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A Population Comparison of *Oncorhynchus mykiss* using Microsatellite Primed PCR

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

by

Warren Nathan Hankinson

August 2000

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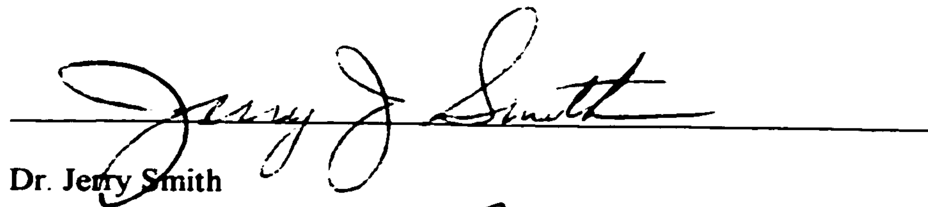

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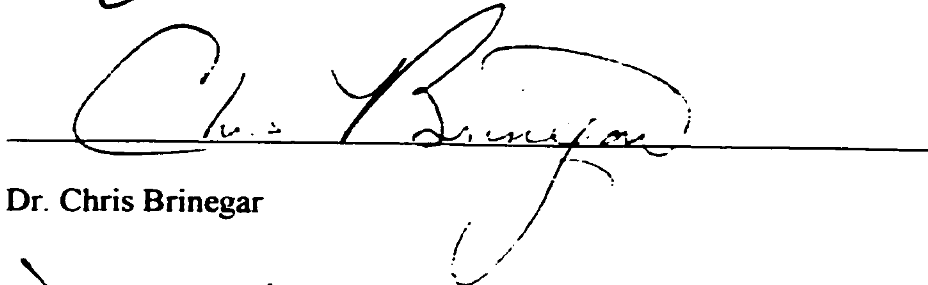

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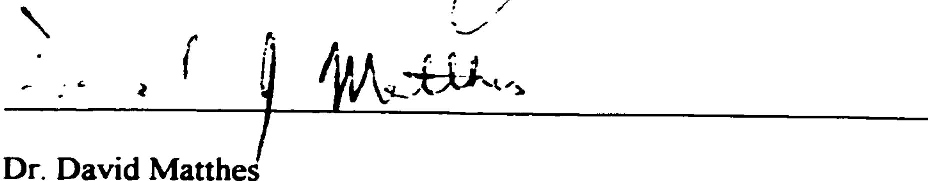

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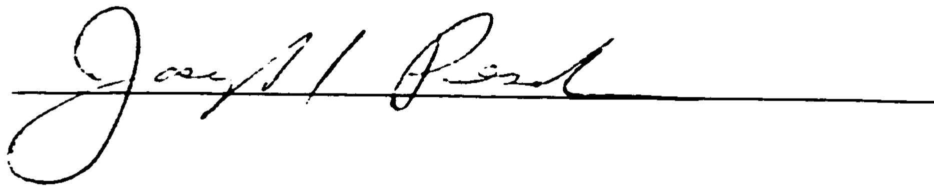

ABSTRACT

A Population Comparison of *Oncorhynchus mykiss* using Microsatellite Primed PCR

by Warren N. Hankinson

Potential genetic differentiation between steelhead (*Oncorhynchus mykiss*) spawning in tributaries flowing into San Francisco Bay and those spawning in streams flowing directly into the Pacific Ocean south of San Francisco Bay was investigated using microsatellite primed PCR (mpPCR) with $(GTG)_5$ as a primer. Five Pacific coastal stream sites and twelve southern San Francisco Bay sites were examined (n=459). Six reliable polymorphic markers were obtained from fin DNA amplified with the $(GTG)_5$ primer. Analysis revealed two population groupings separated by a genetic distance of 0.1 to 0.25. One group appears to represent native San Francisco Bay tributary fish. The second contained all five Pacific coastal stream sites as well as five San Francisco Bay sites which may have been influenced by hatchery fish. Another possible reason for the lack of clear resolution between populations in the two geographic areas was the use of only one primer with only six scoreable markers.

ACKNOWLEDGEMENTS

Much appreciation goes to Dr. Jerry Smith, Dr. Chris Brinegar, and Dr. David Matthes for reviewing this document and providing useful advice and commentary. Dr. Jerry Smith provided assistance with fish samples and ecological insight into some of the phylogenetic results obtained in this study. Dr. Chris Brinegar provided technical and scientific advice and assistance with the molecular work, and provided use of his laboratory and equipment. Funding was provided by the Santa Clara Valley Water District and the Graduate Research Fellowship Program of the Department of Biological Sciences of San Jose State University.

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Introduction

Salmonids have been shown to exist in a complex of genetically distinct local populations which are important to the overall health of the species (Taylor, 1991; Waples 1991b; Utter *et al.*, 1993b). Much concern has arisen recently over the status of steelhead trout (*Oncorhynchus mykiss*) populations, particularly in the southern part of their range (Nehlsen *et al.*, 1991; Busby *et al.*, 1996). In some cases the number of fish returning to spawning streams has fallen and in others entire runs have been eliminated. Among the causes of these declines are overfishing, dams, and water diversions (Wohlfarth, 1986; Nehlsen *et al.*, 1991). Hatcheries designed to mitigate for the declining populations caused by dams create concern that wild fish adapted to localized conditions are being replaced by hatchery fish adapted to hatchery conditions (Wohlfarth, 1986; Waples, 1991a; Hilborn, 1992; Reisenbichler *et al.*, 1992). One difficulty in discerning which populations are in trouble is determining what constitutes the boundaries of an *O. mykiss* population especially considering their extreme phenotypic and life history plasticity (Allendorf and Waples, 1996). Waples (1991b) defined salmonid populations in terms of the evolutionarily significant unit (ESU) which he described as populations which are reproductively isolated from each other and represent an important component in the evolutionary legacy of the species. He stated that the genetic isolation need not necessarily be complete but sufficient to create populations with significantly different allele frequencies.

Many genetic studies have investigated the boundaries of salmonid populations including those of *O. mykiss*. The majority of these studies found that geographically

proximate populations are genetically more similar (Foote *et al.*, 1989; Taylor, 1995). Many concluded that life history (anadromous versus nonanadromous, run time and spawning time differences) and morphological differences have less genetic effect than geography (Chilcote *et al.*, 1980; Currens *et al.*, 1990; Reisenbichler *et al.*, 1992; Utter *et al.*, 1993b) and likely evolved secondarily (Foote *et al.*, 1989; Allendorf and Waples, 1996; Taylor, 1997) or may even be phenotypic expressions of plasticity within a genetically homogenous population (Adkison, 1995). ESU boundaries for salmonids are often found to include multiple drainages within a region rather than individual drainages (Reisenbichler *et al.*, 1992; Busby *et al.*, 1993; Busby *et al.*, 1994). These are generally separated from others by geographic, geological, and climatological barriers and ocean currents and conditions (Waples 1991b; Reisenbichler *et al.*, 1992; Busby *et al.*, 1994). The most significant genetic differences in *O. mykiss* populations appear to be between steelhead and rainbow trout in the Columbia River east of the Cascade Range versus all the coastal populations (Reisenbichler *et al.*, 1992; Nielsen *et al.*, 1994a; Busby *et al.*, 1996). Results of genetic studies in California *O. mykiss* show a genetically very differentiated group (Berg and Gall, 1988; Busby *et al.*, 1996). Fish spawning in streams flowing into San Francisco Bay have been studied very little and their relation to outer coastal populations remains unclear (Busby *et al.*, 1996), although geographic and climatological factors may make one suspect population differentiation.

