
PART I - ADMINISTRATIVE

Section 1. General administrative information

Title of project Preserve Cryogenically the Gametes of Selected Mid-Columbia Salmonid Stocks	
BPA project number	20111
Contract renewal date (mm/yyyy)	
Multiple actions? (indicate Yes or No)	No
Business name of agency, institution or organization requesting funding COLUMBIA RIVER INTER-TRIBAL FISH COMMISSION	
Business acronym (if appropriate)	CRITFC
Proposal contact person or principal investigator:	
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NPPC Program Measure Number(s) which this project addresses	
Section 7.4.E.1 calls for applications of Cryopreservation technology to restore and preserve depleted populations. Section 7.4.E.2 calls for demonstrations of cryopreservation identified in the coordinated habitat and production process.	
Section 7 of the 1994 Columbia River Basin Fish and Wildlife Program calls for immediate efforts to gather data on wild and naturally spawning stocks. The title and goal of Section 7.1 is to "Ensure Biodiversity". Biodiversity being the variety of, and variability in living organisms with respect to genetics, life history, behavior and other fundamental characteristics.	
The Council may have envisioned a cryogenic preservation as a technique for gathering data on wild and naturally spawning stocks, as the application of this technique does fit the program goal. The gametes (eggs and sperm) of spawning salmon contain a vast amount of genetic information about the fish. This genetic information is coded in the deoxyribonucleic acid molecule (DNA). That portion of genetic information contained in the haploid chromosomes of salmon sperm cells can be gathered without impact on spawning salmon stocks. Cryogenic preservation of such "genetic data" is safe (the information is stored without a loss in fidelity) and it is inexpensive. After collection it is proposed that this material be transferred to an internationally recognized salmonid gene bank.	
FWS/NMFS Biological Opinion Number(s) which this project addresses	
n.a.	
Other planning document references	
<i>Wy Kan Ush Me Wa Kush Wit</i> , the Anadromous Fish Restoration Plan of the Nez Perce, Umatilla, Warm Springs and Yakama tribes calls for the reintroduction of salmon to watersheds from which they have been extirpated. While not explicitly cited as a technique of reintroduction, cryogenically preserved gametes predating the loss of a salmon stock in the wild would be a logical choice for use in a reintroduction effort.	

The National Marine Fisheries Service, as condition IX to required ESA permits for handling listed Snake River Salmon, requires a written statement indicating a "Willingness to Cooperate in a Cooperative Breeding Program". Cryogenic preservation is a component of Snake River salmon restoration efforts.

Short description

Collect and cryogenically preserve the gametes of fall chinook and/or steelhead from the Klickitat and Upper Columbia River. Transfer these gametes to a recognized qualified salmon gene banking facility.

Target species

Chinook and Steelhead

Section 2. Sorting and evaluation

Subbasin

Systemwide, Lower Mid-Columbia, Upper Mid-Columbia, Yakima, Klickitat

Evaluation Process Sort

CBFWA caucus		CBFWA eval. process		ISRP project type	
X one or more caucus		If your project fits either of these processes, X one or both		X one or more categories	
X	Anadromous fish		Multi-year (milestone-based evaluation)		Watershed councils/model watersheds
	Resident Fish		Watershed project eval.	X	Information dissemination
	Wildlife				Operation & maintenance
					New construction
					Research & monitoring
				X	Implementation & mgmt

Section 3. Relationships to other Bonneville projects

Umbrella / sub-proposal relationships. List umbrella project first.

Project #	Project title/description
	None. Knowledge and the practical application of cryogenic work is largely absent from this region.

Other dependent or critically-related projects

Project #	Project title/description	Nature of relationship
	CRITFC Kelt reconditioning	Will assist in this effort's cryogenic component.

Section 4. Objectives, tasks and schedules

Past accomplishments

Year	Accomplishment	Met biological objectives?
1998	Cryopreservation of wild spring chinook from the Cle Elum River	This fall 240 .5 ml straws of milt from 24 wild spring chinook randomly trapped at the Rosa dam for brood stock at the Cle Elum supplementation hatchery were cryogenically preserved. The milt from every salmon sampled was successfully collected, transported and preserved at the University of Idaho. However this work was unfunded in 1998, and these few samples can not begin to adequately represent the genetic diversity of this population. In this way, this effort has not met its biological objectives. Funding is required.

