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Genetic analysis of *Oncorhynchus mykiss* in South San Francisco Bay tributaries

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GENETIC ANALYSIS OF *Oncorhynchus mykiss*
IN SOUTH SAN FRANCISCO BAY TRIBUTARIES

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Adam Hurst Gemar

May 2006

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ABSTRACT

GENETIC ANALYSIS OF *Oncorhynchus mykiss* IN SOUTH SAN FRANCISCO BAY TRIBUTARIES

By Adam Hurst Gemar

Five microsatellites were used to analyze the genetic similarities of steelhead and resident trout populations within two Pajaro River tributaries, two coastal streams (San Lorenzo River and Gazos Creek), and at eleven South San Francisco Bay tributary sites. The two San Lorenzo River and two Pajaro River watershed sites clustered most closely. Coastal Gazos Creek was intermediate between them and the San Francisco Bay sites. San Francisco Bay tributaries showed substantial genetic structure, with genetic distance largely unrelated to geographic distances. The differences may be due to natural differentiation among populations in watersheds of different accessibility and rainfall amounts. They may also reflect bottlenecks from natural and man-caused stream dry backs and the effects of past stocking of steelhead or catchable rainbow trout.

Acknowledgements

I dedicate this thesis to my father (Robert A. Gemar), who passed away while I was in the process of finishing this master's thesis. He would have been proud! A heart felt thank you to my wife for the support and planning advice that she offered.

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Introduction

Steelhead populations presently range from Alaska to as far south as Malibu Creek at Santa Monica Bay, California (Nielsen et al., 1994). Historically, West Coast steelhead (*Oncorhynchus mykiss*) populations ranged from Alaska to the Rio del Presidio in Baja California (MacCrimmon, 1971; Needham and Gard, 1959). From the Second World War to the 1980's, steelhead numbers decreased dramatically (Nehlsen et al., 1991; Nielsen et al., 1994), primarily due to water usage, dams, diversions, over fishing, and loss of habitat. The National Marine Fisheries Service (NMFS; referred to at the present time as NOAA-NMFS) listed several groups of steelhead trout populations as threatened or endangered under the federal Endangered Species Act (ESA) in August 1997.

Conservation genetics for management of Pacific salmon and steelhead are important and therefore the species are grouped into geographic areas (Park, et al. 1994). These areas are so called evolutionary significant units or ESUs. ESUs are boundaries to identify populations that are morphologically and genetically distinct from other similar populations. Distinct populations may represent evolutionary significant units if they meet two important criteria. These populations must be “substantially reproductively isolated from other conspecific population units” and they must “represent an important component in the evolutionary legacy of the species” to be defined as an ESU (Waples, 1991a; Moritz, 1994).

Figure 1: Central California Coast steelhead ESU and the 15 Pacific Coast ESUs (insert map).

Map generated from the NOAA/NMFS governmental website.

There are 15 distinct groups, or ESUs, of steelhead, from southern California to the Washington-Canadian border and east through Oregon and Idaho (Figure 1, insert) with six ESUs in California. Steelhead within the Central California Coastal ESU were designated a threatened species by the NMFS (Figure 1). There are approximately 6,516 square miles of river basins containing spawning and rearing habitat in the Central California Coastal ESU in the counties of Alameda, Contra Costa, Marin, Mendocino, Napa, San Francisco, San Mateo, Santa Clara, Santa Cruz, Solano, and Sonoma. The adjacent South Central Coastal ESU, from the Pajaro River in Santa Cruz County south to Santa Barbara County was also designated as threatened. The designation of these ESUs was based upon limited genetic information.

ESUs generally reflect north south environmental changes in rainfall/temperature that are expressed as vegetation changes. However, similar changes also occur geographically in a west to east pattern within the Central Coast ESU because of climate changes through the coast range to the interior valley tributaries of San Francisco Bay.

A variety of molecular markers have been used as indicators of population structure, individual similarity, demographics, family structure, inbreeding, straying, and mode of inheritance, etc. (Beacham, et al. 2003; Ferguson and Danzmann, 1998; Wilson and Ferguson, 2002). The most widely used markers are allozymes, mitochondrial DNA and microsatellite DNA (nuclear DNA). Allozymes are protein-based markers that can be separated by means of electrostatic charges and size of the molecule. However, DNA markers mutate faster than allozymes which make them more useful for closely related species or populations within species (Ferguson and Danzmann, 1998; Kohlmann, *et al.*

2003). Mitochondrial DNA is inherited maternally, has high polymorphism, and is highly differentiated between populations, and shows high mutation rates.

Microsatellites are short, one to six base pairs (bp) in length, tandemly repeated simple sequences of nuclear DNA. Homologous primers can anneal to these conserved flanking regions and specific microsatellites can be amplified by PCR technology and size-fractionated or sequenced on a polyacrylamide gel. Microsatellites are scattered throughout prokaryotic and eukaryotic genomes (Moxon and Wills, 1999) and are highly polymorphic in nature due to the variability in the number of tandem repeats (Benzten, et al. 1996). Microsatellites are inherited in Mendelian, biparental, fashion. Due to the inheritance of microsatellites they are important in salmonid migration and breeding studies.

The regions flanking (unique nucleotide sequences) microsatellite loci have been shown to be highly conserved within phylogenetic families (i.e., Salmonidae). Angers, et al. (1997) observed that from the presumed ancestral locus in huchen (*Hucho perryi*), other members of the salmonid genera contained only five base substitutions among 121 nucleotides forming the left and right flanking regions of the SFO12 microsatellite locus. Conservation of microsatellite flanking regions has been reported in other fish and other animal groups (Bentzen, et al. 1993; Estoup, et al. 1993; McConnell, et al. 1995, Morris, et al. 1996; Pepin, et al. 1993).

Microsatellites show very high polymorphism within salmonids (Estoup, et al. 1998; McConnell, et al. 1995; Nielsen, et al. 1994). This is primarily due to high mutation rates of 10^{-5} to 10^{-3} gametes per generation (Banks, et al. 1996). This rate of mutation is found

to be on the order of 3 to 5 times that of amino acid substitutions within proteins and nucleotide substitutions in mitochondrial or other nuclear DNA.

Microsatellites exhibit three categories in which their nucleotide sequences differ. The sequences are termed perfect, compound, and imperfect repeats (Table 1) (Ashley, et al. 1994). The microsatellites within this steelhead study are perfect dinucleotide repeats. Dinucleotide repeats have been found to be the most common tandem repeats within genomes and are quite numerous.

Table 1: Nucleotide repeat patterns seen in microsatellite nucleotide sequences.

Perfect:	$(GT)_n$
Compound:	$(GT)_n(CT)_n$
Imperfect:	$(GT)_nTTATCT(GT)_n$

Note: In the literature, the above terms may differ. They have also been called pure, compound, and interrupted, respectively (example Jarne and Lagoda, 1996).

Processes (i.e., mutations and genetic drift) acting on alleles must be understood and considered when applying microsatellites to the study of populations. Microsatellites are highly variable in length because they mutate rapidly and are hence, polymorphic. This polymorphism is due to the changes that extend or shorten nucleotides within allele sequences. Two mutation models have been proposed to describe the variation at microsatellite loci, the infinite allele mutation model (IAM) and the stepwise mutation model (SMM). The IAM predicts that mutations leading to new allelic states can involve any number of repeat units. The SMM predicts that mutations occur through the gain or loss of one repeat unit (O'Connell, et al. 1997). Polymerase slippage during DNA

replication is the favored mechanism for SMM. Jarne and Lagoda (1996) reported that small alleles tend to increase in size and large alleles will tend to decrease in size. However, the mutational process can cause homoplasy among alleles. The nucleotide sequence of alleles maybe identical due to different mutational processes, for example, the addition of nucleotides or the deletion of nucleotides which result in the same sequence.

This study was undertaken to determine relationships among steelhead and resident rainbow trout populations in San Francisco Bay tributaries and on the Central Coast. The relationships between steelhead and resident trout genetics within San Francisco Bay tributaries and coastal populations are important due to many factors: west to east habitat changes, past and ongoing hatchery stocking in streams and reservoirs, water transfers and diversions and other habitat alterations, and instream barriers including reservoirs. This study analyzed the same microsatellite markers as a study done in the Pajaro River system (Sundermeyer, 1999). The molecular techniques are proven successful and repeatable when analyzing perfect dinucleotide repeat loci. The relevance for comparison between Sundermeyer's steelhead and resident trout population study and this genetics project is that the Pajaro River system is in an ESU adjacent to the South San Francisco Bay tributaries.

