

I Project Title

Development of sensitive, non-lethal genetic diagnostic systems to discriminate species, sex, and runs of fishes dependent on the Bay-Delta system.

Applicant

Russell Rodriguez, Project Leader, USGS

DWR WAREHOUSE

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Approach/Tasks/Schedule

A .

A combination of Polymerase Chain Reaction (PCR) techniques and a non-lethal tissue collection protocol will be used to identify genetic markers to discriminate species, sex, and runs of various fishes at any life stage.

B .

There are three tasks to the proposed work as described below.

- 1 - apply PCR technologies to several priority fish species to identify and isolate genetic markers to discriminate species, sex, and runs at any life stage.
- 2 - use the genetic markers identified in task 1 to develop a high sensitivity, redundant diagnostic system for the non-lethal monitoring of populations.
- 3 - convert the diagnostic systems developed in task 2 for field utility and rapid data acquisition. Offer training to interested parties so they will be able to perform diagnostic analyses independently.

C .

The proposed research will take 3 years with tasks 1, 2, and 3 being completed in years 1, 2, and 3, respectively.

Justification

This project will involve all of the priority fish species identified by the CALFED 1997 funding priorities. It is critical to develop a number of diagnostic protocols to monitor the restoration efforts in the Bay Delta system. Since some of these species are anadromous, they utilize and are dependent on the health of the fresh water river systems and the estuary. Therefore, monitoring runs and species of fish can provide an indication of the effectiveness of restoration efforts. The proposed genetic diagnostic systems will allow biologists to monitor the distribution, sex ratios, residence times, and habitat usage of priority fish at any life stage.

Budget Costs

The project will be performed as three separate tasks so that the success of one is not contingent on changes in future funding priorities. This research will require a salary for a molecular biologist; supplies for PCR, electrophoresis, and cloning; DNA sequence analysis and PCR primer construction. A uv/fluorescent spectrophotometer will be necessary for converting the diagnostic systems for field applicability. However, the equipment will only appear on the second

year funding request.

Annual Budget:

Molecular Biologist (GS9) salary and benefits	\$42,000
Laboratory Supplies	\$30,000
Overhead (38%)	\$27,360
Total	\$99,360

Applicant Qualifications

Dr. Rodriguez has a Ph.D. in microbiology and has been pursuing research in genetics for 19 years. His laboratory in Seattle has been involved with fish molecular genetics for five years and has involved salmon, sturgeon, and steelhead. The systems completed in the lab include generating non-lethal PCR-based diagnostic systems to 1 - discriminate spring and fall chinook salmon in the Columbia river, 2 - discriminate male and female chinook salmon at any life stage, and 3 - determine the genetic diversity of chinook populations with 25 genetic markers. Projects underway in the lab include sex discrimination in white sturgeon, genetic discrimination of winter and summer run steelhead, and genetic analysis of resistance/susceptibility to BKD.

Monitoring and Data Evaluation

These systems will allow biologists to monitor the distribution, sex ratios, residence times, habitat usage of priority fish at any life stage. Sex discrimination will allow for the assessment of sex ratios generated annually as well as migration patterns of males and females, both of which are unknown in most fish species. This is particularly important for species which develop sexual morphologies over long periods of time such as sturgeon. In addition, if there are hormone-simulating chemicals contaminating the Bay Delta waters then sex ratios may be biased and a DNA based system would help determine if the cause is biochemical or genetic. Run-specific markers will allow for the monitoring of migration patterns of different salmonid runs.

The diagnostic systems will require a small piece of fin tissue, DNA extraction, and PCR amplification with specific primer sets. The data generated will be put into a spread sheet and made available to all interested agencies.

Local Support

The NBSC/USGS will provide all of the equipment and expertise necessary to design and carry out the objectives of this project. Once the systems are developed they will be made available to any interested agencies so they may perform diagnostic analyses. This project addresses research needs and management tool development of all priority fish species identified by CALFED.

II Project Title

Development of sensitive, non-lethal genetic diagnostic systems to discriminate species, sex, and runs of fishes dependent on the Bay-Delta system.