Objectives and tasks

Obj 1,2,3	Objective	Task a,b,c	Task
1	Coordinate initial steelhead and chinook salmon gamete preservation efforts with management agencies and Tribes in the Mid-Columbia region.	a	Coordinate planned chinook salmon gamete preservation activities with state and Tribal management agencies.
		b	Request and integrate agency and Tribal input into the selection of chinook and steelhead populations abundant enough to assure the success of Objective 4.
		c	Coordinate with ongoing cryopreservation research and sampling activities.
2	Define cryopreservation project goals for gene banking of gametes from healthy chinook and steelhead populations in the Mid-Columbia region.	a	Determine chinook and steelhead populations for germplasm sampling.
		b	Review the literature and apply finding to adjust the sample sizes of Task 3.1 to those required to preserve a representative sample of the genetic diversity within the selected populations.
		c	Assess the shortening of the range of time (years) needed to complete cryopreservation collections so that sufficient directly non-related

			individual genetic material is preserved.
3	Apply cryopreservation techniques to chinook and steelhead salmon conservation units at high and or mid levels of abundance.	a	Follow adult sampling protocols and cryopreservation techniques for the collection, preservation, storage and inventory of male salmon germplasm. Initial goals are to preserve the gametes of 500 individual salmon from the Klickitat and mainstem Columbia River (Hanford Reach). Collect detailed biological information on the source individuals, which provide the cyropreserved materials.
		b	Cryopreserve adult male chinook salmon gametes from conservation units identified as being at low levels of abundance and high risk of extirpation. Assess the quality of the collections by conducting fertility tests.
		c	To securely store the collected materials, contract with interim, and then permanent recognized salmonid gene banks in at least two independent locations.
		d	Preserve gamete samples on-site or at the identified independent locations.
4	Transfer of Technology.	a	Subcontract for the participation of two tribal fish agencies in the collection of salmon milt in the field. Encourage other fish agencies to visit and assess the applications of these techniques in other areas. Prepare and provide annual reports summarizing all activities associated with cryopreservation sample collection, preservation and storage.

Objective schedules and costs

Obj #	Start date mm/yyyy	End date mm/yyyy	Measurable biological objective(s)	Milestone	FY2000 Cost %
1	04/2000	04/2001	Coordination leading to agreement on the cryogenic preservation of selected salmon populations in the Mid-Columbia area.	Circulation of the sampling plan to the fisheries community.	10%
2	04/2000	04/2001	Identification of the sample sizes needed to preserve a	Defined goals for gene banking of	5%

Obj #	Start date mm/yyyy	End date mm/yyyy	Measurable biological objective(s)	Milestone	FY2000 Cost %
			representative sample of the genetic diversity within selected populations.	gametes in the Mid-Columbia region	
3	04/2000	03/2001	An 'insurance policy' against the loss of genetic diversity.	Completed collections of straws of frozen milt safely in recognized gene bank facilities.	80%
4	04/2000	04/2001	A transfer of technology sufficient that individual biologists, agencies and tribes will be comfortable with expanding this effort.	Each witnessing of the technique by those who've never seen it.	5%
				Total	100%

Schedule constraints

The window of opportunity for collecting milt from adult salmon is narrow. The design of this proposal is to take milt from males which have already naturally spawned (note that the volumes of necessary for cryopreservation are small, and that spent males remain an adequate source of milt for this purpose). It is envisioned that a consensus process will determine which stocks this project will ultimately select. Of the initial list, a range of schedule constraints exists:

Spring chinook spawning in the Klickitat spawn peaks in late August and early September. Winter Steelhead spawning in the Klickitat occurs from March through June. Summer steelhead, if selected, spawn from January through March.

Fall chinook in the Hanford Reach spawn from late October, peak in mid to late November, and taper off into December.

On a different time scale, the opportunity to collect exclusively wild Spring Chinook from the Rosa Dam on the Cle Elum River will expire in the year 2000.

Ideally preparation for field work should commence in mid 1999.

Completion date

This project is not envisioned as requiring funding past 2001. A second field season should complete the training of agency staff. It is envisioned that this work would continue by individual agencies as a special component of spawning surveys.