Several genetic methods have been used to investigate ESU boundaries in *O. mykiss*. Allozymes as the first genetic method have been used extensively in salmonid research (Utter, 1991; Reisenbichler *et al.* 1992; Utter and Ryman, 1993a) but are not as

sensitive as DNA methods and usually require lethal sampling and careful tissue storage (Allendorf, 1994). Recently more sensitive methods such as seqencing hypervariable regions of mitochondrial DNA or analysis of allele frequency from individual microsatellite loci have become popular. These methods may be fairly time consuming and expensive depending on the laboratory set up. Potential drawbacks of using mtDNA include a lower effective population (Ne), ability to look at only a small percentage of the total amount of DNA, and lack of information on effects of male migration because mtDNA is maternally inherited (Allendorf, 1994).

A method developed recently, the RAPD (random amplification of polymorphic DNA) technique (Williams *et al.*, 1990) has been used for a wide variety of inter- and intraspecific studies. This method uses a random sequence primer, usually of ten bases in length, to amplify polymorphic fragments of DNA from throughout the genome of an individual organism resulting in bands of different lengths caused by insertions or deletions in the region between priming sites or by single base changes in the priming site (Gillet, 1991). These fragments have been shown to be dominant and inherited in a Mendelian fashion (Williams *et al.*, 1990). The RAPD method allows for a more extensive sampling of the genome than allozymes (Heipel *et al.*, 1998), doesn't require sacrifice of the organism, is more sensitive than allozymes (Peakall *et al.*, 1995; Heipel *et al.*, 1998) and possibly more sensitive than mtDNA analysis (Bardacki and Skibinski, 1994). It has been used successfully to separate species, populations, and strains in bacteria (Welsh and McClelland, 1990), fungi (Delye and Corio-Costet, 1997; Malvick and Percich, 1998), plants (Kazan *et al.*, 1993; Williams and St. Clair, 1993; Qu *et al.*,

1996; Johns *et al.*, 1997), and animals (Welsh *et al.*, 1991; Lee and Chang, 1994; Todd *et al.*, 1997; Heipel *et al.*, 1998) including fish (Bardacki and Skibinski, 1994; Alegrucci *et al.*, 1995).

Simple sequence repeat or microsatellite primed PCR is similar to RAPDs except that this method takes advantage of very short (usually two to four base pair) tandem repeats that are selectively neutral (Brohede and Ellegren 1999) and ubiquitous in eukaryotic DNA (Sarkar *et al.*, 1991; Largecranz *et al.*, 1993). Usually fifteen or sixteen base segments of these repeats are used as primers to amplify interrepeat regions of DNA. This method has the simplicity of RAPD's but in theory should be more reliable than standard RAPD analysis due to the high homology between template and primer DNA and the greater length of the primers (Meyer and Mitchell, 1995; Duran et al., 1997). It has been shown to produce more polymorphic markers per experiment than RAPD's (Zietkiewicz *et al.*, 1994) and fewer, and therefore easier to score, bands than RAPD's (Weising *et al.*, 1995). Markers produced from mpPCR also segregate in a Mendelian manner (Zietkiewicz *et al.*, 1994), show positional stability in the genome within closely related taxa (Schlotterer *et al.*, 1991; Zietkiewicz *et al.*, 1994), and are dispersed throughout the genome (Meyer and Mitchell, 1995; Buscot *et al.*, 1996). Like RAPD's, mpPCR has been shown to be useful in species, strain, and population separation in fungi (Meyer and Mitchell, 1995; Buscot *et al.*, 1996), plants (Weising *et al.*, 1995; Duran *et al.*, 1997; Ramser *et al.*, 1997), and animals (Perrring *et al.*, 1993; Fullaondo *et al.*, 1997; Heipel *et al.*, 1998). Although RAPD type methods sample an unknown number of loci and potential bands and are not as clearly interpretable as

looking at individual microsatellite loci which produce at most two bands per locus, they should be useful in preliminary population determination studies.

This study uses mpPCR with $(GTG)_5$ as a primer to investigate possible population differentiation between *Oncorhynchus mykiss* spawning in streams flowing into San Francisco Bay versus those spawning in streams emptying directly into the Pacific Ocean south of San Francisco.

Materials and Methods

Sampling locations and methods

Mostly juvenile fish (n=459) were collected by electroshocking or in migrant traps from seventeen stream locations from fourteen streams and their tributaries in 1997 and 1998 (Table 1). Five of these streams and tributaries [(Gazos Creek, San Lorenzo River at Boulder Creek, Zayante Creek (tributary to the San Lorenzo River), and Dos Picachos and Corralitos Creeks (tributaries to the Pajaro River)] flow directly into the Pacific Ocean and the remainder flow into the southern San Francisco Bay (Fig. 1). Fish were assigned to year classes based upon length frequency at each site. Probable steelhead smolts were identified based upon silvery coloration and black edge to the caudal fin. However, smolt coloration was possible only for the eight samples collected in May. Thirty samples were chosen by a randomly generated numbers program in cases where over thirty samples were available for a population.

Table 1. Year classes, number of fish with smolt coloration, and capture dates of fish used in this study from Central Coast and San Francisco Bay tributaries.

Site / Group #	Location	# of fish	Age			Adults	Smolts	Date of capture
			0+	1+	2+			
1	Dos Picachos Cr.	28	5	13	10		0	Sep-97
2	Corralitos Cr.	25	0	19	6		1	May-97
3	Zayante Cr.	27	14	13	0		0	Jun-97
4	San Lorenzo River at Boulder Cr.	30	5	24	0	1	0	Jun-97
5	Gazos Cr.	28	16	12	1		0	Aug-97
6	San Francisquito Cr.	27	0	26	1		0	Jan-98
7	Los Trancos Cr.	23	0	22	1		0	Jan-98
8	Stevens Cr. at Moffet	17	0	8	0	9	8	March, May 1997
9, 10	Stevens Cr. at McClellan	26	0	26	0		6	May-97
11	Stevens Cr. > Stevens Creek Res.	30	0	30	0		0	Jan-98
12	Saratoga Cr.	30	0	27	2		0	May-97
13	Guadalupe River / Lower Los Gatos Cr.	20	16	0	3	1	1?	May-98
14	Los Gatos Cr. < Lake Elsman	27	12	15	0		0	Jul-98
15	Guadalupe Cr.	26	0	21	4	1	1	May-97
16	Los Alamitos Cr.	41	11	23	7		5	May-97
17	Penitencia Cr.	24	7	13	4		6	May-97
18	Smith Cr.	30	0	24	6		0	Jan-98
	Totals	459	86	316	45	12	28	