Principle Investigator

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Organization

Federal Government, tax exempt

RFP

Group 3

III Project Description

A.

In order to assess the ecological/biological effects of restoration efforts, it is critical to have systems in place to monitor species of interest. I am proposing to develop a series nuclear DNA based diagnostic systems that can be used to non-lethally monitor certain life history patterns of all priority fish species identified by CALFED. Specifically, PCR technologies will be utilized to identify nuclear genetic markers that will discriminate sex in Delta smelt, Longfin smelt, splittail minnow, green sturgeon, and steelhead trout. In addition, DNA from the four runs of salmon will be screened in an attempt to identify run-specific markers. Finally, several species-specific markers will be developed for each fish species so egg deposition and juvenile migration patterns may be monitored. The approach we will take begins with the isolation of DNA from non-lethally collected tissue samples using a protocol developed in my laboratory (see below). The DNA will be analyzed by arbitrarily primed PCR (apPCR) using 600 - 1000 PCR primers. This technique has allowed us to isolated sex specific markers in chinook salmon and run specific markers that discriminate spring and fall chinook in the Columbia river system (see below). These systems will allow biologists to monitor the sex ratios in these populations which can be greatly affected by environmental conditions and chemical pollution. The sensitivity of this technology is allows for non-lethal tissue samples to be collected from individuals at any life stage.

B.

This project encompasses fish that live throughout the Bay Delta system and have no specific geographic boundaries. The diagnostic systems generate by this research will be applicable to priority fish populations anywhere in the system and for individuals at any life stage.

C.

These systems will allow biologists to monitor the distribution, sex ratios, residence times, habitat usage of priority fish at any life stage. As a result, the response of these populations to restoration efforts can be visualized rapidly. In addition, the sensitivity of these systems will allow biologists to determine if the mechanism of any sex ratio bias is genetic or due to chemical contamination.

D.

The approach proposed for developing species, sex, and run-specific genetic markers involves non-lethal tissue collections, efficient DNA extraction, and a technique known as arbitrarily primed Polymerase Chain Reaction (apPCR). The protocols involved in this process and the generation of the proposed diagnostic systems are described below.

Sample Collection and DNA Extraction

My laboratory has been developing nuclear DNA technologies to study various aspects of genetic diversity and genome dynamics of fish for four years. In preparation of studies to identify

run, sex, and species discriminating genetic markers, we have obtained samples from several stocks of the fall and spring/summer chinook in the Snake river, and all four chinook runs in the Sacramento river, and white sturgeon from the Columbia river. These samples were collected by fisheries biologists in the US Fish and Wildlife Service, National Biological Service, National Marine Fisheries Service, and the California State Fish and Game Department. These populations represent populations from different years and locations so both spatial and temporal fidelity of sex and run discriminating markers can be assessed. Samples from the remaining priority species will be obtained by biologists from the agencies listed above.

In order to minimize impacts on threatened and endangered populations, we have developed a non-lethal DNA extraction procedure that generates enough DNA from a small piece of fin tissue for 500-1000 genetic analyses. This protocol takes 4-6 hours to complete, eliminates the need for highly toxic chemicals, and can be accomplished using general laboratory equipment (Rodriguez, 1993; Redman and Rodriguez, 1994). In addition, once tissue samples have been placed in the DNA extraction buffer, the DNA is stable for at least one year at ambient temperatures (Rodriguez et al., 1995). This allows for the collection of samples under field conditions without the need of special equipment.

apPCR

The advent of apPCR [also known as random amplified polymorphic DNA (RAPD) or single-primer PCR analysis] has allowed for the taxonomic discrimination of organisms lacking sufficient numbers of morphological characters (Owens and Uyeda, 1991; Welsch and McClelland, 1990; Williams et al., 1990). This technique utilizes short oligonucleotide primers (10 - 20 base pairs) that anneal to complimentary DNA sequences. When sequences, which are complimentary to a specific primer, are located in opposite orientation on separate strands of DNA, a double stranded DNA product may be synthesized by DNA polymerase. The number and size of amplified products is dependent on the frequency and distribution of primer hybridization sites which is not possible to predict without extensive characterization of the genome. However, the number of apPCR products generated from individual primers ranges from less than 5 to more than 20 depending on primer composition and the reaction conditions. Therefore, more than one hundred genetic markers may be generated by apPCR from 10 - 20 oligonucleotide primers. If greater numbers of genetic markers are required then the number of primers utilized can be increased.