In the only previous study Hankinson (2000) used randomly amplified polymorphic DNA (RAPD) to study the genetic similarities of steelhead trout and resident rainbow trout within South San Francisco Bay tributaries. However, genetic studies using RAPDs have been found to be hard to duplicate and may contain many bands, resulting in scoring

difficulties (Hankinson, 2000). Hankinson reported up to 24 bands, but was only able to use six in scoring because of this scoring uncertainty. Moreover, RAPDs may be unreliable and have questionable repeatability, whereas microsatellites have fewer scoring ambiguities (Ferguson and Danzmann, 1998; Meyer and Mitchell, 1995). Hankinson (2000) found divided population structure within San Francisco Bay and coastal populations that contained two main groups. Group one contained “native” San Francisco Bay tributaries, while the second group contained all of the coastal populations as well as five sites from San Francisco Bay influenced by hatchery stocking.

In the Pajaro River system natural barriers and reservoirs have fragmented populations into anadromous steelhead and resident trout. Sundermeyer (1999) showed an effect of stocking steelhead across boundaries. Habitat accessible to steelhead showed genetic markers consistent with changes due to stocking. As mentioned above, my study used Sundermeyer’s (1999) methods of DNA amplification, the same five microsatellite primers/loci, and analysis. Fish samples were also used from Hankinson’s (2000) obtained from San Francisco Bay tributaries to compare steelhead and resident trout within and among San Francisco Bay tributaries, coastal streams, and Parajo streams using microsatellite DNA.

Materials and Methods

Fish Sampling

Fish samples for this study of San Francisco Bay tributaries and one coastal stream (Gatos Creek) were obtained by electrofishing with a backpack electro-fisher in 1997 or 1998 from 13 sites on 11 streams (Figure 2). All 13 sites were used in the Hankinson (2000) study. The Santa Clara County Water District provided fin clip samples for Los Gatos Creek, Stevens Creek, and Guadalupe River by electrofishing and from adult and smolt migrant traps (Figure 2). Microsatellite data were included from five additional sites (two from San Lorenzo River and three from Pajaro River tributaries) from Sundermeyer (1999). Streams were numerically ordered geographically east to west around San Francisco Bay for the purpose of population proximity (Table 2).

Fin clips ($\sim 0.5 \text{ cm}^2$) were taken from caudal fins of individual fish. The clips were placed in a piece of folded chromatography paper and air-dried over night. They were stored in coin envelopes (labeled by size class-standard length, location, date, and sample number) at room temperature in a dark, dry location. Care was taken to prevent cross contamination of samples. The fish were allowed to recover in a live car and were returned to the areas in which they were captured (Hankinson, 2000).

Fish were defined as resident trout (above barriers) and as steelhead (adults and smolts) or “non-smolts,” which included resident trout and/or immature steelhead without smolt coloration. Fish were identified as smolts if they possessed silver bodies with a black edge on the caudal fin.

Figure 2: South Francisco Bay tributaries and coastal streams sampled in this study.

Table 2: Sample locations, drainages, date captured, resident/steelhead trout and sample size of fish used in this study from South San Francisco Bay (S.F.B) and Central Coast tributaries. Group # represents location from east to west around SFB.

<u>Stream Site (Group #)</u>	<u>Drainage</u>	<u>Date</u>	<u>Resident Trout</u>	<u>Steelhead Trout</u>	<u>Sample Size</u>
Smith Creek (1)	Alameda Creek	January 1998	X		33-35
Penitencia Creek (2) Nonsmolts	East side of S.F.B. Coyote Creek	May 1997	X	X	20-22
Penitencia Creek (2) Smolts	East side of S.F.B. Coyote Creek	May 1997		X	14-15
San Felipe Creek (3)	East side of S.F.B. Coyote Creek	July 1998	X		28-30
Guadalupe River (4) and lower Los Gatos Creek	West side of S.F.B. Guadalupe River	December 1998, April-May 1999		X	22-29
Almaden Lake (5) Smolts	West side of S.F.B. Guadalupe River	April 2000		X?	26-30
Alamitos Creek (6)	West side of S.F.B. Guadalupe River	May 1997, July- October 1997	X		30
Guadalupe Creek (7)	West side of S.F.B. Guadalupe River	May 1997	X		28-30
Los Gatos Creek (8) Above Lexington Res.	West side of S.F.B. Guadalupe River	July 1998	X		25-28
Stevens Creek (9) below reservoir (McCellan) Nonsmolts	West side of San Francisco Bay	May 1997	X	X	30-31
Stevens Creek (9) below reservoir (McCellan/Evergreen) Smolts	West side of San Francisco Bay	May 1997, June 1998, April-May 1999		X	30-31
Stevens Creek (10) above Reservoir	West side of San Francisco Bay	January 1998	X		30-31
San Francisquito Creek (11)	West side of San Francisco Bay	January 1998		X	25-30
Gazos Creek (12)	Coastal	August 1997		X	36
San Lorenzo River (13) @ Boulder Creek	San Lorenzo River	June 1997		X	* 30
Zayante Creek (14)	San Lorenzo River	June 1997		X	* 30
Sprig Lake (15)	Pajaro River	June 1997	X catchables		* 12
Uvas Creek (16) @ Croy	Pajaro River	June 1997	X		* 29-30
Dos Picachos (17)	Pajaro River	September 1997	X	X?	* 30-33

* Sundermeyer (1999) samples.

Polymerase Chain Reaction (PCR) and Electrophoresis

DNA Extraction:

A 5% Chelex (BioRad) suspension (in HPLC grade water) was prepared. Razor blades were used (one razor blade/fin) to cut small pieces ($\sim 2 \text{ mm}^2$) of fin clip from each sample and were placed in an individual 1.5 ml centrifuge (labeled) tubes with 200 μl of 5% Chelex. Samples were incubated in a water bath at 65°C for 20 minutes, vortexed for 10 seconds, incubated at 95°C for 10 minutes, vortexed for 10 seconds, and microcentrifuged for 3 minutes. The DNA extract was stored at -80°C until samples were used.

PCR:

PCR conditions were optimized for all loci, changing three variables, MgCl_2 concentrations, annealing temperatures, and number of cycles for 25 μl master mix(es). The variables tested: MgCl_2 concentrations were 1.0mM, 1.75mM, 2.5mM with annealing temperatures of 50°C, 52°C, and 54°C, and number of cycles as 28, 30 and 32 (Appendix A). Final tests were run at optimized MgCl_2 concentrations, temperatures, and cycles as described below.

DNA samples were thawed at room temperature and microcentrifuged for 3 minutes. For each locus, 2 μl of sample DNA were added to a PCR master mix of 23 μl in 0.5 μl PCR tubes. HPLC water was used as a negative control for each PCR set. The optimized concentrations of reagents for each locus were 1x PCR buffer (10x, MgCl_2 free; Promega), 1.75mM MgCl_2 (except for locus One μ 2, for which I used 1.0mM MgCl_2)

(Promega; 25mM), 0.5 μ M each (forward & reverse) primer (Operon), 0.2mM dNTP (Promega, 10mM premixed), 1 unit *Taq* DNA polymerase (Promega; 5 units/ μ l), and HPLC grade H₂O (Appendix A).

A simple pre-PCR contamination test was conducted at the start of this study to test for primer specificity. Two control (H₂O) PCR tubes were subjected to contamination by rubbing vigorously the opening of a PCR tube through a beard or coughing into the tube. Although the results of the contamination tests were negative, caution was taken prior to PCR.

The DNA for the five dinucleotide microsatellite loci (Table 3) were amplified in an Eppendorf Master Gradient thermocycler. The thermocycler was programmed for DNA denaturation at an initial 94°C for 2 minutes, followed by 30 or 32 cycles (for locus One μ 2) at 94°C for 40 seconds, DNA annealing at 52°C (54°C for One μ 2) for 1 minute, DNA extension at 72°C for 1 minute, and held at 4°C until used. Once the DNA amplification was complete, 4 μ l of 6x DNA dye was added to each 25 μ l PCR product. The PCR products were frozen at -20°C for future use.