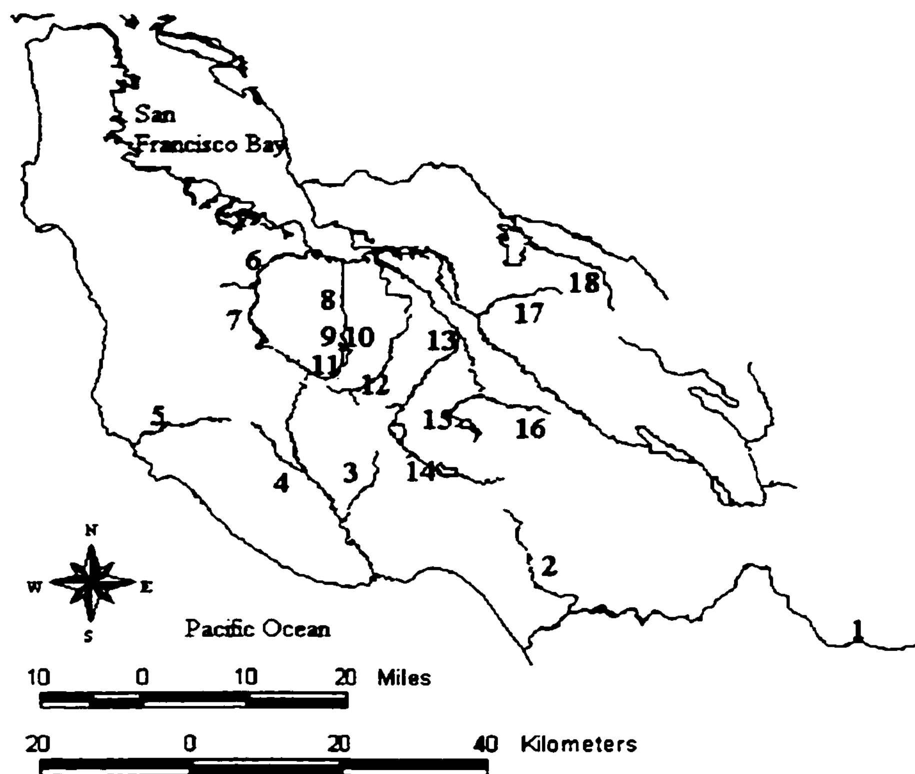

Figure 1. Sampling locations: 1. Dos Picachos Creek, 2. Corralitos Creek, 3. Zayante Creek, 4. San Lorenzo River at Boulder Creek, 5. Gazos Creek, 6. San Francisquito Creek, 7. Los Trancos Creek, 8. Stevens Creek at Moffet, 9. Stevens Creek at McClellan smolts, 10. Stevens Creek at McClellan nonsmolts, 11. Stevens Creek above reservoir, 12. Saratoga Creek, 13. Guadalupe River, 14. Los Gatos Creek below Lake Elsman, 15. Guadalupe Creek, 16. Los Alamitos Creek, 17. Penitencia Creek, 18. Smith Creek

DNA Extraction and PCR Procedure

Partial caudal fin clips were cut from fish with cleaned scissors. Most fin clips were dried for several hours on filter paper and then placed on the filter paper in individual envelopes at room temperature until used. A portion of the fin clips supplied by the Santa Clara Valley Water District were frozen. Many of these samples were not usable because of degraded DNA from freezing and thawing. For DNA extraction samples were placed in sterile 1.5 ml microcentrifuge tubes with 200 µl of 5% Chelex – 100 resin in sterile water (BioRad, Richmond, CA). Samples were heated at 65 to 70°C for 20 minutes, vortexed, then heated at 95 to 100°C for 15 minutes and vortexed again. Finally, the samples were centrifuged for three minutes at 10,000 rpm and placed in a -80°C freezer until used for PCR.

Optimization of PCR conditions was performed as follows: PCR was performed with chelexed fin DNA using 0.5 µM $(GTG)_5$ (Operon, Alameda, CA) dissolved in TE buffer with varying (1-5 mM) concentrations of $MgCl_2$ at 42, 50, and 58°C (Fig. 2). Samples were run on a 3% Metaphor-XR agarose gel (FMC, Rockland, ME) in 1x TBE buffer at 150 V for two hours. The banding pattern at 58°C with 2 mM $MgCl_2$ was chosen as optimal. Samples were next screened for optimal primer concentration using the optimal temperature and $MgCl_2$ concentration from the first experiment and $(GTG)_5$ concentrations ranging from 0.2 to 0.8 µM. Results appeared similar for primer concentrations from 0.4 to 0.8 µM $(GTG)_5$.

PCR for the population comparison was performed in 0.6 ml microcentrifuge tubes in an MJ Research (Waltham, MA) PTC-100 thermal cycler. Total reaction volume