By utilizing oligonucleotide primers 15 - 16 bp in length and composed of simple sequence repeats [eg. (CAG)₅, (GACA)₄], we have found that in several taxa 80 to 100% of the DNA products generated from apPCR amplification will be shared among individuals of the same species for any given primer (Freeman and Rodriguez, 1995; Freeman et al., 1993; Perring et al., 1993; Rodriguez and Owen, 1992; Van der Knaap et al., 1993). Alternatively, individuals from different species share between zero to 20% of the DNA products generated from these primers. As a result, we have found that apPCR band patterns are species specific and may be used for unequivocal taxonomic identification.

Several laboratories have reported difficulties in reproducing band patterns using apPCR

(Ellsworth et al., 1993; Tommerup, et al., 1995, Waugh and Wayne, 1992) and we have performed extensive analyses to identify the cause(s) of non-reproducibility. There have been many reports concerning the optimization of apPCR by modification of the reaction buffer, primer length and composition, source of the DNA polymerase enzyme, the quality of template DNA, and the programming of thermocyclers (Yu and Pauls, 1992; Ellsworth et al., 1993). While all of these modifications may greatly impact apPCR results, we have found that a major source of non-reproducibility in apPCR is based on the accuracy of the thermocyclers. This was determined with twenty thermocyclers from nine companies as described in the methods section. A scanning thermocouple thermometer was connected to 12 temperature probes that were placed in 0.5 ml tubes containing 20µl PCR reactions. All of the machines tested were precise; however, only two brands were accurate with regard to the programmed denaturing temperatures. In fact, some machines never came within 10° C of the programmed denaturing temperatures. The inability to reach denaturing temperature results in incomplete DNA denaturation so primer annealing sites may not be available. The ability of thermocyclers to achieve programmed denaturing temperatures was directly correlated with the reproducibility of amplified product patterns from the different reaction tubes. As a result of these analyses, all of the data presented in this section was produced on thermocyclers manufactured by Barnstead/Thermoloyne.

Species-Specific Markers

In order to determine the impacts of restoration efforts on priority fish species, it is necessary to develop techniques for monitoring of individual species at any life stage. We have begun to address this by combining the species specific band patterns of apPCR and the specificity of dual primer PCR (dpPCR). After a series of species-specific apPCR fingerprint patterns have been generated, it is possible to clone and sequence individual products in order to design marker specific PCR primers for dpPCR analysis. Most of the DNA products generated by apPCR represent species specific sequences which can be verified by DNA hybridization analysis (Fani et al., 1993; Hardys et al., 1992). Therefore, primers will be generated from the sequences on each end of species specific bands and used for dpPCR to specifically amplify the band(s) of interest. The sensitivity and specificity of dpPCR is greater than apPCR and have allowed us to isolate DNA and perform diagnostics in an 8 - 10 hour period. As we continue to develop these techniques to achieve greater levels of sensitivity and resolution we hope to decrease the analysis time to 4 hours or less. All of the markers generated in this portion of the study will be tested for species-specificity by PCR amplification of DNA from closely related fish species.

Sex-Specific Markers

DNA samples representing known sexes of the priority species will be analyzed by apPCR with 600 - 1000 primers. Experiments from our laboratory indicate that sex-specific products may be visualized with fewer than 100 primers (Clifton and Rodriguez, 1997). Sex specific PCR products will be cloned and sequenced so marker-specific primer sets can be constructed as described above. The sex-specific primer sets will then be tested on 50 - 100 samples representing

known sex of each species. The sex-specific marker generated for chinook salmon has been incorporated into field research projects where "take limits" have been imposed on spring salmon prior to the development of sexual morphologies. The sex-specific marker allowed researchers to ensure equal sex ratios of the fish collected.