Gel Preparation:

PCR products were run on a BIO-RAD *PROTEAN II*xi (30cm) electrophoresis system. The electrophoresis glass plates (front and back) were cleaned by Alconox and distilled water and dried. A follow up cleaning of the plates was by 95% ethanol and Kimwipes. The electrophoresis system was setup according to the BIO-RAD *PROTEAN*

*I*xi protocol using 1.0mm spacers. Loci were run on 8% polyacrylamide-bis (29:1) gels, except One μ 2 (6% gel), using 1% Spreadex[®] Polymer NAB

Table 3. Microsatellite Loci, Primers sequences (Forward & Reverse), Annealing Temperatures/Number of Cycles.

Locus	Primer Pair (5'-3') used in this study	Anneal*/cycles	Primer Source
Ssa14	CCT TTT GAC AGA TTT AGG ATT TC CAA ACC AAA CAT ACC TAA AGC C	52°C/32	McConnell et al. 1995
Ssa289	CTT TAC AAA TAG ACA GAC T TCA TAC AGT CAC TAT CAT C	52°C/32	McConnell et al. 1995
Omy77	CGT TCT CTA CTG AGR CAT GGG TCT TTA AGG CTT CAC TGC A	52°C/30	Morris et al. 1996
Omy207	ACC CTA GTC ATT CAG TCA GG GAT CAC TGT GAT AGA CAT CG	52°C/30	O'Connell et al. 1997
One μ 2	GGT GCC AAG GTT CAG TTT ATG TT CAG GAA TTT ACA GGA CCC AGG TT	54°C/30	Scribner et al. 1996

*All annealing temperatures and cycles used in this study differed from published source.

(Elchrom Scientific) (Table 4, a and b). Solutions were mixed in a 50ml centrifuge tube before transferring between the plates. Using a serological pipette, the acrylamide was brought up to 2-3 cm below the top of the front (short) plate. A 50% methanol overlay was carefully placed on the acrylamide and the gel was allowed to polymerize (approximately 30 minutes). After polymerization of the gel, the overlay was poured off and a Kimwipe was used to absorb any excess methanol from the surface of the acrylamide gel. A 4% gel was used to form the wells (25-30 well comb) and make the DNA bands even (Table 4, c). Using a 1000 μ l pipette, the 4% gel was placed over the

higher-percentage gel (6 or 8%), and a 25 or 30 well comb was placed into the gel. The gel was allowed to polymerize for 15-30 minutes and the comb was removed. The gel was inverted and any liquid left in the wells was absorbed using a Kimwipe.

TAE buffer (1x) was poured into the lower buffer chamber (~1500ml), and the gel was inserted into the buffer chamber at an angle so that all the air bubbles were forced out from the lower gel surface. The upper buffer chamber was filled with 1x TAE so that the upper portion of the gel was covered. One microliter of Elchrom M3 Marker (75-622 bp), 1.0 μ l of 50 bp ladder and 3.5 μ l known microsatellite standards were used to call unknown DNA sample bands (4.0 μ l). Gels were run at 200 volts for 3.5 hours. The gels were stained with ethidium bromide for 10 minutes and rinsed with water for 5 minutes.

Gels were photographed (entire gel and close-ups of two halves) and saved via computer (Gel Doc – UV Transilluminator 1000, Bio-Rad). Photographs (Appendix A, III-V) of gels were scored (DNA bands sized) twice. Unclear/unreadable bands were generally run again or re-Chelexed and rerun. However, since the fin clips (samples) were run previously in Hankinson's study as well, some samples contained only enough remaining tissue for one run. They could not be re-amplified or scored; hence the sample sizes were slightly different for different loci (Table 2).

Statistical Analysis

Allele frequencies were calculated using GENEPOP version 3.1b (Raymond and Rousset, 1995). Hardy-Weinberg equilibrium and genotypic linkage disequilibrium

were calculated in GENEPOP.

Table 4. Volume of stock solutions to mix a 30ml gel. a) 8% Acrylamide / 1% Spreadex[®], b) 6% Acrylamide / 1% Spreadex[®], and c) 4% gel.

a)

6ml	3ml	1.25ml	19.5ml	150µl	15µl
40% Stock Acrylamide (29:1)	10 % Spreadex [®] Polymer	20X TAE "Running Buffer"	diH ₂ O (millipore)	10% APS*	TEMED

b)

4.5ml	3ml	1.25ml	21.0ml	150µl	15µl
40% Stock Acrylamide (29:1)	10% Spreadex [®] Polymer	20X TAE "Running Buffer"	diH ₂ O (millipore)	10% APS*	TEMED

c)

500µl	250µl	4.25ml	50µl	8µl
40% Stock Acrylamide (29:1)	20X TAE	diH ₂ O (millipore)	10% APS*	TEMED

*APS: Ammonium Persulfate (made fresh everyday)

Due to the large number of multiple tests, the probability of a Type I Error increases; therefore, the P-values were corrected using Bonferroni correction for $\alpha = 0.05$ within Hardy-Weinberg and linkage disequilibrium. The Bonferroni correction equation for α value is $(n - i + 1) * P\text{-value} = p'$, where n = the number of tests, $i = i^{th}$ P-value, and p' = adjusted P-value. GENEPOP was also used to calculate expected and observed heterozygosity and homozygosity, using Levene's correction, for each population.

Samples from Sprig Lake, a catchable rainbow trout fishing lake, was used as an outgroup because of its genetic dissimilarity to any other site in the Sundermeyer (1999) study. Cavalli-Sforza's chord distances (D_c) (Cavalli-Sforza and Edwards, 1967) were

calculated in GENDIST and used in the CONTML tree, a program within PHYLIP version 3.5c (<http://evolution.genetics.washington.edu/phylip/html>). An assumption of Cavalli-Sforza is that the mutation process of microsatellites is IAM, an Infinite Allele Model. A phylogenetic tree was constructed through CONTML, a program within PHYLIP (Felsenstein, 1981), by using allelic frequencies generated in GENEPOP to produce genetic chord distances (Appendix A, IV). CONTML estimates phylogenies by the restricted maximum likelihood method based on the Brownian motion model and is based on the model of Cavalli-Sforza and Edwards (CONTML program documentation).

A phylogenetic tree was also constructed through NEIGHBOR (PHYLIP program) using Neighbor-Joining and UPGMA methods. NEIGHBOR constructs a tree by successive clustering of lineages, setting branch lengths where the lineages join. The tree does not assume an evolutionary clock (NEIGHBOR, program documentation). This program generated a neighbor-joining tree from the Cavalli-Sforza chord distances created from GENDIST.

The two trees (CONTML and NEIGHBOR) were generated because Sundermeyer (1999) found that the CONTML tree showed the best formation of the branches, yet found similar results with the NEIGHBOR tree as well. Felsenstein (1981) suggested using two trees (CONTML and NEIGHBOR), one tree measures gene frequency data by maximum likelihood under a model in which all divergence is due to genetic drift (CONTML) and a tree second that uses Nei's genetic distance by matrices (NEIGHBOR).

Both the CONTML and Neighbor-Joining trees were viewed through TREEVIEW 5.1.2 (1998), <http://taxonomy.zoology.gla.ac.uk/rod/rod.html>. Trees in TREEVIEW were “ladderize-left ordered” (“Tree” option) for clarity.

Results

Microsatellite Loci Description

The number of alleles from the five microsatellite loci examined within the South San Francisco Bay tributaries in this study ranged from 11 in Ssa289, 19 in Ssa14, 20 in Omy77, 34 in Omy207, and 39 in One μ 2. These numbers were 6% to 42% greater than for the Pajaro and San Lorenzo River systems. Although the same scoring standards were used in the Pajaro and San Lorenzo River systems the number of alleles were 8 in Ssa289, 18 in Ssa14, 18 in Omy77, 24 in Omy207, and 33 in One μ 2 (Sundermeyer, 1999). Although each population contained a high allelic diversity, the majority of the alleles were uncommon (Figure 3). Locus Ssa14 showed 19 alleles between 126bp-170bp, but a single allele (150bp) accounted for one-third ($f=0.33$) of the sampled alleles. For Ssa289, 11 alleles ranged from 100bp-126bp but 108bp ($f=0.32$) and 110bp made up two-thirds of the alleles in the population. The allele distribution for Omy77 was 94bp-144bp, yet 70% of the alleles were 104bp ($f=0.19$), 106bp ($f=0.27$), and 110bp ($f=0.24$). The alleles at locus Omy207 ranged from 82bp-162bp, but 4 alleles (100bp, 104bp, 114bp, and 156bp) made up 48% of sampled alleles. For locus One μ 2, the allele sizes ranged from 202bp-340bp, with the main distribution of alleles between 232bp-242bp and 254bp-260bp; the most common allele size was 260bp ($f=0.14$) (Figure 3).

