 units/ $\mu$ L), and 100 ng of sample DNA. Primer concentrations were 0.125  $\mu$ M for One2, 0.05  $\mu$ M for One6 and 0.1  $\mu$ M for One8 in duplex, and 0.075  $\mu$ M for Omy77. The PCR

profile for Omy77 amplifications was 95°C for 3 minutes (pre-dwell), 35 cycles of 1 minute at 95°C (denature), 1 minute at annealing temperature, 2 minutes at 72°C (extend), followed by 5 minutes at 72°C (post-dwell). The annealing temperature was 51°C for Omy77. One2 and the One6, One8 duplex were run in 'touchdown' PCR conditions with the same pre-dwell, denature, extension, and post-dwell parameters, but with the 1 minute annealing steps as follows: 2 cycles each at 62°C, 60°C, and 58°C followed by 30 cycles at 55°C anneal.

Microsatellite alleles for each sample and locus were separated by 5% denaturing polyacrylamide gel electrophoresis on the ABI 377 Sequencing system (Perkin Elmer). Prior to gel electrophoresis, samples were diluted in deionized formamide, blue dextran dye, and Genescan ROX-500 and denatured for 2 minutes at 95°C. Sizing of microsatellite alleles was determined with Genescan ROX-500 size standard run within each sample and analyzed with Genescan and Genotyper software (Perkin Elmer). Allele sizes in base pairs include the total size of the PCR product. Allele frequencies for each population were calculated in F-STAT (Goudet, 1999) for each locus. Observed and expected heterozygosities ( $H_o$  and  $H_e$ , respectively) were calculated with Genetic Data Analysis (GDA) software (Lewis and Zaykin, 1999) for each locus and over all loci for each population. Using F-STAT, alleles were randomized across populations to assess deviation from expected heterozygosity under Hardy Weinburg equilibrium. The proportions of randomizations giving larger  $H_e$  than  $H_o$  were calculated and are reported as significance levels for testing whether observed and expected heterozygosities are significantly different. Significance level has been set at  $p=0.05$ . The genetic distance between pairs of samples was calculated according to Nei (1987) and unweighted pair-group method with arithmetic mean (UPGMA) genetic distance trees were drawn in GDA. Data were analyzed both with separated and combined Pahsimeroi hatchery and natural fish.

## **RESULTS and DISCUSSION**

### *Mitochondrial Haplotypes*

Using the conserved salmonid primers S-phe and P2, mitochondrial DNA of 101 samples from five populations throughout the Snake River Basin were successfully sequenced. Amplification of this highly variable 3' end of the mitochondrial control region produced a fragment 232 base pairs long including primers. This region has been studied extensively in southern steelhead by Nielsen et. al. (1994a, 1994b, 1997) and correspond to base pairs 965 to 1149 in Digby et. al. (1992) plus 5 base pairs of the adjacent phenylalanine tRNA gene. The analyzed sequence includes 193 base pairs containing four variable sites in the studied populations. These variable sites revealed five mitochondrial haplotypes (Table 2) with maximum sequence divergence between haplotypes at 1.6% and a mean distance of 0.9% (Table 3). Two of the haplotypes, ST19 and ST21, are unique to the populations studied. ST19 was found in populations from the Little Sheep Creek, Oxbow Hatchery and Pahsimeroi River Wild samples. A single individual from Oxbow Hatchery had haplotype ST21. The most common haplotype found in the study was ST1, which was found in 54.5% of the population. The remaining haplotype frequencies were ST9 25.7%, ST19 9.9%, ST2 8.9%, and ST21 1.0%.

**Table 2.** Mitochondrial Haplotype Variability for *Oncorhynchus mykiss* collected from the Snake and Salmon River drainages. \*Numbers correspond to Digby et al., 1992. Variable nucleotide sites are indicated in red. ST19 and ST21 are mitochondrial haplotypes unique to study (shaded).