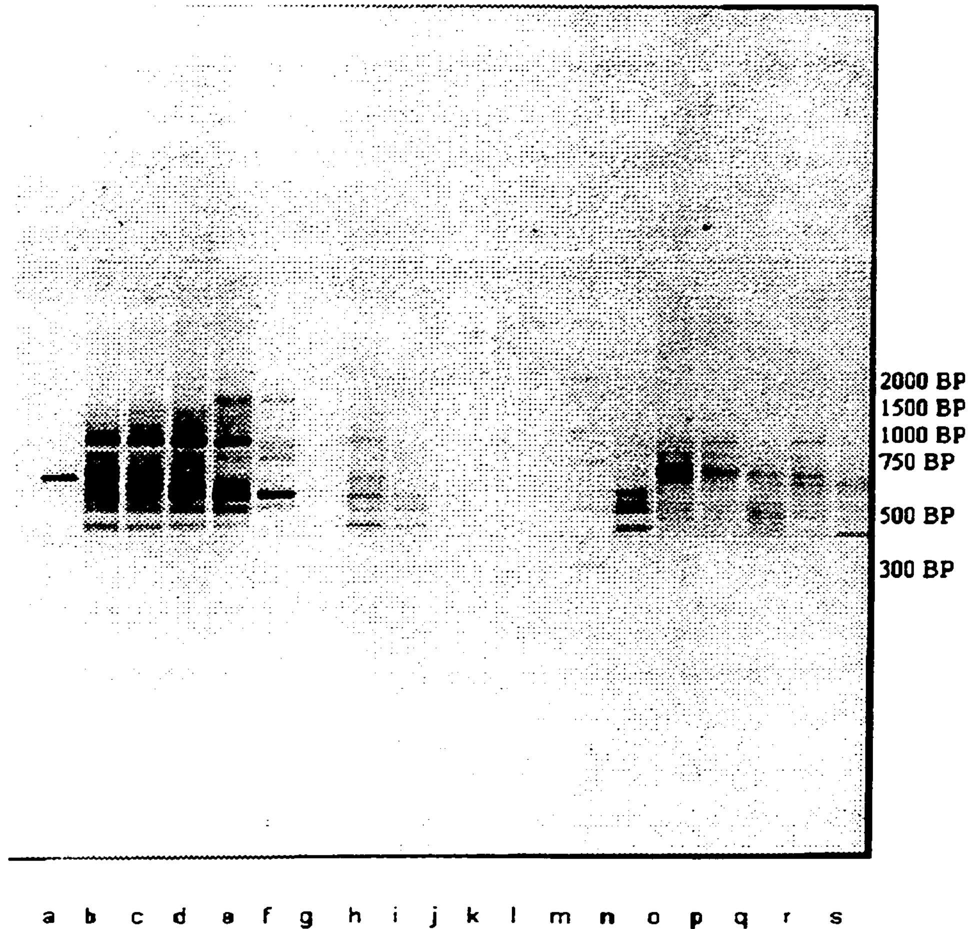

Figure 2. Optimization of MgCl2 concentration and annealing temperature using DNA from an *O. mykiss* sample. Lane a: deionized water control; lanes b-f: 5-1 mM MgCl2 at 58 C; lane g: deionized water control; lanes h-l: 5-1 mM MgCl2 at 50 C; lane m: PCR marker; lane n: deionized water control; lanes o-s: 5-1 mM MgCl2 at 42 C.

was 25 μl and included: 1 μl chelexed DNA, 2.5 μl 10x PCR buffer (PCR Buffer II, PE Biosystems, Foster City, CA), 2.0 μl of 25 mM $MgCl_2$ (final concentration 2 mM), 0.5 μl of 10 mM dNTP's (final concentration of each dNTP was 0.2 mM), 1 μl of 10 μM $(GTG)_5$ (final concentration 0.4 μM), 17.8 μl of autoclaved distilled deionized water and 0.2 μl of 5 unit/μl AmpliTaq Gold DNA polymerase (PE Biosystems, Foster City, CA) (1 unit final). The thermal cycler was programmed to activate the polymerase at 93°C for 9 minutes, denature the DNA at 93°C for 30 seconds, anneal the primer at 58°C for 1 minute, allow primer extension at 72°C for 2 minutes, repeat the previous three steps for a total of 40 cycles, perform a final extension of unfinished products at 72°C for 6 minutes, then refrigerate products at 4°C until removal.

Electrophoresis and Visualization

Samples were run on a 20 cm x 20 cm x 3.5 mm gel of 3% Metaphor-XR agarose in 1x TBE buffer. Five μl of 6x DNA loading buffer were added to each 25 μl sample and mixed. Samples (9.5 μl) were loaded into each well of a 40 well gel along with three wells containing PCR DNA markers (AMRESCO, Solon, OH) and one or two wells with a negative control (chelex blank and/or water blank). The gel was run at 133 volts for 3 to 3.5 hours, after which it was stained in 1 μg/ml ethidium bromide for 30 minutes and rinsed in deionized water for 30 minutes before visualization on a BioRad (Hercules, CA) Gel Doc 1000 image analyzer with Molecular Analyst software version 2.1.1. Photographs were taken of the entire gel and of each third of a gel.

Band Scoring and Statistical Analysis

A minimum of nineteen gels was used in scoring all fish. In most cases samples were run in duplicate on different gels, and in some cases PCR was performed in duplicate. As many as twenty four bands were visible in the 400 to 1,500 base pair range in some of the gels; however, only bands in the 400 to 875 base pair range were considered consistently scoreable in all gels. Six of the fourteen bands seen in this range were used in statistical comparison (Fig. 3). The remainder were not used because they were too close in size to be consistently separated with confidence, there was excessive variation in band intensity, or they were not consistently reproducible between samples run from duplicate PCR's. One or the other negative controls produced bands only occasionally, with none of the same size as any marker used in the analysis.

Data for the six bands on all fish tested were entered as a presence absence matrix. Unweighted Pair Group Method with Arithmetic Averages (UPGMA) dendrograms were produced based on Nei's original genetic distance (Nei, 1972) and Nei's unbiased genetic distance (Nei, 1978) with Popgene software version 1.31 (Yeh *et al.*, 1997). These distance measures as well as Wright's modification of Roger's genetic distance (Wright, 1978) and a distance measure based on the coancestry coefficient (Reynolds *et al.*, 1983) were also used to produce dendrograms using Tools for Population Genetic Analysis© software version 1.3 (Miller *et al.*, 1997). All dendrograms were created with bootstrapping (2000 permutations). Due to the dominant nature of RAPD type data, Hardy-Weinberg equilibrium was assumed in data analysis.

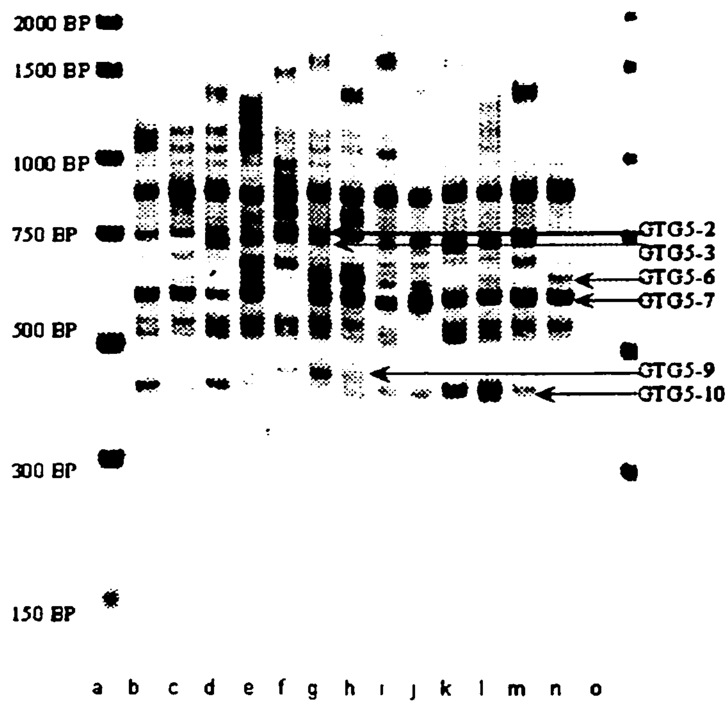

Figure 3. Gel showing the six bands used for analysis. Lane a shows the bands resulting from the PCR marker, lanes b through n show 13 different *O. mykiss* from the San Lorenzo River, Boulder Creek location, and lane o is the result of a chelex control PCR.