The average heterozygosity of all sites, including San Lorenzo and Pajaro River populations, ranged from 0.7067 in Alamitos Creek to 0.9027 in Penitencia Creek. For most of the populations at each site and locus, the expected and observed heterozygosity and homozygosity generally agreed. However, there were a few discrepancies between

expected and observed values in some populations. In the Hardy-Weinberg equilibrium test (probability-test), the P-values were calculated and corrected with the Bonferroni correction for the large number of comparisons (Table 5). Out of ninety-five tests within a population at a single locus, thirty-eight P-values were significantly different from Hardy-Weinberg. After the Bonferroni correction only eighteen P-values showed significant deviation from Hardy-Weinberg at a α -level of 0.05, and 12 of the 18 site/loci were from San Francisquito Creek, Almaden Lake and the three Stevens Creek populations. However, for each of the five, while one locus showed an excess of homozygotes another showed a deficiency of homozygotes (Tables 5 and 6). The other six loci with Hardy-Weinberg deviations were for only 1 of the 5 loci at a site.

In an analysis for genotypic disequilibrium (the tendency for two alleles to be present on the same chromosome), thirteen of one hundred and ninety tests showed P-values of less than α -level 0.05 (Table 7). However, after the Bonferroni corrections for the large number of multiple tests, Zayante Creek at Ssa289/Omy77 was the only P-value that showed a statistical significance below the α -level of 0.05 (Table 7).

Distances and Phylogenies

In the CONTML (Maximum Likelihood method) tree (Figure 4), the Sprig Lake population was the most different from any of the other populations, with the largest chord distances relative to all other sites ($D_c=0.0797-0.1039$) (Table 8). Sprig Lake was a seasonal fishing lake, stocked with catchable trout from (an) unknown source(s).

Figure 3: Average allele frequency at each locus within all populations.

Using Sprig Lake as an outgroup in the CONTML tree, the two San Lorenzo sites (Boulder Creek and Zayante Creek) and the two Pajaro sites (Uvas @ Croy and Dos Picachos) ($D_c=0.0210-0.0385$) (Tables 8 and 9) cluster most closely together and the only other coastal site (Gazos Creek) clustered as an intermediate between the San Lorenzo, Pajaro sites and the San Francisco Bay sites (Figure 4 and Table 9). Geographically, Gazos Creek lies between the San Lorenzo River, Pajaro River sites and the San Francisco Bay tributaries (Figure 2).

The CONTML tree revealed that the steelhead populations within the tributaries of San Francisco Bay had much structure and formed a very bushy pattern with long branch lengths (Figure 4). The long branches within and the short divergences among “clusters” suggest little confidence in the pattern of branching. In addition the general branching pattern showed little relation to geographical pattern of the sample sites.

Within the San Francisco Bay, the Stevens Creek sites (smolts below and non-smolts below the reservoir, and the resident trout above the reservoir) showed the most distinct clustering with relatively short branch lengths and a long “trunk” (Figure 4). The chord distances were low (Table 8 and 10), among all three Stevens Creek groups. The Stevens Creek non-smolts (which probably included younger steelhead) below the reservoir were closest to the Stevens Creek smolts below the reservoir ($D_c=0.0181$). Stevens Creek upper residents were closest to Stevens Creek smolts below the reservoir ($D_c=0.0272$) and non-smolts below the reservoir ($D_c=0.0273$) (Tables 8 and 10). The Stevens Creek samples were also relatively dissimilar to other sites; chord distances of the three Stevens Creeks samples to other San Francisco Bay tributaries all were at least 0.0378.

Table 5. Average heterozygosity for south San Francisco Bay and for Central Coast tributaries. Expected and observed heterozygosity and homozygosity are given for each locus and population. N is the sample size.

	Average Heterozygosity	Ssa14			Ssa289			Omy77			Omy207			Onej2			N					
		Hetero	Obs	Exp	Homo	Hetero	Obs	Exp	Homo	Hetero	Obs	Exp	Homo	Hetero	Obs	Exp		Homo				
Smith Creek	0.8864	31.20	34	3.80	1	25.38	31	6.62	1	25.26	29	7.74	4	27.34	28	6.66	8	29.22	28	3.78	5	32-35
	0.8809	17.58	18	4.42	4	14.38	18	4.62	1	17.08	20	2.92	0	20.30	21	1.70	1	17.51	13	1.49	6	19-22
	0.9027	13.03	13	1.97	2	8.92	12	4.08	1	11.81	12	2.19	2	13.19	14	0.81	0	13.48	13	1.52	2	13-16
	0.7746	23.56	27	6.44	3	20.16	19	8.84	10	20.23	20	8.77	9	21.96	25	4.04	1	24.92	20	5.08	10	26-30
San Felipe Creek	0.8063	22.89	18	4.11	9	21.60	24	6.40	4	19.36	16	3.64	7	24.34	24	2.66	3	24.41	24	1.59	2	23-27
Guadalupe/Los Gatos	0.7124	25.29	24	4.71	6	21.05	25	7.95	4	21.19	15	8.81	15	26.12	22	3.88	8	21.49	16	2.51	8	24-30
Almaden Lake	0.7067	24.81	28	5.19	2	22.27	27	7.73	3	22.92	17	7.08	13	24.25	20	5.75	10	20.03	14	9.97	16	30
Alamitos Creek	0.7653	23.56	24	4.44	4	12.33	11	16.67	18	17.84	19	8.16	7	24.00	26	5.00	3	22.68	26	4.32	1	26-29
Los Gatos Creek	0.8377	16.98	24	10.02	3	19.11	23	7.89	4	20.94	21	6.06	6	21.45	23	3.55	2	24.95	21	3.05	7	25-28
Stevens B/NS	0.7637	24.46	22	5.54	8	21.79	31	9.21	0	19.25	23	11.75	8	26.81	22	3.19	8	27.41	22	3.59	9	30-31
Stevens B/S	0.7845	31.86	27	7.14	12	28.38	40	11.62	0	27.95	28	10.05	10	32.39	34	6.61	5	31.74	23	5.26	14	38-40
Stevens Upper	0.7228	23.02	20	7.98	11	23.65	31	8.35	1	23.25	24	8.75	8	21.97	19	10.03	13	26.10	21	5.90	11	31-32
San Francisquito Cr.	0.7751	21.21	23	5.79	4	23.22	29	6.78	1	20.81	21	8.19	8	20.04	14	2.96	9	24.14	21	4.86	8	28-30
Gazos Creek	0.7617	20.15	25	13.85	9	22.83	25	12.17	10	27.18	21	5.82	12	30.94	32	3.06	2	26.30	25	5.70	7	32-35
San Lorenzo River	0.8391	22.46	23	7.54	7	23.14	23	6.86	7	25.11	25	3.89	4	27.59	26	2.41	4	28.25	28	1.75	2	29-30
Zayante Creek	0.7200	16.71	17	13.29	13	21.22	21	8.78	9	23.46	14	6.54	16	26.03	27	3.97	3	26.85	29	3.15	1	30
Spring Lake	0.8436	8.00	9	1.00	0	9.29	10	1.71	1	8.13	6	3.87	6	9.29	10	1.71	1	8.37	9	1.63	1	9-12
Uvas @ Croy	0.7345	22.47	21	6.53	8	22.95	22	6.05	7	26.12	20	3.88	10	26.07	20	2.93	9	27.02	25	2.98	5	29-30
Dos Picachos	0.7123	21.31	23	11.69	10	23.62	19	8.38	13	21.98	19	10.02	13	27.97	27	4.03	5	26.19	25	3.81	5	30-32

Table 6. Estimated exact P values and standard errors for Hardy-Weinberg equilibrium tests for each microsatellite locus at south San Francisco Bay, Parajo, and San Lorenzo River sites. Boxed values indicate significant deviations. Only 18 P-values are significant after corrected by Holm's Sequential Bonferroni levels of $\alpha = 0.05$ (bold/boxed).