|       |    | Variable sites* |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
|-------|----|-----------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|       |    | *               | 1021 | 1032 | 1042 | 1050 | 1052 | 1086 | 1103 | 1104 | 1106 | 1109 | 1113 | 1124 | 1133 | 1147 | 1149 |
| mtDNA |    |                 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| type  | N  | 60              | 71   | 81   | 89   | 91   | 125  | 142  | 143  | 145  | 148  | 152  | 163  | 172  | 186  | 188  |      |
| ST1   | 51 | T               | G    | G    | T    | T    | T    | A    | G    | A    | G    | A    | A    | T    | G    | C    |      |
| ST2   | 9  | C               | G    | G    | T    | T    | T    | A    | G    | A    | G    | A    | A    | T    | G    | C    |      |
| ST9   | 25 | T               | G    | G    | T    | T    | T    | A    | G    | A    | G    | A    | A    | T    | A    | C    |      |
| ST19  | 10 | T               | G    | G    | T    | T    | T    | A    | G    | A    | G    | A    | A    | C    | G    | C    |      |
| ST21  | 1  | T               | G    | G    | T    | T    | T    | A    | G    | A    | T    | A    | A    | T    | A    | C    |      |

**Table 3.** Pairwise distance matrix for 5 steelhead mtDNA haplotypes. Numbers above diagonal are percent sequence divergence; numbers below diagonal are the number of differences between mtDNA haplotypes.

| mtDNA haplotype | 1 | 2   | 9   | 19  | 21  |
|-----------------|---|-----|-----|-----|-----|
| 1               | - | 0.5 | 0.5 | 0.5 | 1.0 |
| 2               | 1 | -   | 1.0 | 1.0 | 1.6 |
| 9               | 1 | 2   | -   | 1.0 | 0.5 |
| 19              | 1 | 2   | 2   | -   | 1.6 |
| 21              | 2 | 3   | 1   | 3   | -   |

In their studies of southern steelhead Nielsen et. al. (1994b) found a strong frequency cline in ST1 and ST2 with increasing frequencies from south to north. The northern California populations had frequencies of 58.3% for ST1 and 13.9% for ST2 haplotype. These correspond to the observed haplotype frequencies found in our study. Within the studied populations mitochondrial haplotype diversity is greatest in the Oxbow Hatchery and Little Sheep Creek, with each having four mitochondrial haplotypes, ST1, ST9, ST19, ST21 and ST1, ST2, ST9, ST19, respectively (Figure 1). The high mitochondrial haplotype diversity at these locations indicates a good representation of the source populations, provided no additional supplementation from outside sources has occurred. The inadvertent advancement of run timing at Oxbow Hatchery and the subsequent attempts to return the run to more natural timing may have altered frequency distribution for mitochondrial haplotypes. Unfortunately this cannot be determined since return rates of native fish to Hells Canyon Dam, the collection source for the hatchery, are extremely low. Comparisons of the wild and hatchery fish from Little Sheep Creek may be needed to ensure the status of this ESA-listed run. To ensure that the hatchery frequency distributions are representative of wild populations, sampling of wild and hatchery fish could be performed for comparison of mitochondrial haplotype frequencies.

Samples from Dworshak Hatchery contained only two mitochondrial haplotypes, ST1 and ST9. This population is unusual in having ST9 as the most prevalent mitochondrial haplotype. This distinctive mitochondrial frequency distribution is consistent with allozyme studies that have unique frequencies in this population (Williams, 1994).

Outmigrants from Johnson Creek showed three haplotypes, ST1, ST2, and ST9. All three of these haplotypes are found in both steelhead and resident rainbow populations that have access to the ocean (Nielsen et. al., 1997). Determination as to whether these are precocial steelhead or resident rainbow therefore cannot be made without comparative sampling of known anadromous steelhead and resident rainbows from the system.

Individuals from Pahsimeroi River and Hatchery exhibited differences in haplotype diversity and frequency. The natural out-migrants had three haplotypes, ST1, ST9, and ST respectively (Figure 1 in main report). This differed from the hatchery samples that had haplotypes ST1, ST2, and ST9. The haplotype information for the hatchery fish is indicative of the stock collection from the Snake River, but includes one haplotype, ST2, not found in the natural samples. The natural Pahsimeroi samples include one haplotype, ST19, not found in the hatchery samples. This lack of ST2 in the hatchery population, along with the inclusion of ST19 in the natural population deserves further study to determine whether natural fish are escaped hatchery fish spawning naturally.

The most common mitochondrial haplotype found in the redband trout samples was ST20. This haplotype is unique to this study and was found in all collection sites for redband trout. Other haplotypes found were ST12. The inclusion of haplotype ST1 may be indicative of past supplementation of rainbow trout into the Blitzen River population.