Section 5. Budget

FY99 project budget (BPA obligated):	n.a.
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FY2000 budget by line item

Item	Note	% of total	FY2000 (\$)
Personnel	Fishery Scientist, .25 FTE Fishery Scientist, .1 FTE	15	\$9,897 \$3,299
Fringe benefits	@ 31.5 %	5	\$4,157

Supplies, materials, non-expendable property			\$9,000
Operations & maintenance		10	\$600
Capital acquisitions or improvements (e.g. land, buildings, major equip.)		1	
NEPA costs			
Construction-related support			
PIT tags	# of tags:		
Travel		3	\$2,469
Indirect costs		12	\$11,151
Subcontractor	YIN, U of I, BIA	55	\$49,000
Other			
TOTAL BPA REQUESTED BUDGET			\$89,573

Cost sharing

Organization	Item or service provided	% total project cost (incl. BPA)	Amount (\$)
Bureau of Indian Affairs	Personnel	5%	\$4,800
Total project cost (including BPA portion)			\$94,373

Outyear costs

	FY2001	FY02	FY03	FY04
Total budget	\$75,000	\$0	\$0	\$0

Section 6. References

Watershed?	Reference
	Ashwood-Smith, M.J. (1980). Low temperature preservation of cells, tissue, and organs. In: Low Temperature Preservation in Medicine and Biology. M.J. Ashwood-Smith and J. Farrant, eds. Pitman Medical Limited, Turnbridge Wells, Kent, Eng. pp. 19-44.
	Cloud, J. G., and C. Osborne. 1997. Cryopreservation of salmonid sperm. Department of Biological Sciences, University of Idaho. Moscow, ID
	Cloud, J. G. and G. H. Thorgaard, editors. Genetic Conservation of Salmonid Fishes. Series A: Vol. 248, NATO Advanced Science Institute Series, Plenum Press, New York and London
	Mounib, M.S. 1978. Cryogenic preservation of fish and mammalian spermatozoa. Journal of Reproductive Fertilization, 53: 13-18.
	Northwest Power Planning Council. 1994. Columbia River Basin Fish and Wildlife Program. Portland, OR.
	Stoss, J. 1983. Fish gamete preservation and spermatozoan physiology. In: Fish physiology Vol. 9 Part B. Hoar, W.S., D.J. Randall, and E.M. Donaldson eds. Academic Press, New York. pp. 305-350.
	Whittingham, D.G. 1980. Principles of embryo preservation. In: Low Temperature

	Preservation in Medicine and Biology. M.J. Ashwood-Smith and J. Farrant, eds. Pitman Medical Limited, Turnbridge Wells, Kent, Eng. pp. 65-83.

PART II - NARRATIVE

Section 7. Abstract

The world faces changing environmental and political conditions, and these conditions do not always maintain native fish stocks at levels which maintain genetic diversity. Detrimental conditions causing the decreases in salmon stocks can be improved in some cases, but time is required. The 1994 Columbia Basin Fish and Wildlife Program calls for demonstrations of cryopreservation identified in the coordinated habitat and production process, as well as ensuring biodiversity.

Cryopreservation of sperm is a proven technique for preserving fish germ plasm for extended periods of time (200+ years), and therefore can serve as a partial insurance against the loss of genetic diversity. Cryopreservation is already in use in programs designed to store rare fish germ plasm, but it is unapplied in the regions proposed. Biologists working with endangered stocks urge that the collection and storage of salmon gametes be conducted when stocks are at healthy levels. The end product of these approaches differ in that collections made on endangered stocks genetically sample a subset of the few individuals left in a population. The goal of this project is to demonstrate that representative genetic samples of the biodiversity from healthy populations may be economically made and preserved. This project may be monitored and evaluated based upon an assessment of the representativeness of the collections made and fertility evaluations of the stored product. Cryopreservation programs do not address habitat problems, but they are the simplest and most economical means to store genetic information contained in the DNA of male salmon from today's stocks.

Section 8. Project description

a. Technical and/or scientific background

The 1994 Columbia Basin Fish and Wildlife Program calls for demonstrations of cryopreservation identified in the coordinated habitat and production process, as well as ensuring biodiversity. This technique has been successful with many fish species including steelhead, chinook, coho, chum salmon, pink salmon, sockeye, Atlantic salmon, Pacific herring, Atlantic halibut, grey mullet, black porgy, brown trout, channel catfish, black grouper, Atlantic croaker, common carp, marine puffer, milkfish, barramundi, and summer whiting.