	Ssa14		Ssa289		Omy77		Omy207		Oneμ2	
	P-value	S.E.	P-value	S.E.	P-value	S.E.	P-value	S.E.	P-value	S.E.
Smith Creek (1)	0.0260	0.0089	0.1873	0.0116	0.0042	0.0023	0.2146	0.0147	0.0136	0.0047
Penitencia N (2)	0.6565	0.0158	0.5179	0.0125	0.5715	0.0142	0.1342	0.0269	0.0023	0.0018
Penitencia S (2)	0.1833	0.0117	0.3099	0.0181	0.4100	0.0204	0.0551	0.0154	0.1285	0.0156
San Felipe Creek (3)	0.0846	0.0075	0.3615	0.0246	0.3319	/	0.1397	0.0178	0.0334	0.0072
Guadalupe River/Los Gatos (4)	0.0000	0.0000	0.0055	0.0018	0.2892	0.0255	0.3493	0.0321	0.0186	0.0109
Almaden Lake (5)	0.3609	0.0195	0.0018	0.0010	0.0230	0.0049	0.1151	0.0163	0.0009	0.0007
Alamitos Creek (6)	0.1019	0.0158	0.0028	0.0012	0.0154	0.0034	0.1669	0.0191	0.0000	0.0000
Guadalupe Creek (7)	0.0000	0.0000	0.0054	0.0012	0.7192	0.0103	0.6837	0.0231	0.0833	0.0151
Los Gatos Creek (8)	0.0082	0.0025	0.0464	0.0052	0.6834	0.0099	0.0239	0.0077	0.0000	0.0000
Stevens B/N (9)	0.0355	0.0064	0.0000	0.0000	0.8768	0.0120	0.0000	0.0000	0.0058	0.0053
Stevens B/S (9)	0.0188	0.0046	0.0000	0.0000	0.3139	0.0245	0.0000	0.0000	0.0000	0.0000
Stevens Upper (10)	0.0137	0.0041	0.0000	0.0000	0.0113	0.0021	0.0000	0.0000	0.0000	0.0000
San Francisquito Creek (11)	0.0000	0.0000	0.1641	0.0104	0.1183	0.0075	0.0013	0.0011	0.1910	0.0221
Gazos Creek (12)	0.7006	0.0204	0.5322	0.0223	0.0514	0.0121	0.5494	0.0369	0.3471	0.0142
San Lorenzo River (13)	0.3072	0.0218	0.1605	0.0121	0.4251	0.0326	0.0078	0.0047	0.6313	0.0371
Zayante Creek (14)	0.1217	0.0212	0.9728	0.0031	0.0000	0.0000	0.8195	0.0245	0.0856	0.0139
Sprig Lake (15)	0.9123	0.0089	0.8782	0.0058	0.5485	/	0.1290	0.0144	0.0183	0.0037
Uvas Creek @ Croy (16)	0.0267	0.0054	0.7775	0.0105	0.0031	0.0021	0.0927	0.0133	0.3090	0.0368
Dos Picachos (17)	0.9307	0.0159	0.1540	0.0077	0.1140	0.0157	0.5519	0.0179	0.1789	0.0268

Table 7: Genotypic disequilibrium: P-values and standard errors (S.E.) between paired loci.
Significant values at Holm's corrected levels, $\alpha = 0.05$ are boxed.
After Bonferroni correction only one was significantly different.

	Ssa14/Ssa289	S.E.	Ssa14/Omy77	S.E.	Ssa14/Omy207	S.E.	Ssa14/Omy2	S.E.	Ssa289/Omy207	S.E.
Smith Creek	0.0135	0.0082	0.4389	0.0424	0.7619	0.0371	1.0000	0.0000	0.5194	0.0360
Penitencia NS	0.2445	0.0233	1.0000	0.0000	0.0390	0.0153	1.0000	0.0000	1.0000	0.0000
Penitencia S	0.8541	0.0180	0.2649	0.0316	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000
San Felipe Creek	0.7852	0.0263	0.8303	0.0218	0.9621	0.0115	0.4119	0.0386	0.9408	0.0172
Guadalupe River/Los Gatos	0.1282	0.0250	1.0000	0.0000	1.0000	0.0000	0.0709	0.0224	0.5881	0.0420
Almaden Lake	0.5643	0.0323	0.6371	0.0366	0.3877	0.0464	1.0000	0.0000	0.0667	0.0177
Alamitos Creek	0.9889	0.0038	0.7465	0.0286	0.1775	0.0323	0.2052	0.0247	0.1572	0.0203
Guadalupe Creek	0.1931	0.0203	0.0586	0.0126	0.0215	0.0115	0.0651	0.0176	0.0938	0.0158
Los Gatos Creek	0.5525	0.0278	0.8751	0.0169	0.0123	0.0059	0.3842	0.0335	0.4365	0.0368
Stevens B/NS	0.1954	0.0228	0.3803	0.0318	0.2091	0.0333	0.7179	0.0366	0.1683	0.0207
Stevens B/S	0.5362	0.0253	0.3854	0.0398	0.1052	0.0252	0.7829	0.0366	0.2713	0.0274
Stevens Upper	0.2884	0.0223	0.0234	0.0105	0.2459	0.0342	0.5682	0.0419	0.4259	0.0272
San Francisquito Creek	0.3396	0.0346	0.9974	0.0017	0.7573	0.0330	0.8615	0.0234	1.0000	0.0000
Gazos Creek	0.5667	0.0307	0.3760	0.0365	1.0000	0.0000	0.0204	0.0073	0.9190	0.0228
San Lorenzo River	0.7420	0.0285	0.7352	0.0356	0.1692	0.0299	1.0000	0.0000	0.6999	0.0392
Zayante Cr	0.7166	0.0238	0.3046	0.0348	0.4282	0.0355	0.0166	0.0107	0.2834	0.0346
Sprig Lake	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000
Uvas Creek @ Croy	0.0520	0.0133	0.8498	0.0285	1.0000	0.0000	0.4761	0.0464	1.0000	0.0000
Dos Picachos	0.2072	0.0312	0.8437	0.0283	0.6114	0.0432	1.0000	0.0000	0.7975	0.0343

(Table 7 - continued):

	Ssa289/Omy77		Ssa289/Oneµ2		Omy207/Oneµ2		Omy77/Omy207		Omy77/Oneµ2		S.E.	
		S.E.		S.E.		S.E.		S.E.		S.E.		S.E.
Smith Creek	0.2146	0.0291	0.7037	0.0365	1.0000	0.0000	0.8156	0.0325	0.2688	0.0367		0.0367
Penitencia NS	0.7517	0.0236	1.0000	0.0000	1.0000	0.0000	0.2470	0.0359	1.0000	0.0000		0.0000
Penitencia S	0.6734	0.0246	0.4586	0.0310	1.0000	0.0000	0.1748	0.0217	1.0000	0.0000		0.0000
San Felipe Creek	0.9520	0.0128	0.5799	0.0352	0.0247	0.0089	0.0840	0.0166	0.1976	0.0276		0.0276
Guadalupe River/Los Gatos	0.7526	0.0285	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000		0.0000
Almaden Lake	0.0187	0.0069	0.7933	0.0293	0.1134	0.0259	0.7164	0.0375	0.4716	0.0401		0.0401
Alamitos Creek	0.2880	0.0204	0.2251	0.0146	0.9293	0.0150	0.5445	0.0344	0.8405	0.0143		0.0143
Guadalupe Creek	0.9831	0.0035	0.2417	0.0214	0.0583	0.0188	0.4929	0.0332	0.8373	0.0194		0.0194
Los Gatos Creek	0.4543	0.0346	0.8271	0.0276	0.0326	0.0124	0.1373	0.0236	0.3090	0.0373		0.0373
Stevens B/NS	0.2878	0.0219	0.6776	0.0315	1.0000	0.0000	0.7491	0.0224	0.3151	0.0350		0.0350
Stevens B/S	0.1756	0.0204	0.6234	0.0282	0.4151	0.0439	0.3586	0.0376	0.4086	0.0411		0.0411
Stevens Upper	0.1528	0.0131	0.4633	0.0301	0.6763	0.0416	0.0358	0.0115	0.8944	0.0206		0.0206
San Francisquito Creek	0.1102	0.0192	1.0000	0.0000	0.2904	0.0404	0.2947	0.0321	0.5770	0.0366		0.0366
Gazos Creek	0.6474	0.0387	0.9672	0.0076	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000		0.0000
San Lorenzo River	0.7520	0.0331	1.0000	0.0000	1.0000	0.0000	0.2146	0.0353	1.0000	0.0000		0.0000
Zayante Creek	0.0416	0.0131	0.0193	0.0089	0.0410	0.0190	0.3039	0.0427	0.3450	0.0403		0.0403
Sprig Lake	1.0000	0.0000	1.0000	0.0000	0.0692	0.0104	0.4601	0.0233	0.1062	0.0103		0.0103
Uvas Creek @ Croy	0.1074	0.0249	0.2369	0.0387	0.0118	0.0101	1.0000	0.0000	0.2200	0.0400		0.0400
Dos Picachos	0.9107	0.0208	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	0.0281	0.0132		0.0132