### *Microsatellites*

For each population, allele frequencies and observed and expected heterozygosities for each microsatellite locus have been analyzed. The microsatellites chosen for this study were highly polymorphic in steelhead trout. Microsatellite diversity has been published have been previously for only one of the populations in this study -- the Dworshak National Fish Hatchery steelhead from the Clearwater River (Ostberg and Thorgaard, 1998). The most common allele in Ostberg and Thorgaard (1998) for the Clearwater steelhead was 254 bp for One6 and 118 or 128 bp for Omy77. For this study, the most common allele for Clearwater steelhead was 254 bp (45% of fish collected), while the most common allele for Omy77 was 132 bp (32% of samples). These independent samples of Clearwater steelhead in 1992 and 1999 thus appear to coincide in allelic distribution.

Over all loci combined, all steelhead trout populations sampled by the Nez Perce Tribe had lower observed heterozygosities than that expected assuming a random-mating, large population. This was not true for the redband trout populations collected from southeastern Oregon. None of the Oregon redband trout populations deviated from the expected heterozygosities, but it must be noted that samples sizes were small for these populations. Deviation from expected heterozygosities could be indicative of inbreeding, small population sizes, or the presence of null microsatellite alleles. If a null or nondetected microsatellite allele exists for these populations,

individuals that possess the null allele would be erroneously scored as a homozygote, thereby decreasing the value of observed heterozygosity. Those samples for which no PCR product was obtained are indicated in the raw data in the Appendix.

The genetic distances and UPGMA distance diagrams drawn from these populations are the most informative in discerning the relationships among the Snake and Salmon River steelhead populations sampled. Nei's genetic distances (1988) calculated in this study are an expression of the probability that a randomly chosen allele from two different populations will be identical (or different in this case) relative to two randomly chosen alleles from the same population. Distance trees with the Pahsimeroi River population combined and separated into natural and hatchery components are presented in Figures 2 and 3, respectively.

**Figure 2.** UPGMA phenogram for *Oncorhynchus mykiss* from the Snake River basin and eastern Oregon drainages with the Pahsimeroi River hatchery and natural samples combined. The bottom axis is average genetic distance.

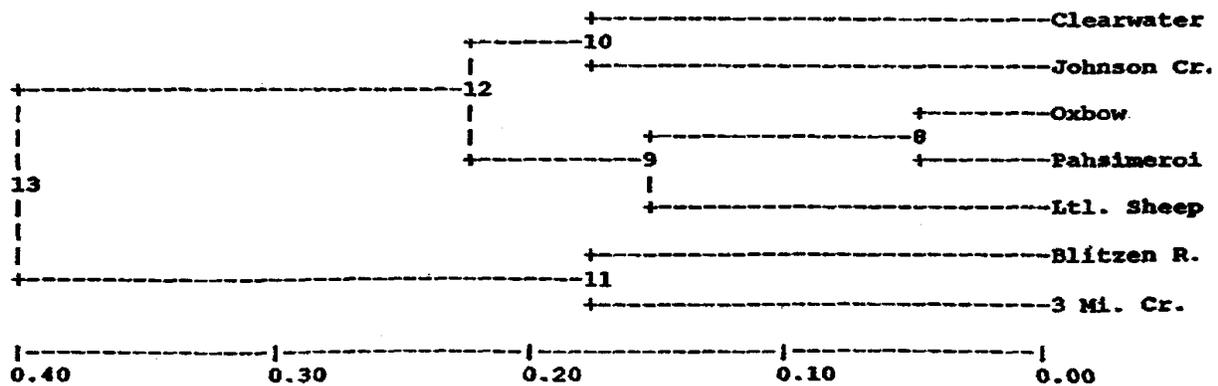
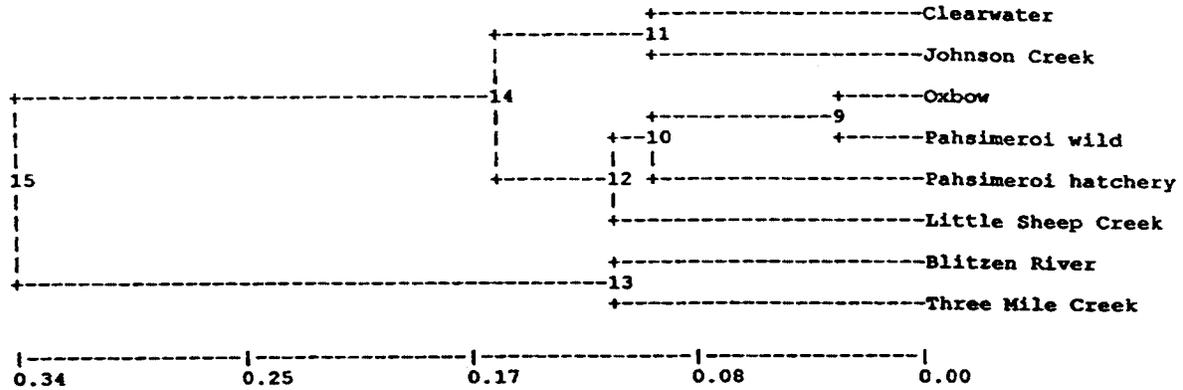