Cryopreservation is already in use in portions of the Snake River. Since 1992 the Nez Perce Tribe (NPT) has contributed 565 cryopreserved samples in a local gene banking effort. The NPT was funded in 1998 by the Bonneville Power Administration to coordinate and initiate gene banking of adult male gametes from listed spring and summer chinook in the Snake River basin. The WDFW is working in the lower Snake River tributaries. These collections are stored in duplicate at two separate facilities, the University of Idaho and Washington State University. This gene banking effort is not without applications, in 1997 ODFW sought and received NMFS approval for the transfer of some of these NPT cryopreserved sperm samples to their Snake River spring/summer chinook captive broodstock program.

The products of this project may, at some point in the future be used to mitigate losses of native salmon in place and in kind. A proposal to do this is not being made, such a proposal will hopefully never have to be made. That is the nature of cryopreservation, it is a partial "saving of the pieces" to insure against the failure of society to protect a localized salmon population. This project is a logical component of the work called for in the conference titled "The Establishment of a Germ Plasm Repository for Threatened and Endangered Fish". This conference was hosted July 23, 1997 by the University of Idaho and supported by Washington

Sea Grant Program and the University of Idaho. Speakers at this conference represented the Smithsonian Institute, the USGS, the NPT, IDFG, Louisiana State University and WSU.

b. Rationale and significance to Regional Programs

The 1994 Columbia Basin Fish and Wildlife Program calls for demonstrations of cryopreservation identified in the coordinated habitat and production process, as well as ensuring biodiversity. This technique has been successful with many fish species including steelhead, chinook, coho, chum salmon, pink salmon, sockeye, Atlantic salmon, Pacific herring, Atlantic halibut, grey mullet, black porgy, brown trout, channel catfish, black grouper, Atlantic croaker, common carp, marine puffer, milkfish, barramundi, and summer whiting.

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c. Relationships to other projects

Relationship with The Nez Perce Fisheries Resources Management Department.

This department has been collecting milt from threatened or endangered spring and summer chinook salmon in Idaho since 1992. This agencies staff has provided valuable advice based on their years of experience with the procedures involved. Their staff also have been supportive of this project's timing (i.e., its being preformed, if possible, while stocks are abundant). Two advantages come from such timing. The stocks are logistically easier and less expensively sampled, and larger collections made on an abundant population may better represent that population genetically.

Relationship with Confederated Tribes and Bands of The Yakama Indian Nation, Fisheries Program

The YIN Fisheries program offers an excellent infrastructure under which this project may be implemented. Supervisory staff and technicians are geographically situated to sample the Klickitat and Hanford Reach with minimal logistical complications. Their supervisor staff and crews are familiar with the river, and they are willing to participate in the pilot project.

d. Project history

This project was rated “Tier 2” in FY 99. It was one of seven projects which the Independent Science Review Board recommended elevating to “Tier 1” in FY 99, but the project remained unfunded. Unfunded demonstration work was completed in the fall of 1998, and successfully cryopreserved some wild Spring Chinook from the Cle Elum River. The principal investigator has been active in promoting this work since 1994.

e. Proposal objectives

The following tasks are patterned after the NPT's "Listed Stock Chinook Salmon Gamete Preservation project.

OBJECTIVE 1. Coordinate initial steelhead and chinook salmon gamete preservation efforts with management agencies and Tribes in the Mid Columbia region.

Task 1.1 Coordinate planned chinook salmon gamete preservation activities with state and Tribal management agencies.

Task 1.2 Request and integrate agency and Tribal input into the selection of chinook and steelhead populations abundant enough to assure the success of Objective 4.

Task 1.3 Coordinate with ongoing cryopreservation research and sampling activities.

OBJECTIVE 2. Define cryopreservation project goals for gene banking of gametes from healthy chinook and steelhead populations in the Mid Columbia region.

Task 2.1 Determine chinook and steelhead populations for germplasm sampling.

Task 2.2 Review the literature and apply finding to adjust the sample sizes of Task 3.1 to those required to preserve a representative sample of the genetic diversity within the selected populations.