The weaker clustering among Alamitos Creek, Smith Creek, and Almaden Lake (Figure 4) showed chord distances of 0.0248 (Alamitos Creek and Almaden Lake), 0.0277 (Alamitos Creek and Smith Creek) and 0.0369 (Almaden Lake and Smith Creek) (Table 10). Alamitos Creek and Almaden Lake were genetically close and are also geographically adjacent (Figure 2). However, the association between Smith Creek, Alamitos Creek and Almaden Lake is harder to reconcile since Smith Creek is a Mt. Hamilton tributary to the Alameda Creek system (Figure 2). Both Almaden Lake and Alamitos Creek were also genetically relatively close to the nearby Guadalupe River/Los Gatos Creek steelhead samples ($D_c=0.0285$ and 0.0435) (Table 10). However, the adjacent Guadalupe Creek resident trout population clustered weakly elsewhere (Figure 4) and was relatively genetically distant from all three ($D_c=0.0428 - 0.0556$) (Table 8 and 10).

San Francisquito Creek steelhead were most closely related to Almaden Lake fish ($D_c=0.0326$), coastal Gazos Creek steelhead ($D_c=0.0372$) and Guadalupe River/Los Gatos Creek steelhead ($D_c=0.0380$), but in the CONTML tree were weakly aligned with Almaden Lake, Alamitos Creek, and Smith Creek fish (Figure 4). The non-smolt population within Penitencia Creek showed a much closer relationship to other watersheds, including Guadalupe River/Los Gatos ($D_c=0.0328$), Los Gatos Creek ($D_c=0.0370$), Almaden Lake ($D_c=0.0419$) and San Francisquito Creek ($D_c=0.0419$), than to the smolt population of Penitencia Creek ($D_c=0.0498$) (Table 10). The two Penitencia Creek samples were very weakly clustered with upper Los Gatos samples (Figure 4) and were only weakly related ($D_c=0.0370-0.0480$) to that population.

Figure 4: CONTML phylogenetic tree for South San Francisco Bay, Gazos Creek, San Lorenzo, and Pajaro watersheds based on five microsatellite loci. The tree is rooted with Sprig Lake, a catchable trout fishing lake.

Table 8. Cavalli-Sforza chord distance (Dc) for steelhead and trout populations within South San Francisco Bay and Coastal tributary sites. Distances are based on 5 microsatellite loci (Ssa14, Ssa289, Omy77, Omy207, and Omy2).

[illegible]

Table 9: Cavalli-Sforza chord distance similarities (D_c) generated by five microsatellites between coastal sites and San Francisco Bay sites.

Coastal Sites	Most Similar Sites		
Gazos Creek (12)	San Francisquito Creek 0.0372	San Lorenzo River 0.0377	Zayante Creek 0.0410
San Lorenzo River @ Boulder (13)	Zayante Creek 0.0210	Dos Picachos 0.0360	Uvas Creek @ Croy 0.0366
Zayante Creek (14)	San Lorenzo River 0.0210	Uvas Creek @ Croy 0.0385	Gazos Creek 0.0410
Uvas Creek @ Croy (16)	Dos Picachos 0.0355	San Lorenzo River 0.0366	Zayante Creek 0.0385
Dos Picachos (17)	Uvas Creek @ Croy 0.0355	San Lorenzo River 0.0360	Zayante Creek 0.0416

*Sprig Lake = site 15

Table 10: Cavalli-Sforza chord distance similarities (D_c) generated by five microsatellites for San Francisco Bay sites and coastal sites.

San Francisco Bay sites	Most Similar Sites			
Smith Creek (1)	Alamitos Creek 0.0277	Almaden Lake 0.0369	Guad River/Los Gatos 0.0431	San Francisquito Creek 0.0469
Penitencia Creek NonSmolts (NS) (2)	Guad. River/Los Gatos 0.0328	Los Gatos Creek 0.0370	Almaden Lake 0.0419	San Francisquito Creek 0.0419
Penitencia Creek Smolts (S) (2)	Los Gatos Creek 0.0480	Penitencia Creek NS 0.0498	Gazos Creek 0.0579	Guadalupe Creek 0.0611
San Felipe Creek (3)	Guad. River/Los Gatos Cr. 0.0399	Almaden Lake 0.0407	Los Gatos Creek 0.0429	Guadalupe Creek 0.0475
Guad. River/Los Gatos Cr. (4)	Almaden Lake 0.0285	Penitencia Creek NS 0.0328	Stevens Creek Upper 0.0378	San Francisquito Creek 0.0380
Almaden Lake (5)	Alamitos Creek 0.0248	Guad. River/Los Gatos Creek 0.0285	San Francisquito Creek 0.0326	Smith Creek 0.0369
Alamitos Creek (6)	Almaden Lake 0.0248	Smith Creek 0.0277	Guad. River/Los Gatos Cr. 0.0432	Penitencia Creek NS 0.0445
Guadalupe Creek (7)	Stevens Creek B/S 0.0405	Almaden Lake 0.0428	San Felipe Creek 0.0475	Guad. River/Los Gatos Creek 0.0497
Los Gatos Creek (8)	Penitencia Creek NS 0.0370	Guad. River/Los Gatos 0.0382	Almaden Lake 0.0423	San Felipe Creek 0.0429
Stevens Creek B / NS (9)	Stevens Creek B / S 0.0181	Stevens Creek Upper 0.0273	San Francisquito Creek 0.0491	Almaden Lake 0.0492
Stevens Creek B / S (9)	Stevens Creek B / NS 0.0181	Stevens Creek Upper 0.0272	Guad. River/Los Gatos Creek 0.0397	Guadalupe Creek 0.0405
Stevens Creek Upper (10)	Stevens Creek B / S 0.0272	Stevens Creek B / NS 0.0273	Guad. River / Los Gatos Creek 0.0378	Almaden Lake 0.0414
San Francisquito Creek (11)	Almaden Lake 0.0326	Gazos Creek 0.0372	Guad River/Los Gatos 0.0380	Penitencia Creek N.S 0.0419

The Guadalupe River/Los Gatos Creek tributary was set off by itself in the CONTML tree because it showed the closest relationship to members of three dissimilar clusters. The closest relationships were to Almaden Lake ($D_c=0.0285$), Penitencia Creek smolts ($D_c=0.0328$) and Upper Stevens Creek ($D_c=0.0378$). Guadalupe Creek and San Felipe Creek were not very close genetically ($D_c=0.0475$), but very weakly clustered in CONTML because they were relatively dissimilar to all other sites ($D_c>0.0399$).

Both tree formations (CONTML and Neighbor-Joining) formed the same basic patterns between coastal and San Francisco Bay tributaries. In the Neighbor-Joining tree, the San Lorenzo and the Pajaro sites also clustered closely and Gazos Creek was intermediate between them and the San Francisco Bay tributaries (Figure 5). The two trees differed only slightly for the poorly clustering San Francisco Bay tributaries. San Francisquito Creek is the closest San Francisco Bay tributary to the Golden Gate and the coast (Figure 2) and was intermediate between the Gazos Creek and other San Francisco Bay tributaries within the Neighbor-Joining Tree (Figure 5). However, San Francisquito Creek weakly clustered with Almaden Lake, Smith Creek, and Alamitos Creek in the CONTML tree (Figure 5). The remaining tributaries clustered similar to that of the CONTML tree.