Run-Specific Markers

The biology of chinook salmon indicate that run timing is genetically determined and unique for each run. The dynamics and precise timing of each run are affected by environmental conditions (Levings, 1993). For example, a run of chinook salmon may occur over a six week period with the number of individuals migrating over time reflecting a bell-shaped curve. In order to identify DNA sequences that are linked to or responsible for run timing, it is necessary to scan and compare the nuclear genomes of several stocks representing individual runs. Using DNA from the stocks described above, apPCR will be used to scan chinook genomes to identify run-specific genetic markers. The intent of these studies is to develop a number of run discriminating markers that can be used in concert to determine spatial and temporal life history aspects of juvenile chinook. This will allow for the rapid identification of chinook salmon stocks and runs in the Snake and Sacramento rivers so that the status and trend of these populations can be assessed.

Laboratory Facilities

Our laboratory has all of the equipment necessary to perform DNA extractions, Cloning, PCR analysis, agarose and acrylamide gel electrophoresis, and DNA sequence analysis. We have been performing these types of studies for several years and have developed protocols and expertise in generating diagnostic system to discriminate sex, species, and populations of a variety of species.

E.

Development of the proposed diagnostic systems will be accomplished with molecular technology that we routinely use in our laboratory. There are three tasks associated with this project, all of which are consistent with the 1997 funding priorities. The first task is to identify sex specific and species-specific genetic markers in all of the non-salmon priority fish species. This task will be completed in the first year and result in the generation of at least one manuscript in a peer-reviewed scientific journal. The first task also involves analyzing DNA representing the different salmon runs in order to screen for run-specific genetic markers. The success and time required for this task will depend on the genetic basis of run timing. If there is an evolutionarily conserved basis for run timing (which is suggested by life histories) then identifying genetic markers responsible for or linked to genes involved in the process is simply a matter of time and screening. If however run timing is determined by something other than genetics (there is no evidence for this) then there may be no genetic markers to pursue. Alternatively, if different runs hybridize at a high frequency then no run-specific markers will be visualized. Although there has been some evidence for run hybridization in hatchery populations, it has not been observed in wild salmon and the genetic implications of the hatchery hybrids are poorly understood.

The second task will involve DNA sequence analysis and the generation of marker-specific

PCR primers. This task will be performed in the second year of this project and result in at least one manuscript in a peer-reviewed scientific journal. Once the PCR primers are tested, they will be made available to agencies for monitoring purposes.

The third task involves the conversion of the diagnostic systems for field applicability. This will make analysis very rapid, simple, and cost effective and will be pursued in the third year. Since the future funding cycles are not yet known, this proposal only addresses task #1 which will be pursued in the first year.

F.

The proposed genetic systems will allow biologists to monitor the distribution, sex ratios, residence times, and habitat usage of priority fish at any life stage. As a result these systems can be used to monitor the effectiveness of restoration programs. In fact, these types of systems will be critical to determine local and distant ecological/biological effects of restoration efforts anywhere in the system. The systems will be established so redundancy will negate the possibilities of false positive or negative results and the data will be represented as the presence or absence of markers. Therefore the data can be added to any data bases that are being developed for monitoring purposes.

G.

There are no legal or regulatory conflicts associated with the implementation of the proposed genetic systems.

References

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- Yu, K. and Pauls, K.P. 1992 Nucleic Acids Research 20:2606.

IV Cost Schedule

A.

This project involves highly technical work requiring specialized training. Therefore a GS9 salary is requested to accomplish tasks 1 & 2. Material needs involve supplies for DNA extraction, PCR analysis, electrophoresis, and cloning.

Task	Labor Hours	Salary/ Benefits	Overhead	Materials	Misc.	Total cost
1	1 year	42,000	27,360	30,000	0	99,360

B.