Figure 2 indicates that the Pahsimeroi River hatchery and natural samples combined are most closely related to the Oxbow Hatchery fish sampled from Hells Canyon Dam on the Snake River. This grouping reflects the historical origin of the Pahsimeroi River hatchery stocks, which were derived from the Hells Canyon/Snake River steelhead when the Pahsimeroi hatchery opened. Moreover, when the Pahsimeroi natural and hatchery fish are separated in the analysis (Figure 3), the seven natural fish are more similar to Oxbow fish than the natural fish are to the hatchery fish. The sample size for the natural fish is extremely small to make any firm generalizations, but it appears at first that these fish may be progeny of historical hatchery stocks that are naturally spawning in the wild; however, this does not explain why the hatchery and natural stocks are not more similar to each other. The relationship between Little Sheep Creek steelhead and the Pahsimeroi and Oxbow fish further implies that the Pahsimeroi fish are more related to mainstream Snake River stocks of steelhead than they are to the other population sampled in the Salmon River basin, namely the Johnson Creek fish. Johnson Creek fish were juveniles sampled at a screw trap in the spring. Johnson Creek fish were most similar to Clearwater steelhead. There is some question as to whether these fish were resident rainbow or precocious steelhead, but without data from resident rainbow populations from these basins, it is difficult to identify the life history ecotype of these fish. Furthermore, the relationships of the Pahsimeroi steelhead to other stocks in the Salmon River basin may be better resolved with tissues collected from other known steelhead stocks in this basin. Since it was not known whether the Johnson Creek

fish are steelhead or rainbow trout, future samples of both ocean-run and resident *O. mykiss* from the Salmon River system would be useful in resolving the relationships of the Pahsimeroi steelhead and Johnson Creek fish.