Figure 5: Neighbor-Joining phylogenetic tree for South San Francisco Bay, Gazos, San Lorenzo, and Pajaro watersheds, based on five microsatellite loci. The tree is rooted with Spring Lake, a catchable trout fishing lake.

Discussion

Hardy-Weinberg Equilibrium

If the observed values in the Hardy-Weinberg equilibrium show a significant difference from the expected values there was a violation of one or more assumptions. These assumptions are: 1) population size is large, 2) mating is non-random in the population (the most common deviation results from inbreeding), 3) males and females have similar allele frequencies, 4) there are no mutations or migrations affecting the allele frequencies in the population, and 5) the genotypes have equal fitness (i.e., there is no selection). As mentioned in the Materials and Methods section, the Bonferroni correction was used to lower the chance of making a type one error-incorrectly declaring a difference based on chance alone due to a large number of comparisons.

Eighteen out of ninety-five tests for departures from Hardy-Weinberg equilibrium were found to be significant (Table 6), with too many heterozygotes (interbreeding) or homozygotes (inbreeding). However, no site showed a consistent trend towards interbreeding or inbreeding at two or more loci, so the results probably have no biological significance.

Scoring errors could have contributed to the high number of departures at One μ 2, which accounted for 6 of the 18 deviations from Hardy-Weinberg. One μ 2 had very high polymorphism (39 alleles) and a large range between alleles (202-340bp) which made this locus difficult to score. All six significant One μ 2 departures were observed to have excess homozygotes. Due to this high polymorphism, errors in scoring could be made by failure to detect a second band because of the close proximity of alleles to one another

and to differences in band strength (Figure 3). Hankinson (2000) noted variation in “excessive band intensity” as well, which influenced bands used within his study.

Genotypic Disequilibrium

With only one significant P-value at Zayante Creek (0.0416) for genotypic disequilibrium the different loci were not linked. Although no significant genotypic disequilibrium was found within this study, some factors that influence genotypic disequilibrium include changes in population demographics (such as population growth, bottlenecks, geographical subdivision, and migration) and selective forces (Hill and Robertson, 1966).

Distances and Phylogenies

The phylogenetic trees (CONTML and Neighbor-Joining) and distances (D_c) in this study should be interpreted with caution due to the small sample sizes and the small number of loci used to examine these populations. Sample sizes of 50-100 individuals are desired for more precise estimation of genetic distances and more than five loci should be used to analyze microsatellite data (Ruzzante, 1998).

In both trees generated, the overall pattern shows relatively close association between the San Lorenzo and Pajaro sites, with Gazos Creek an intermediate between them and the highly differentiated San Francisco Bay tributaries (Figure 4). In Sundermeyer's (1999) study, Dos Picachos and Uvas Creek @ Croy (Pajaro sites) showed only moderate clustering and were highly separated compared to the San Lorenzo sites and to Pajaro

sites that had apparently been affected by planting of San Lorenzo fish. However, the two relatively distinct Pajaro populations clustered with the two San Lorenzo sites in this study simply because they were more similar to each other than to any South San Francisco Bay population (Tables 9 and 10). The Cavalli-Sforza chord distance between the Pajaro populations (Dos Picachos and Uvas Creek @ Croy) in my study was 0.0355. Sundermeyer (1999) reported the Cavalli-Sforza chord distance at 0.0425. This difference in chord distance is most likely caused by comparisons among different populations within the two studies.

Gazos Creek still seems to remain a native steelhead run because of its separation from South San Francisco Bay tributaries (Figure 4 and 5). The closest similar tributaries to Gazos Creek were San Francisquito Creek ($D_c=0.0372$), San Lorenzo River ($D_c=0.0377$), and Zayante Creek ($D_c=0.0410$) (Table 9). Gazos Creek, unlike tributaries within San Francisco Bay, did follow a geographically based pattern in its genetic makeup (Table 9 and Figure 2).

San Felipe Creek was presumed to be a native resident trout population. However, San Felipe Creek is a perennial stream that flows into Anderson Reservoir and the Department of Fish and Game has stocked catchable trout in Anderson Reservoir and in Coyote Reservoir (upstream of Anderson) and those fish can migrate into San Felipe Creek (Leidy, et al. 2005). Guadalupe Creek, which has been historically stocked (1950's) (Jerry Smith, personal communication), clustered with San Felipe Creek ($D_c=0.0399$)

One of the closest relationships ($D_c=0.0248$) was found between Alamitos Creek and Almaden Lake (an old quarry). Alamitos Creek flows out of Almaden Reservoir and into Almaden Lake. A barrier downstream of Almaden Lake was presumed to prevent steelhead access until laddering in the fall of 1999. However, the fish sampled (April 2000) in Almaden Lake consisted of smolts and two adult steelhead. Smolts from anadromous adults should not have been in the system for at least another year. Adult fish may have passed the barrier during El Niño storms in 1998 or may have been placed above the barrier by fisherman. Alternatively, residual smolt production may have been occurring within Alamitos Creek fish.

Smith Creek (a tributary to the Alameda Creek system) also was closely related to the Alamitos Creek ($D_c=0.0277$) and Almaden Lake populations ($D_c=0.0369$). Geographically, these populations (Smith Creek and Alamitos Creek) are quite separated. One possible explanation for their genetic similarities may be the stocking of catchable trout, from a similar hatchery source, into these systems. The three weakly clustered with San Felipe and Guadalupe creeks, which appear to have hatchery influence, in the neighbor-joining tree, but not the CONTML tree.

Stevens Creek contained three population samples: resident trout above the reservoir (upper), resident trout and young steelhead below the reservoir (B/NS), and steelhead adults or smolts below reservoir (B/S). Hankinson (2000), using RAPD's (randomly amplified polymorphic DNA) to analyze the same samples used in this study, found restricted gene flow below the reservoir between the steelhead (smolts) and the nonmolt population. The two samples below the reservoir clustered into two separate branches of

the trees. However, in this study, the three Stevens Creek populations clustered. The closest relationship was between smolts and non-smolts below the dam ($D_c=0.0181$). The clustering suggests that gene flow (introgression) may in fact occur between these two populations. Stevens Creek Reservoir was historically stocked with catchable trout allowing hatchery introgression with resident trout above the reservoir and with steelhead and resident trout downstream of the reservoir. The lower portion of the stream dries up in the summer months and during severe drought years may never completely flow to the Bay. During dry years introgression between the resident fish (Table 2) and the landlocked steelhead could occur and could explain the clustering and the chord distances ($D_c=0.0181-0.0273$) (Table 10). The results of Hankinson's study (2000) and this study differed for Stevens Creek populations. The RAPD markers used in Hankinson's study detected differences in the Stevens Creek populations (i.e. populations had restricted gene flow); whereas, the microsatellites used in this study showed no difference in the populations (i.e. possible introgression is occurring).

The San Francisco Bay populations did not show isolation by geographic distance, as did the coastal populations within this study. This isolation by distance was seen in more extensive studies of coastal populations as well (Bjorkstedt, et al. 2005). The geographically inconsistent clustering of tributaries within the San Francisco Bay could be because of past stocking of catchable rainbow trout from various sources. Also, some tributaries have previously been planted with catchable trout while others have not and some reservoirs within San Francisco Bay tributaries are presently being stocked. One would expect to see a geographic arrangement among the South San Francisco Bay

tributaries according to their east to west ordering as eastern watersheds are drier and warmer than those of the cooler, moister western watersheds. In addition, straying of steelhead into adjacent streams should be more likely than to distant streams, but there was no evidence of geographically-ordered gene flow. Genetic drift may also have played a part in the structure seen in this study within the San Francisco Bay tributaries. During severe dry years, populations may have gone through bottlenecks which severely altered the allele frequencies of a population.

No geographic patterns were seen within this study among populations around San Francisco Bay. Mutations could play a part in the pattern of the clustering among fish populations. Salmonids display a great variation in the number of repeat units. This is unusual when compared with other vertebrates, but is similar to the distribution seen in other teleosts (Morris et al., 1996). Brooker et al. (1994) speculates that this may be a result from higher rates of slippage during DNA replication because the organisms are poikilothermic and living in a cold-water environment. However, the stepwise geographic patterns shown in the Bjorkstedt et al. (2005) and Sundermeyer (1999) studies indicates that mutation rates do not vary enough to alter genetic patterns.