This project will begin soon after funding is available because there is a large pool of Ph.D. level scientists at the University of Washington who have expressed interest in such a project. Development of species-specific and sex-specific markers will be completed one year from the start date and identification of run-specific markers will depend on the factors described earlier in this proposal.

C.

This research will provide diagnostic tools that may be used to monitor priority fish species and to ensure equal sex ratios in those populations. As a result, the impacts of any restoration efforts made by third parties on these species can be evaluated.

V. Applicant Qualifications

Dr. Rodriguez has been performing genetic analysis of fish and other organisms for more than 19 years. His laboratory has developed diagnostic systems for sex discrimination of chinook salmon, run discrimination of spring and fall chinook salmon in the Columbia River, detection of the salmon pathogen *Ceratomyxa shasta*, and a series of genetic markers that evaluate genetic diversity in chinook salmon populations. In order to work with threatened and endangered species he has developed a non-lethal protocol for collecting tissue samples in the field and extracting DNA of sufficient quality and quantity for thousands of PCR analyses. In addition, his laboratory has also developed similar diagnostic systems in fungi, insects, nematodes, plants, and bacteria. An abbreviated curriculum vitae for Dr. Rodriguez is described below.

RUSSELL J. RODRIGUEZ

EDUCATION:

Ph.D. – Oregon State University (Corvallis, OR), May 1983

Major – Microbial Physiology Emphasis – Membrane Physiology in Fungi

B.S. – University Of California (Davis, CA), June 1978

Major – Bacteriology Emphasis – General Bacteriology

EXPERIENCE:

Microbiologist/Project Leader (GS-13) – US Geological Survey, December 1992 to present.

Affiliate Associate Professor Dept. Botany, University of Washington 1995 to present

Assistant Professor – University of California at Riverside, Department of Plant Pathology, December 1988 to April 1993, (Affiliate Status until 6/97)

Postdoctoral Fellow – Cornell University, Department of Plant Pathology, May 1985 – May 1988.

Postdoctoral Fellow/Research Associate – Oregon State University, Department of Microbiology, August 1983 – December 1984.

Graduate Research Assistant – Oregon State University, December 1979 – July 1983.

GRANTS AWARDED:

NSF - "Assessing nematode biodiversity". Co-PI. 7/91 – 7/92, \$50,000.

USDA - "Genetics of host-parasite interactions between plants and fungal pathogens in the genus *Colletotrichum*". NCR-173 A cooperative grant for annual workshops. Co-PI. 11/91-11/95

USDA "Identifying pathogenicity genes in *Colletotrichum magna*". PI. 9/92 – 9/94, \$100,000.

NSF, DOE, USDA "Genetic basis for pathogenicity in the genus *Colletotrichum*". Co-PI. 10/92 – 10/97, \$250,000.

USDA "Identifying pathogenicity genes in *Colletotrichum magna*". PI. 9/94 – 9/96, \$100,000.

US-Israel BARD. "The interaction between nonpathogenic mutants of *Colletotrichum* and *Fusarium*, and the plant host defense system.". Co-PI. 1/97 – 1/00, \$300,000.

USFS "Population genetics, phylogeny, and ecology of *Bridgeoporus nobilissimus*". Co-PI. 1/97-1/98, \$5000.

USGS/BRD "Effects of stand composition and structure on forest productivity, and species diversity. Co-PI. 6/97-6/99, \$45,000.

PUBLICATIONS: (representative from the last 4 years, total since 1983 is 45)

- Freeman, S., Pham, M.H., and Rodriguez, R.J. 1993 Genotyping *Colletotrichum* species using a nuclear DNA repetitive element, restriction enzyme digestion patterns of A + T rich DNA, and arbitrarily primed PCR. *Exper. Mycol.*, 17:309-322.
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- Rasmussen, C., Holloway, J., and Rodriguez, R.J. 1997 Identification of a nuclear DNA marker that discriminates spring from fall chinook salmon in the Columbia and Snake river systems. In preparation.