This assumption was supported in four of the Pacific coastal sites also used in a microsatellite study of *O. mykiss* by Sundermeyer (1999).

Results

The presence or absence of the six bands was variable from one fish to another with the exception of one band, which was absent in only two of the fish sampled. Allele frequencies for most of the loci were highly variable between populations (Table 2).

All four distance measures produced very similar population groupings. Each revealed two major groupings containing identical populations separated by a genetic distance of 0.1 to 0.25, depending on the measure used (Figs. 4-7). One group contained only eight groups of fish from streams emptying into the San Francisco Bay. The cluster included resident trout populations (Los Alamitos, Los Gatos, and Saratoga creeks) and steelhead populations (Guadalupe River, San Francisquito Creek, Los Trancos Creek, one site on Stevens Creek, and fish with smolt coloration at another Stevens Creek site). The other group contained the five coastal Pacific steelhead streams as well as five San Francisco Bay stream locations, including two on Stevens Creek. Other San Francisco Bay streams that clustered with the coastal populations were Smith and Guadalupe creeks with resident trout and Penitencia Creek, which has both steelhead and resident trout.

Table 2. Allele frequencies of mpPCR markers for steelhead / rainbow trout populations in Central Coast and San Francisco Bay tributaries.

Site / Group #	Location	Allele Frequency (Band Presence) Locus					
		GTG5-2	GTG5-3	GTG5-6	GTG5-7	GTG5-9	GTG5-10
1	Dos Picachos Cr.	0.40	0.62	0.58	1.00	0.58	0.35
2	Corralitos Cr.	0.65	0.47	0.80	1.00	0.55	0.43
3	Zayante Cr.	0.39	0.46	0.46	1.00	0.57	0.42
4	San Lorenzo River at Boulder Cr.	0.34	0.55	0.37	0.82	0.16	0.68
5	Gazos Cr.	0.50	0.62	0.47	0.81	0.35	0.62
6	San Francisquito Cr.	0.36	0.81	0.57	1.00	0.49	0.57
7	Los Trancos Cr.	0.22	1.00	0.49	1.00	0.37	0.37
8	Stevens Cr. at Moffet	0.23	0.76	0.58	1.00	0.46	0.46
9	Stevens Cr. at McClellan (smolts only)	0.29	1.00	0.59	1.00	0.29	0.42
10	Stevens Cr. at McClellan (nonsmolts)	0.61	0.55	0.37	1.00	0.19	0.68
11	Stevens Cr. > Stevens Creek Res.	0.82	0.59	0.63	1.00	0.32	0.48
12	Saratoga Cr.	0.12	1.00	0.39	1.00	0.32	0.48
13	Guadalupe River	0.16	1.00	1.00	1.00	0.37	0.45
14	Los Gatos Cr. < Lake Elsman	0.16	1.00	1.00	1.00	0.42	0.39
15	Guadalupe Cr.	0.24	0.56	0.41	1.00	0.48	0.24
16	Los Alamitos Cr.	0.12	1.00	0.69	1.00	0.51	0.62
17	Penitencia Cr.	0.39	0.59	0.39	1.00	0.71	0.59
18	Smith Cr.	0.48	0.37	0.59	1.00	0.45	0.27

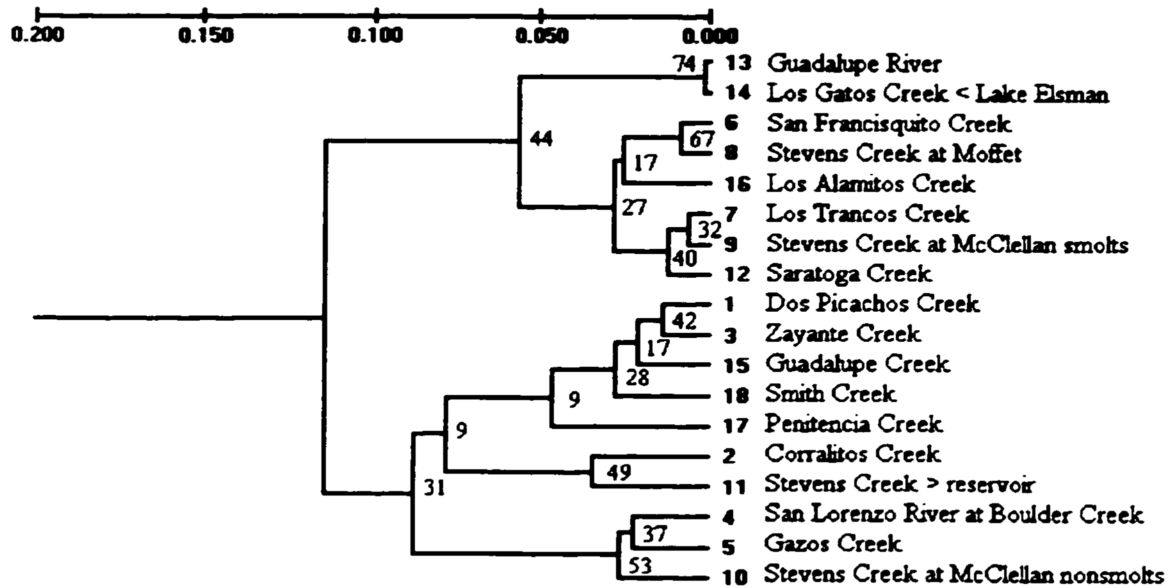

Figure 4. Dendrogram for *O. mykiss* populations produced from Nei's original genetic distance (Nei 1972) with bootstrapping using Tools for Population Genetic Analysis software version 1.3. Numbers at branch points are percentages of similar replicates.