Errors in scoring alleles may have influenced the structure of the phylogenetic trees within San Francisco Bay. However, the polymer Spreadex[®] separated the alleles well, and staining and photographing the gels allowed clear resolution between base pairs. The allele “stutter” was used as a 2 base pair “ladder” between adjacent alleles. However, there were some problems with the clarity with the larger alleles, as in One μ 2 (<340bp). A 6% acrylamide gel was used to accommodate the large allele size (Spreadex[®],

protocol manual). Size standard alleles via ABI automatic sequencing obtained from Sundermeyer's (1999) research were also used in this study.

The sampling from various years (spring-fall 1997, winter 1998, spring 1999, and spring-summer 2000) may have affected the outcome of this population genetics study. In addition, the number of year classes in the samples varied from population to population (site to site). Both sampling from various years and different age classes, may have contributed to allele distribution differences.

Management Implications

Unlike Sundermeyer's 1999 Pajaro River study, the San Lorenzo River hatchery fish have no stocking history within the San Francisco Bay tributaries and they did not cluster together. The chord distances, from the San Lorenzo River system to South San Francisco Bay tributaries, are some of the highest (0.0544-0.0743). Past hatchery stocking of catchable trout from various sources (Hankinson, 2001), small population bottlenecks, and barrier removals have apparently affected the population genetics within the South San Francisco Bay tributaries. Some possible ongoing sources for the hatchery fish may have been from Parkway Lakes (Coyote Creek), Coyote Reservoir, Lexington Reservoir, Stevens Creek and the percolation ponds along Los Gatos Creek. This may contribute to both the great genetic structure and the non geographic pattern shown by both CONTML and Neighbor-Joining phylogenetic trees (Figure 4 and 5). Numerous studies involving comparisons of hatchery and wild populations have yielded strong evidence that hatchery fish have caused losses of genetic variability between and within

anadromous populations of salmonids (Waples, 1991b and Hilborn, 1992). It seems, in this study, that historical hatchery fish in Steven Creek, Anderson and Lexington Reservoirs may be one reason for the apparent gene flow between the Stevens Creek, San Felipe Creek and Upper Los Gatos Creek populations.

The use of five microsatellites to genetically analyze the populations of steelhead and resident trout within South San Francisco Bay, two Pajaro River tributaries, and two coastal streams showed a lack of geographical pattern. The San Francisco Bay tributary populations had a wide range of genetic structure; while the two Pajaro River populations clustered most closely to one another and the coastal streams clustered between the two groups (the Bay sites and the Pajaro sites). These differences may reveal natural causes, the effect of man's influence of stocking and water diversions or a combination of both. The genetic structure (non-clustering) within the Bay tributary trout populations leave many questions unanswered. Further genetic studies of San Francisco Bay populations will help assist in the knowledge and management of *Oncorhynchus mykiss*.

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APPENDIX A:

PCR Master Mix Concentrations

Electrophoresis (Gel) Photos

Phylogenetic tree (CONTML and Neighbor-Joining) Computer Settings

Master mix concentrations for Ssa14, Ssa289, Omy77, and Omy207; (25 µl/reaction)

Stock Reagents	[Desired]	Volume of Stock (25µl/rxn)
Buffer (MgCl ₂ free) 10X	1X	2.5µl
MgCl ₂ (25mM)	1.75mM	1.75µl
*X (10µM)	0.5µM	1.25µl
*X (10µM)	0.5µM	1.25µl
DNTP (10mM)	0.2mM	0.5µl
H ₂ O (HPLC, 0.2µm filtered)	—	15.55µl
Taq (5 units/µl)	1 unit	<u>0.2 µl</u>
		23 µl
DNA		<u>2 µl</u>
		25 µl total/rxn

* X = Ssa14, Ssa289, Omy77 and Omy207

Master mix concentrations for Oneµ2; (25 µl/reaction)

Stock Reagents	[Desired]	Volume of Stock (25µl/rxn)
Buffer (MgCl ₂ free) 10X	1X	2.5µl
MgCl ₂ (25mM)	1.00mM	1.00 µl
Oneµ2 (10mM)	0.50µM	1.00µl
Oneµ2 (10mM)	0.5µM	1.25µl
DNTP (10mM)	0.2mM	0.5µl
H ₂ O (HPLC 0.2 µm filtered)	—	16.30 µl
Taq (5 units/µl)	1 unit	<u>0.2 µl</u>
		23 µl
DNA		<u>2 µl</u>
		25 µl total/rxn

CONTML settings for generating the phylogenetic tree.

U	Search for best tree?	Yes
C	Gene frequencies or continuous characters?	Gene frequencies
A	Input file has all alleles at each locus?	Yes
O	Outgroup root?	Yes, (Sprig Lake)
G	Global rearrangement?	Yes
J	Randomize input order of species?	Yes
	Random seed...	5001
	Number of times jumble...	50
M	Analyze multiple data sets?	No
0	Terminal Type	ANSI
1	Print out the data at start of run?	Yes
2	Print indications of progress of run?	Yes
3	Print out tree?	Yes
4	Write out trees onto tree file?	Yes

NEIGHBOR settings for generating the phylogenetic tree.

N	Neighbor-joining or UPGMA tree?	NJ
O	Outgroup root?	Yes (Sprig Lake)
L	Lower-triangle data matrix?	Yes
R	Upper-triangle data matrix?	No
S	Subreplications?	No
J	Randomize input order of species?	Yes
	Random seed...	5001
M	Analyze multiple data sets?	No
0	Terminal type?	ANSI
1	Print out the data at start of run?	Yes
2	Print indications of progress of run?	Yes
3	Print out tree?	Yes
4	Write out trees onto tree file?	Yes

Alleles of a) Ssa14 and b) Ssa289. Results from an 8% acrylamide/1% Spreadex gel, run 3 hours @ 200V. Markers used were c) M3 (76-522 base pairs) and M50 (50bp).

Alleles of a) Omy 77 and b) Omy207. Results from an 8% acrylamide/1% Spreadex gel, run 3 hours @ 200 V.

a)

b)

Alleles of One μ 2. Results from a 6% acrylamide/1% Spreadex gel, run for 3 hours @ 200V

An optimization run. MgCl₂ concentrations are 1.0mM, 1.75mM, 2.5mM; temperature 48°C, 50°C, 52°C, 54°C and 56°C.

APPENDIX B:

Microsatellite allele frequencies

Frequency Distribution for Locus Ssa14 at each San Francisco Bay Population.

cont. Frequency Distribution for Locus Ssa14 for each Population.

Frequency Distribution for Locus Ssa289 at each San Francisco Bay Population.

cont. Frequency Distribution for Locus Ssa289 for each Population.

Frequency Distribution for Locus Ssa289 at each San Francisco Bay Population.

San Jose State University
Institutional Animal Care and Use Committee

LETTER OF OFFICIAL PROTOCOL REVIEW

Date: October 15, 1999

Dear JERRY SMITH:

The animal care and use portion of your research proposal indicated below was reviewed by the Institutional Animal Care and Use Committee (IACUC). The IACUC Protocol Number indicated below should be used when ordering animals for this study, and on grant and contract proposals to fund this study. This protocol number may be used **ONLY** by the principal investigator and other participants included in the protocol. **The IACUC must be notified in writing of any proposed changes to this approved protocol, and approval must be granted in writing before any change is instituted.**

If you have any questions, please contact Dr. Miriam Saltmarch at **924-3118**.

APPROVAL FOR THE IACUC COMMITTEE

(JDM)
Miriam Saltmarch, Ph.D., Chair, IACUC

PROTOCOL #: 687

INVESTIGATOR NAME: JERRY SMITH

TITLE OF PROTOCOL: Genetic Differences Among Populations of Steelhead/Rainbow Trout (Oncorhynchus mykiss)

Approval Date: October 14, 1999 Expiration Date: October 31, 2002

Species To Be Used: Trout Total No. of Animals: 700-1000

Principal Investigator: JERRY SMITH

Department: Biological Sciences Phone: 4-4855

Co-Investigator: N / A
XXXXX This application was re-approved without modification.

 This application was approved with the following mandatory changes:

 This application was **not** approved for the following reasons:

cc: University Animal Care Office
Chair: Investigator's Department, or Departmental Animal Committee