Task 2.3 Assess the shortening of the range of time (years) needed to complete cryopreservation collections so that sufficient directly non-related individual genetic material is preserved.

OBJECTIVE 3. Apply cryopreservation techniques to chinook and steelhead salmon conservation units at high and or mid levels of abundance.

Task 3.1 Follow adult sampling protocols and cryopreservation techniques for the collection, preservation, storage and inventory of male salmon germplasm. Initial goals are to preserve the gametes of 500 individual salmon from the Klickitat and mainstem Columbia River (Hanford Reach). Collect detailed biological information on the source individuals which provide the cryopreserved materials.

Task 3.2 Cryopreserve adult male chinook salmon gametes from conservation units identified as being at low levels of abundance and high risk of extirpation. Assess the quality of the collections by conducting fertility tests.

Task 3.3 To securely store the collected materials, contract with interim, and then permanent recognized salmonid gene banks in at least two independent locations.

Task 3.4 Preserve gamete samples on-site or at the identified independent locations.

OBJECTIVE 4. Transfer of Technology.

Task 4.1 Subcontract for the participation of two tribal fish agencies in the collection of salmon milt in the field. Encourage other fish agencies to visit and assess the applications of these techniques in other areas. Prepare and provide annual reports summarizing all activities associated with cryopreservation sample collection, preservation and storage.

f. Methods

The numbers of fish proposed for collection are similar to those of other salmonid gene banking projects (50 individuals/river or large tributary). However the sample size will be revisited and justified based on what is known about the regions proposed.

Fish handling protocol training will be provided to all personnel prior to collection and handling of adult male salmon to minimize handling stress. Each team member will be assigned a specific duty to improve the efficiency of sample collection. All adult male salmon sampled were collected by hand or net. Pre-measured MS-222 will be used to anaesthetize all adult salmon, along with a sodium bicarbonate buffering compound to buffer the acidic effect of the MS-222. Extra care will be taken during milt collection to ensure the quality of preserved samples. The abdomen of the anesthetized male salmon will be thoroughly dried and stripped gently to reduce or eliminate contamination of the semen samples.

Fish biological information (length, general condition, external marks) will be recorded following semen collection. Caudal fin tissue may be collected for genetic (DNA) analysis. Scales will be taken for scale pattern analysis to determine wild or hatchery origin and age classes. Following sampling and data collection the anesthetized salmon will be immediately returned to a slow water area and assisted until recovered. Concurrently, the semen samples will be placed in two separately labeled Whirl Pak7 bags, oxygenated, and placed in a covered insulated cooler on wet ice on top of newspaper. Cryopreservation will occur within a 24 period, followed by shipping and storage at independent repositories. The duplicate repositories will serve as a safeguard against a catastrophic event that could destroy all germ plasm samples if they were stored at one facility.

Sperm evaluation is an important component of the cryopreservation program in order to cull poor quality sperm samples prior to freezing, and to estimate the fertility of the stored sperm post-thaw.

Fertility will be evaluated by:

- *fertilization rate - proportion of eggs fertilized by a given number of spermatozoa;
- *sperm motility - percentage of motile sperm following the addition of a sperm activating solution (Mounib 1978).

There are four stages in the cooling sequence of cryopreservation of cells:

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 - During this phase, there is a net movement of water out of the cells as the temperature is constantly being reduced. The cooling rate during this phase needs to be slow enough to allow water to move out of the cells, but it must be fast enough to protect the intercellular environment from the effect of the high salt concentrations. The success of cryopreservation is dependent upon required cryoprotectants (such as DMSO) in the freezing solution. These small compounds enter the cells and protect the cells during dehydration. The rate at which the sperm is cooled is a critical factor in the success of the cryopreservation process; For salmonid sperm, cooling rates of -20 to -30°C/minute appear to be optimal (Stoss 1980), down to approximately -79°C.

4) reduction to liquid nitrogen temperature - The frozen milt is then plunged into liquid nitrogen at -196°C.

The amount of sperm cryopreserved varies greatly by individual fish. Many of the fish sampled have been actively spawning for several days and sometimes very little or no sperm is available. A 2 ml sample can fertilize up to 100 eggs (Cloud and Thorgaard, 1993).