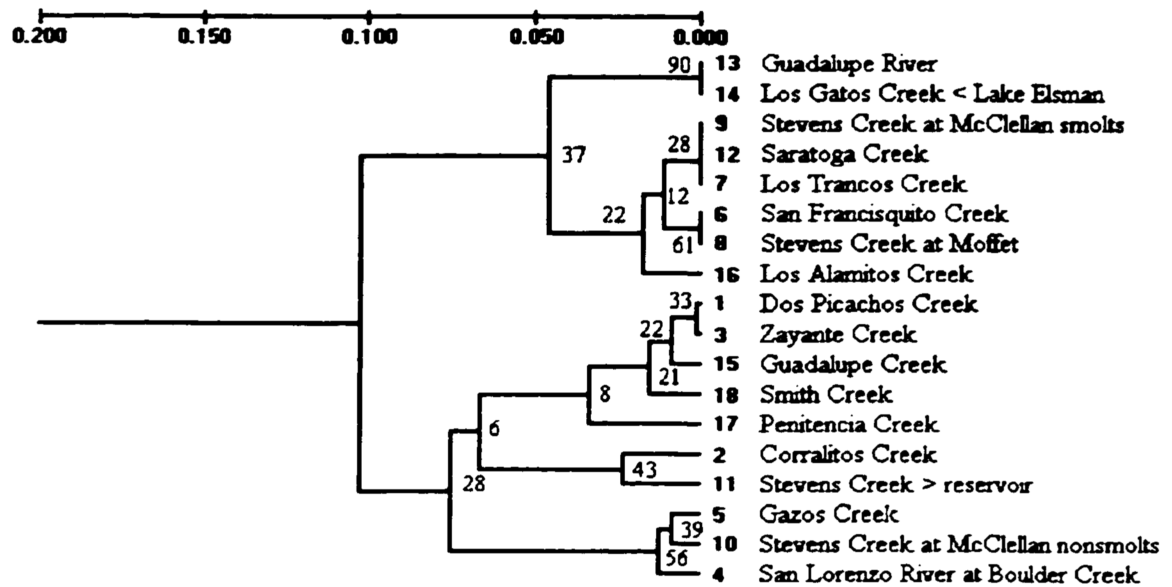

Figure 5. Dendrogram for *O. mykiss* populations produced from Nei's unbiased genetic distance (Nei 1978) with bootstrapping using Tools for Population Genetic Analysis software version 1.3.

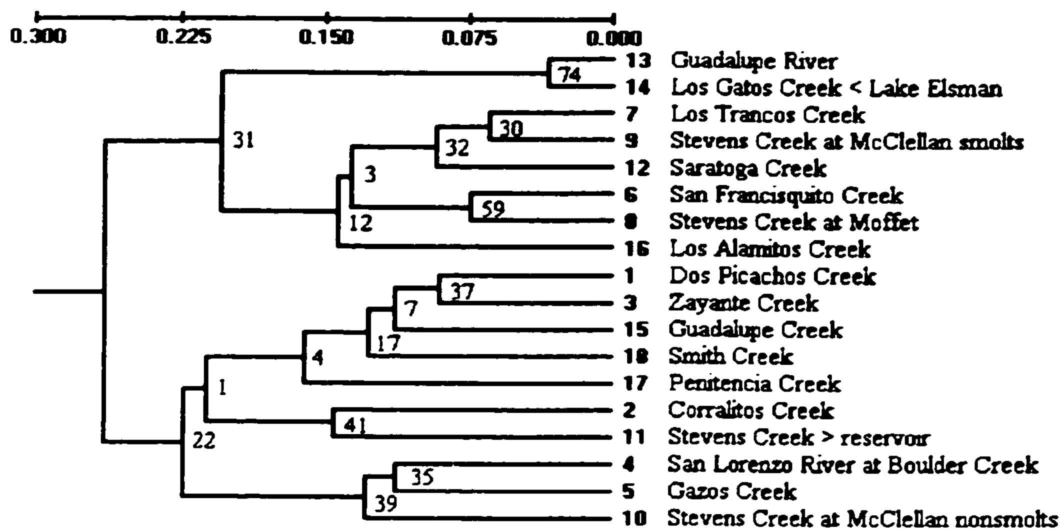

Figure 6. Dendrogram for *O. mykiss* populations produced from Wright's modification of Roger's genetic distance (Wright 1978) with bootstrapping using Tools for Population Genetic Analysis software version 1.3.

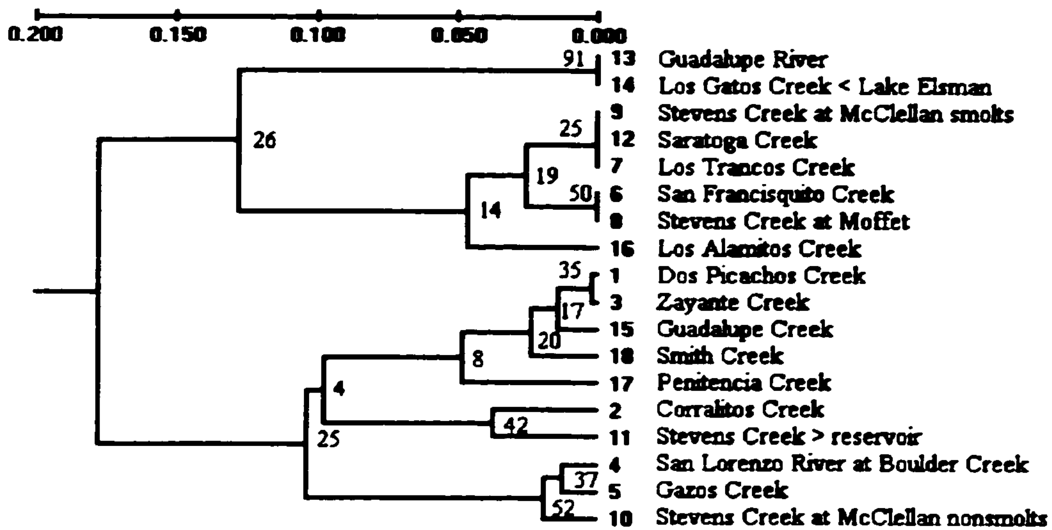

Figure 7. Dendrogram for *O. mykiss* populations produced from a genetic distance based on the coancestry coefficient (Reynolds et al.,1983) with bootstrapping using Tools for Population Genetic Analysis software version 1.3.

Discussion

Four different genetic distance measures of three different types were used in population comparison of *O. mykiss* in the present study. The first two distance measures, Nei's (1972) original (standard) distance and Nei's (1978) standard distance corrected for bias are among the most widely used. These measures estimate the number of codon differences per locus from allele frequency data. Nei's unbiased (1978) genetic distance corrects for bias introduced by small ($n < 50$) sample size by substituting sample gene identities with unbiased estimates of population gene identities which consider sample size. In his model of population differentiation for these distances, Nei assumed an ancestral population at equilibrium with respect to effects of mutation, selection, and drift is split into two and subsequent populations undergo independent evolution, with each new mutation assumed to be different from those preexisting (Nei 1972, 1974, 1976). Populations are assumed to be almost completely isolated since separation, and nucleotide substitutions are assumed to be random, independent, and to occur at a constant rate. Probability of identity of genes (alleles) chosen at random from each of two populations is based on observed data and requires no assumptions about selection, mutation and migration (Nei 1973, 1975). Nei's distance measures are linearly related to time after divergence of two populations if the rate of codon substitutions per year is constant, and are linearly related to geographic distance in certain migration models (Nei 1972, 1973, 1975). Nei's distances are useful over a wide range of genetic distances, from the population to the species level, and are only unreliable when genetic identity is close to zero (genetic distance is very large) (Nei 1973). Nei's standard distance coupled

with UPGMA was found to produce the most accurate branch lengths for phylogenetic trees in a computer model because of its linear relationship with the number of gene substitutions (Nei *et al.*, 1983). Nei (1973, 1975) mentions that although distance measures are most accurate with data from a large number of loci, useful relative values of distance may be obtained with fewer loci. Nei (1973, 1976) argued that these distance measures are superior to geometric representations of genetic distance because those have no clear biological interpretation.