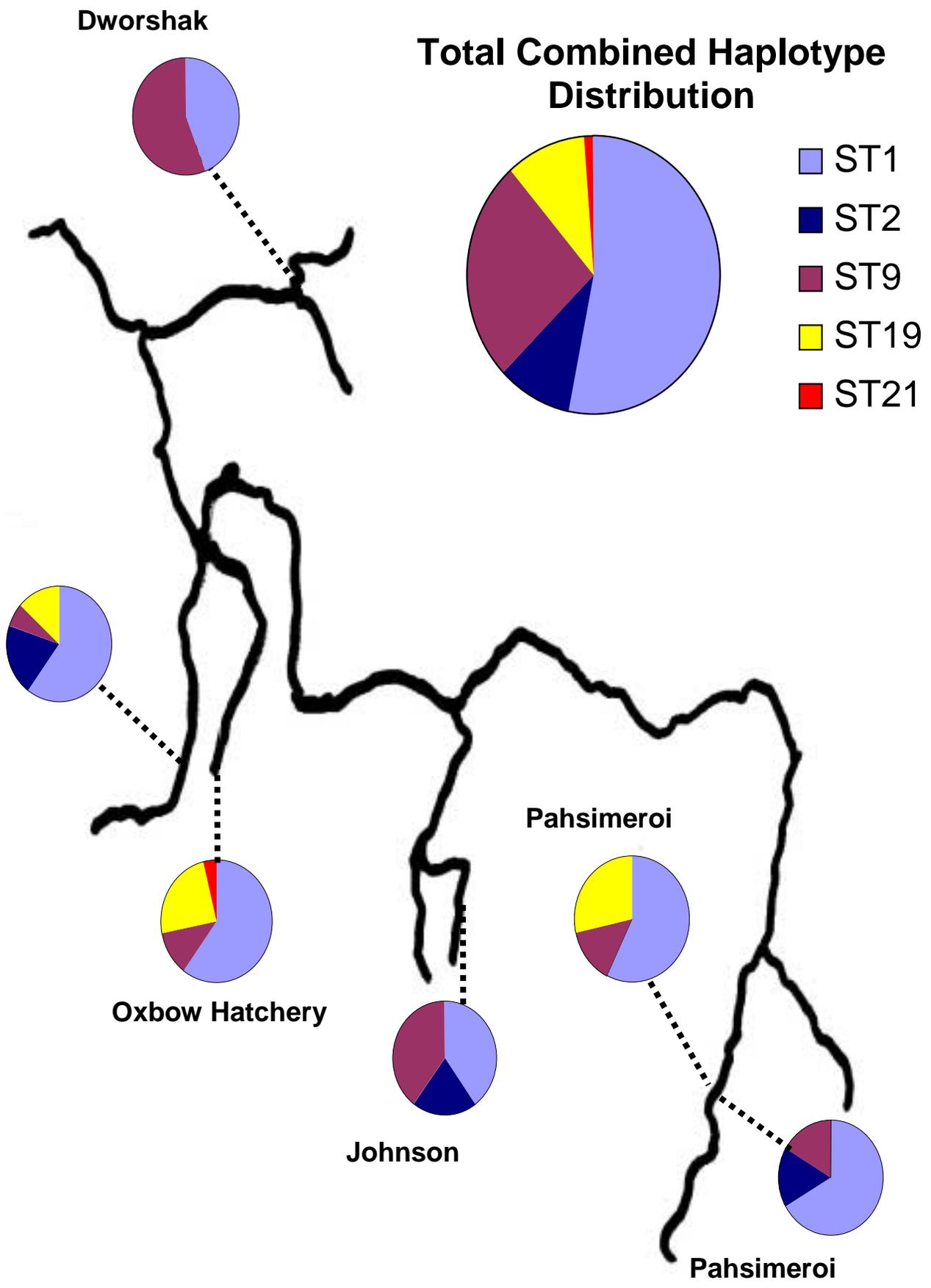
The three populations of redband trout sampled in southeastern Oregon were quite different from the steelhead populations sampled in the Snake River drainage (Figures 2 and 3). These populations of redband trout historically had access to the Snake and Columbia River systems when lake levels in the Oregon basins were high in the late Pleistocene (Behnke, 1992). However, the precise relationship of these fish with Columbia and Snake River steelhead and rainbow trout may be difficult to determine without larger sample sizes and inclusion of redband, rainbow, and steelhead trout from a greater sampling area.

**Figure 3.** Genetic distance phenogram drawn from Nei's genetic distance data for *O. mykiss* populations with Pahsimeroi River samples separated into natural and hatchery populations. The bottom axis is average genetic distance.



*Conclusion*

The mitochondrial DNA haplotype and microsatellite allelic distribution appear to agree on the similarities and relationships among the Snake River basin steelhead trout collected by the Nez Perce Tribe. The most striking result is the relationship among the Pahsimeroi River natural and hatchery fish and the Oxbow Hatchery steelhead. The microsatellite allele frequency data suggests that natural fish may be more similar to Oxbow fish than they are to Pahsimeroi hatchery steelhead. Likewise, the fact that hatchery and natural Pahsimeroi fish each have a mitochondrial type not shared by the other and the striking similarities in mitochondrial haplotypes for Oxbow and Pahsimeroi natural fish further support the microsatellite data. These relationships certainly reflect the historical makeup of the hatchery stocks, but are not conclusive for the natural stocks without larger sample sizes of natural fish and more information from other steelhead stocks in the Salmon River system. Any conclusions that are drawn from this particular relationship should be cautiously done since the sample sizes were so small for these natural fish. Finally, the ecotype identity of the Johnson Creek juveniles could not be resolved without genetic information from other steelhead and rainbow trout from the area.



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