Safety considerations from the handling of liquid nitrogen have been considered. The practices recommended by the National Research Council (1995) will be followed, as published in their "Prudent Practices in the Laboratory Handling and Disposal of Chemicals".

An objective assessment of factors that may limit success of the project and/or critical linkages of the proposal with other work.

The success of a cryogenic preservation project has two parts. The first is the successful collection and storage of representative samples of a population. The training of technicians to freeze milt has been demonstrated and is feasible. The trickier part of this process lies in the hands of those who, in the future will need to thaw this milt and fertilize eggs in future field situations. In nature salmonid sperm swim for 30 seconds after deposition. In short, time is of the essence, and low fertilization rates will be the consequence of any unpolished procedures.

Facilities and equipment

The office facilities and office equipment for coordinating this work already exist at CRITFC, the YIN, and the BIA. The vehicle needs of this project will be met by sharing the costs of maintaining annually leased vehicles with other projects. Cryopreservation has specialized equipment requirements. The amount of hardware required depends on whether or not fish samples are frozen in the field, or shipped live to a lab for storage. Objective 4 (technological transfer), dictates that demonstrations of freezing techniques be made. Therefore at a minimum the following items will be required:

Budget

No special or high cost equipment is required for this work. A liquid nitrogen container, liquid nitrogen dry shipper, liquid nitrogen vapor freezer, microscope, and Cryopreservation database software are required. None of these individual items exceeds \$1,600. The personnel costs of the budget include two fishery scientists who will supervise and design the sampling project. Operations include the annual cost of maintaining samples in liquid nitrogen. Travel costs cover trips from the Portland area to the spawning reaches of selected streams. Subcontracts cover the cryogenic related expenses of spawner survey crews of the YIN. Another subcontract will include the freezing of milt at the U of I and another facility.

Section 9. Key personnel

Keith Hatch has an MS in Fisheries from OSU. He studied the genetic structure of coastal Oregon steelhead using electrophoresis, and publishing the thesis titled "Phenotypic Comparison of Thirty-eight Steelhead (*Oncorhynchus mykiss*) populations from Coastal Oregon". He edited the five volume "Stock Summary Reports for Columbia River Anadromous Salmonids", published in 1992 by the BPA. He has training and some practical experience in the freezing of milt, it's thawing, and it's use in fertilizing eggs by completion of the University of Idaho's Cryopreservation of Salmonid Sperm workshop in 1997.

Dr. Talbot has a particularly strong background in linking environmental data with variability in production, producing habitat-based predictive models of fish production for major rivers in Eastern Canada. His research education and experience provide the necessary skills for a critical appraisal of methodologies employed in sampling designs, monitoring systems, research programs and resource management. He has extensive experience in length-based analytical and statistical fisheries methods. From the design of experiments to data collection to statistical analysis, he has assisted tropical and temperate research teams to develop research programs and management plans in fisheries and aquaculture. He has experience in teaching, through workshops and university courses. Dr. Talbot held an Associate Scientist position at the University of Québec at Chicoutimi, directing a firm specializing in international development, with particular interest in fisheries resource monitoring, population dynamics, aquaculture and genetics.

George Lee is a Supervisory Biologist with the YIN. He will be devoting 1/4 FTE to the project during the spawning season. He will provide field support and work on the technology transfer aspects of the project within the YIN and with other fish agencies.

Section 10. Information/technology transfer

The knowledge of how to perform the collection of salmon milt in the field, how it is frozen, and how to transport it will be transferred to the tribes and local fish agencies by way of its direct demonstration. Objective 4 (technology transfer) is a minor budgetary portion of this project, but it is one of the bigger goals of the whole project. Personnel unfamiliar with cryopreservation work will be trained in a short course held at the U of I. Staff from other fishery agencies will be encouraged to visit and assess the applications of these techniques. Presentations on the project will be made at relevant professional society meetings (for example, The Native American Fish and Wildlife Society). The target audience for this technology transfer is specific in that it is limited to fishery biologists and fishery managers. It is not the intent of this project to inform the general public about the need for, or even the existence of gene banking programs. A low public profile for this gene banking initiative is proposed. This is to prevent it from being cited by that segment of society that rationalizes salmon habitat destruction.