Wright's (1978) modification of Rogers' (1972) genetic distance is of the class of distance measures in which genetic distance is defined as the geometric distance, calculated by the extended Pythagorean theorum, between two populations represented as points in a multidimensional (Euclidian) space. Rogers' distance uses allelic frequencies as coordinates for points of a locus and the arithmetic average of the locus distances for an array of loci as the final distance calculation. Wright's modification uses the arithmetic average of the square root of the squared loci distances thereby using the geometric distance concept more consistently. This adjustment gives less weight to loci with small differences in allelic frequencies than does Rogers' original distance. This distance measure and Nei's (1972, 1978) distances assume random genetic drift and mutation are the factors affecting population differentiation.

The third type of distance measure is that of Reynolds *et al.* (1983) which is based on the coancestry coefficient. This measure is based on a model where genetic drift is the only force operating and mutation and other forces affecting gene frequencies are excluded. The original ancestral population is not inbred, essentially infinite, in Hardy-

Weinberg equilibrium at each locus, and at linkage equilibrium at every pair of loci. Replicate populations are taken as independent random samples from the original population at time zero. Generations are assumed discrete and replicate populations are assumed to remain isolated, constant in size, and maintained by random mating. With these assumptions the coancestry coefficient is the probability that a random pair of genes at the same locus within a randomly chosen population are identical by descent. This distance measure estimates the coancestry coefficient between populations and for short term evolution is linearly related to time. For the pure drift model this distance measure has an advantage over Nei's and Rogers' distances in that no estimation of initial gene frequencies in the ancestral population is required. In a computer simulation study both this distance and Nei's unbiased distance were unaffected by linkage of loci (Reynolds *et al.*, 1983). However, Nei's distance was greatly affected by initial allelic frequencies in contrast to the distance of Reynolds *et al.*, which was not.

Dendrograms from these distance measures in this study should be interpreted with caution, due to the relatively small number of loci analyzed. However, it is notable that populations clustered similarly and that the two major population groupings were present in dendrograms produced by all three types of distance measure.

Most studies of *O. mykiss* have found geographic distance to dominate genetic structuring (Chilcote *et al.*, 1980; Foote *et al.*, 1989; Currens *et al.*, 1990; Reisenbichler *et al.*, 1992; Utter *et al.*, 1993b; Taylor, 1995). Nielsen *et al.* (1994a) found California *O. mykiss* were roughly separable into three North to South geographic regions based on

sequence differences of a variable mtDNA segment and data from a microsatellite locus. However, another study found a lack of geographic congruence between microsatellite and mtDNA data from California *O. mykiss* (Nielsen *et al.*, 1997). The National Marine Fisheries Service (NMFS) broke the California populations into four coastal and one central valley ESU (Busby *et al.*, 1996). The streams in the present study were within the NMFS Central ESU (Russian River to Soquel Creek on the coast plus the San Francisco Bay tributaries) and the South Central ESU (from the Pajaro River system south to Pt. Conception).

The sharp separation of the five coastal populations from 8 of the 13 San Francisco Bay tributary groups in this study supports the expectation of population division due to geographic distance and physical and ecological barriers. Straying between coastal and San Francisco Bay groups should be relatively weak, and the brackish estuarine habitat of the bay may be a significant selective factor for early ocean life of *O. mykiss*. However, there is no geographical pattern to the five San Francisco Bay populations which cluster with the coastal populations. Four factors may help explain these results.

The first factor includes the various potential influences of hatchery fish. These include the swamping of populations with hatchery alleles (Hindar *et al.*, 1991; Felsenstein, 1997; Schluter, 1997), which may be significantly different from those of wild populations (Waples 1991a; Danzmann *et al.*, 1994; Nielsen *et al.*, 1994c), and subsequent reduction of interpopulation variation (Riesenbichler and Phelps 1989; Riesenbichler *et al.*, 1992; Thomas and Mathisen, 1993). In two studies, hatchery fish

increased the number of mtDNA haplotypes in comparison with native populations (Nielsen *et al.*, 1994b; Williams *et al.*, 1996). In some cases hatchery and wild fish show similar allele frequencies (Nielsen *et al.*, 1994b). In others hatchery fish may have little permanent affect on wild trout populations within a stream system (Campton and Johnson, 1985). There is some evidence that hatchery and wild *O. mykiss* may remain reproductively isolated and may segregate within a stream with wild fish in some portions and hatchery or hatchery x wild hybrids in another (Williams *et al.*, 1997). Some reservoirs and streams in Santa Clara County are stocked with hatchery reared catchable rainbow trout which are a mixture of *O. mykiss* strains from throughout the state, including some strains from coastal streams (Sid Poe and Jerry Ayers, California Department of Fish and Game, personal communication). Those sites presently stocked with catchables include Coyote Creek, Stevens Creek Reservoir, and percolation ponds adjacent to Los Gatos Creek. Catchable trout have also been planted in Penitencia and Guadalupe creeks in the past.

The Stevens Creek sites upstream and downstream (McClellan) from the reservoir cluster with the coastal populations and appear to reflect the heavy stocking of catchable trout in the reservoir. However, steelhead smolts and adults from the seasonal lower portion of the stream clustered with the San Francisco Bay tributary group, as did a subset (n=6) of McClellan fish which showed smolt coloration. It appears that a small "native" steelhead run may coexist with a predominately hatchery origin resident trout population in Stevens Creek. The clustering of Guadalupe and Penitencia Creek populations with the coastal populations may also reflect past stocking of hatchery reared

catchable rainbow trout. When Penitencia Creek smolts (n=6) and nonsmolts were analyzed separately they grouped closely together when analyzed by Nei's unbiased (1978) genetic distance and by Reynolds *et al.'s* (1983) coancestry coefficient based distance. However, the smolts separated from the other populations by a genetic distance of 0.15 and 0.3, respectively, and were placed on a separate branch from the two major population groupings when analyzed by Nei's original (1972) genetic distance and Wright's (1978) modification of Roger's genetic distance (not shown in Figs. 4-7). These results may not be surprising in the case of Nei's distances, as Nei's unbiased (1978) distance is a correction for small sample size and likely more accurate in this instance. A larger sample size would further clarify these results. The clustering of the Smith Creek population with the coastal group is harder to explain in terms of hatchery fish, as past stocking of catchables on this remote stream seems less likely. Upper Los Gatos, Saratoga, Los Trancos, and San Francisquito creeks have no apparent history of hatchery stocking and cluster quite tightly. They may represent native *O. mykiss* strains. The close relationship of the Guadalupe River and lower Los Gatos Creek steelhead smolts, adults, and juveniles with those of upper Los Gatos Creek appears to suggest they are also "native". Los Alamitos Creek also contains primarily native fish, although the adjacent and connected Guadalupe Creek clusters with the coastal group, apparently reflecting past stocking of hatchery reared fish.

A factor that may or may not have had some affect on the observed population divisions is the year class of fish sampled. Felsenstein (1997) showed that random genetic drift can occur from one generation to the next in wild populations. Studies have shown

evidence for (Reisenbichler *et al.*, 1992) and against (Taylor, 1995) significant genetic differences between year classes in *O. mykiss*, and one study suggests that hatchery fish in particular may have significant genetic year class differences (Butler and Cross, 1996). Fish in the current study were predominantly juveniles; however, these covered more than one year class. Some populations also include a large proportion of adult or 2+ year class fish (Stevens Creek at Moffet and Dos Picachos Creek).

Another possible factor in explaining the clustering of fish from two streams, Penitencia and Smith Creeks, flowing out of the dry hills of east San Francisco Bay, with the Pacific coastal fish is the inclusion of only two sites from East Bay locations. It might be suspected that fish from these drier, warmer hills separated from the other San Francisco Bay populations by the Santa Clara Valley and the San Francisco Bay might be genetically differentiated from fish from the cooler, moister West Bay tributaries. East Bay fish may cluster with the Pacific coastal fish by chance, due to historical stocking differences, or perhaps because there is more straying between Pacific coast fish and fish in the East Bay due to geographic or oceanographic conditions. Perhaps if more sites in the East Bay hills were sampled a third population cluster might develop which was separated from or within the Pacific coastal cluster.

The fourth possible factor is the RAPD and mpPCR method itself. Despite their successful use in a wide range of organisms, these methods have drawbacks. Among these are difficulties associated with the multilocus, multiple banding pattern results (Baker *et al.*, 1992). These include ambiguous bands, possible linkage of loci, the dominant expression of markers, and the possibility that bands of equal size from

different individuals may be nonhomologous (Black, 1993; Riesberg, 1996). A conservative approach was used in deciding which bands to use in this study. Only reproducible, clear and clearly separable polymorphic bands were scored. This eliminated a large number of bands leaving six usable markers, GTG5-2, 3, 6, 7, 9, 10 (Fig. 3). The problems of linkage and nonhomologous markers have been investigated in other RAPD and mpPCR studies and it has been shown that only a very small percentage of markers are linked or nonhomologous (Williams and St. Clair, 1993; Meyer and Mitchell, 1995), although the situation with this single primer in *O. mykiss* is not known. Another concern with RAPD's and mpPCR is reproducibility between labs and even among different instruments, reagents, or differing quality of target DNA (Black, 1993; Bardacki and Skibinski, 1994; Meyer and Mitchell, 1995 Weising *et al.*, 1995). However, reproducibility within studies is usually good (Alegrucci *et al.*, 1995; Meyer and Mitchell, 1995; Buscot *et al.*, 1996; Heipel *et al.*, 1998; Malvick and Percich, 1998). It has been demonstrated that some RAPD and mpPCR products are the result of imperfect matching between the primer and template, and that this is independent of temperature (Welsh and McClelland, 1990; Williams *et al.*, 1990; Huang *et al.*, 1992; Weising *et al.*, 1995; Gillings and Holley, 1997). In this study reproducibility was generally good with the exception of three bands which were not scored. A problem developed towards the end of this study in which no results were obtained from PCR products until new polymerase and nucleotides were used and freshly prepared DNA was amplified. After this, gel bands remained less distinct perhaps due to a problem with the gel apparatus. Therefore results from an additional population were not used.

Due to time and resource constraints only six markers from one mpPCR primer were scored in this preliminary study. Sundermeyer (1999) used five microsatellite loci to analyze the four San Lorenzo River and Pajaro River watershed sites examined in this study and got substantially different results. In this study Zayante Creek, a tributary of the San Lorenzo River, and Dos Picachos Creek, a distant tributary of the Pajaro River clustered very closely (Figs. 4-7). Corralitos Creek (Pajaro River watershed) and the San Lorenzo River at Boulder Creek were on distinct subbranches of the coastal / San Francisco Bay group. Sundermeyer (1999) found the two San Lorenzo River sites to cluster very closely (as might be expected), and the Corralitos Creek site, which has received hatchery steelhead plants of San Lorenzo River fish, to cluster closely with them. The isolated Dos Picachos Creek site was quite distinct. Sundermeyer's much more intensive microsatellite results closely matched the geographical and stocking history relationship of the four sites, while the results in this limited study with a single mpPCR primer matched poorly.

An additional potential problem with mpPCR compared to microsatellite analysis is constructing a genetic tree based upon genetic differences. Microsatellite trees are sensitive to assumptions about mutational relationships of alleles at each microsatellite locus (Sundermeyer, 1999). For RAPD's the source of the genetic differences is unknown (one or more microsatellites plus other genetic material), and there is no conceptual basis on which to pick a model to assess genetic distances.

The experience gained with this one primer could have allowed additional primers to be run and scored at a reduced time. Successful RAPD / mpPCR studies typically use

several primers resulting in dozens of markers, although correct strain or population division has been determined with fewer. One study showed that 20 randomly chosen RAPD markers separated 95 common bean landrace accessions nearly as well as all 106 markers (Johns *et al.*, 1997). The success rate declined rapidly with fewer than 20 markers although several individual markers made three or fewer misclassifications of all accessions. In another study just two mpPCR primers, $(GTG)_5$ and $(GACA)_4$ produced 30 markers that successfully separated all 47 coconut genotypes analyzed (Duran *et al.*, 1997). Perhaps the use of fewer samples and more primers producing more genetic markers as suggested by Nei (Nei, 1978; Hallerman and Beckmann, 1988) would have improved resolution of populations in the current study.

This attempt to separate *O. mykiss* populations using mpPCR appears to show some promise as evidenced by the clustering of all five Pacific coast samples into the same division of dendrograms created using Nei's genetic distances (Nei, 1972, 1978), Wright's modification of Roger's genetic distance (Wright, 1978), and a genetic distance based on the coancestry coefficient (Reynolds *et al.*, 1983). Although the separation was imperfect, with several San Francisco Bay stream populations in the Pacific coastal cluster, the results were notable considering they were obtained with only one primer resulting in six markers.

Further studies taking advantage of lessons obtained from this study might prove mpPCR a useful preliminary method to help determine ESU boundaries for *O. mykiss*. Due to the lack of a database and the difficulties in interpretation of mpPCR results mentioned earlier, this method is perhaps best used as a screening tool to identify

possible ESU boundaries quickly and inexpensively. Recommendations for future similar studies would include: 1) screening for a minimum of three or four primers for clear, reproducible polymorphic markers and using fewer fish per location if time and resources dictate, and 2) quickly identifying and using only the most unambiguous and reproducible markers from each primer, rather than wasting time attempting to score all bands, which was a mistake made in this study. Further studies of *O. mykiss* population division in the San Francisco Bay and nearby Pacific coastal drainages might include additional streams from the East and North Bay and coastal streams north of the Golden Gate and might include additional genetic methods.

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