

Conservation Genetics of the Endangered Delta Smelt (*Hypomesus transpacificus*) in the
San Francisco Estuary

By

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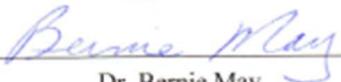
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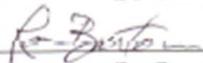
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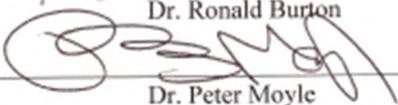
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ABSTRACT

Delta smelt (*Hypomesus transpacificus*) are small, planktivorous, pelagic fish endemic to the upper San Francisco Estuary (SFE). The SFE is a highly urbanized ecosystem that is affected by the introduction of nonnative species, water diversions, contaminants, and the creation of an extensive levee system, which are all considered threats to delta smelt. Delta smelt were relatively abundant prior to 1980, with populations declining dramatically after this time. Delta smelt were listed as threatened by both federal and state agencies in 1993, and sustained record-low abundance indices prompted their listing as endangered under the California Endangered Species Act in 2009. In response to this decline, a captive breeding program for delta smelt has been established at the UC Davis Fish Conservation & Culture Laboratory to preserve this species in the event of extinction in the wild. The overall goal of this dissertation is to inform conservation efforts for delta smelt by 1. developing a genetic management and monitoring plan for the captive delta smelt population, and 2. exploring the genetic diversity of this species in its native range.

To assess wild delta smelt population genetics and to genetically manage the captive population, I developed 24 microsatellite loci for delta smelt that also cross-amplified in longfin smelt (*Spirinchus thaleichthys*) and wakasagi smelt (*Hypomesus nipponensis*) (Chapter 1). In Chapter 2, I describe the development and implementation

of a genetic management plan for the delta smelt captive population. I used 12 microsatellite markers to annually reconstruct the captive pedigree and perform a modified version of minimal kinship selection to recommend pair crosses of captive delta smelt. I found that the founders of the captive population were unrelated based on simulations, with the exception of one half-sibling pair. The goals of the genetic management plan are to equalize founder representation and maximize the captive effective population size. In Chapter 3, I assess the genetic diversity within the captive delta smelt population using 16 microsatellite markers and compare it to the wild delta smelt population to determine the utility of the genetic management plan. I found that the genetic diversity was sufficiently retained in the genetically managed captive population, as significant population divergence was not detected and 90% of the genetic diversity present in the captive population will be maintained for 100 years. In Chapter 4, I conducted a population genetic study using 16 microsatellite markers to assess the geographic and temporal population structure of delta smelt, detect genetic bottlenecks and estimate the wild effective population size from delta smelt collected from 2003 to 2009. I found reduced effective population size and a significant genetic bottleneck in all sampling years. An ephemeral geographic and temporal genetic signal was present but inconsistent, leading to the conclusion that delta smelt exist in the SFE as a single, panmictic population. The results of this study demonstrate the utility of population genetics in prioritizing conservation management of this imperiled species. Collectively, the significance of this body of work includes the application of novel hatchery genetic management techniques and an increased understanding of delta smelt in their native range to inform conservation management of this species.

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TABLE OF CONTENTS

**CHAPTER 1 – CHARACTERIZATION OF 24 MICROSATELLITE LOCI
IN DELTA SMELT, *HYPOMESUS TRANSPACIFICUS*, AND THEIR
CROSS-SPECIES AMPLIFICATION IN TWO OTHER SMELT SPECIES
OF THE OSMERIDAE FAMILY**

ABSTRACT	1
INTRODUCTION.....	2
METHODS	2
RESULTS AND DISCUSSION.....	3
REFERENCES	8

**CHAPTER 2 – HATCHERY GENETIC MANAGEMENT TECHNIQUES
FOR ENDANGERED SPECIES USING THE DELTA SMELT CAPTIVE
REFUGIAL POPULATION AS A MODEL**

ABSTRACT	9
INTRODUCTION.....	10
METHODS	13
RESULTS	21
DISCUSSION.....	22
CONCLUSION.....	29
REFERENCES	37

CHAPTER 3 – FISH HATCHERY OR ZOO POPULATION? GENETIC ANALYSIS OF THE DELTA SMELT CAPTIVE BREEDING PROGRAM

ABSTRACT	43
INTRODUCTION.....	44
METHODS	49
RESULTS	53
DISCUSSION.....	56
CONCLUSION.....	61
REFERENCES	69

CHAPTER 4 – PRIORITIZING CONSERVATION MANAGEMENT WITH POPULATION GENETICS: A CASE STUDY OF THE ENDANGERED DELTA SMELT

ABSTRACT	75
INTRODUCTION.....	76
METHODS	78
RESULTS	83
DISCUSSION.....	86
CONCLUSION.....	91
REFERENCES	98

LIST OF FIGURES

CHAPTER 2

Figure 2.1 San Francisco Estuary Map	30
Figure 2.2 Founder pairwise relatedness coefficients.....	31
Figure 2.3 Founder representation	32
Figure 2.4 Mean kinship histogram	33
Figure 2.5 Standard operating procedure	34

CHAPTER 3

Figure 3.1 Sampling map of San Francisco Estuary.....	63
Figure 3.2 Neighbor-joining tree	64

CHAPTER 4

Figure 4.1 CDFG Spring Kodiak Trawl Sampling Map	93
Figure 4.2 Abundance index and N_e	94

LIST OF TABLES

CHAPTER 1

Table 1.1 Microsatellites developed for delta smelt	6
Table 1.2 Microsatellite cross-species amplification	7

CHAPTER 2

Table 2.1 Microsatellite loci used	35
Table 2.2 Simulated relatedness coefficients and variances	36

CHAPTER 3

Table 3.1 Genetic diversity of captive and wild delta smelt	65
Table 3.2 Heterozygosity of captive and wild delta smelt	66
Table 3.3 Captive and wild pairwise R_{ST} values	67
Table 3.4 Estimates of N_e	68

CHAPTER 4

Table 4.1 Wild delta smelt genetic diversity	95
Table 4.2 Pairwise population R_{ST} values	96
Table 4.3 Bottleneck test results	97

CHAPTER 1

**CHARACTERIZATION OF 24 MICROSATELLITE LOCI IN DELTA
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ABSTRACT

We characterized 24 polymorphic tetranucleotide microsatellite loci for delta smelt (*Hypomesus transpacificus*) endemic to the San Francisco Bay Estuary, CA, USA. Screening of samples ($n= 30$) yielded two to twenty-six alleles per locus with observed levels of heterozygosity ranging from 0.17 to 1.0. Only one locus deviated from Hardy-Weinberg equilibrium, suggesting these individuals originate from a single panmictic population. Linkage disequilibrium was found in two pairs of loci after excluding the locus out of Hardy-Weinberg equilibrium. Twenty-two primer pairs cross-amplified in Wakasagi smelt (*Hypomesus nipponensis*), and fifteen primer pairs cross-amplified in longfin smelt (*Spirinchus thaleichthys*).

INTRODUCTION

The delta smelt (Osmeridae: *Hypomesus transpacificus*) is an annual planktivorous fish endemic to the Sacramento-San Joaquin River delta and upper San Francisco Bay Estuary of central California (Moyle *et al.* 1992). Delta smelt have been in rapid decline since they were listed as threatened by the U.S. Fish and Wildlife Service (USFWS) under the U.S. Endangered Species Act in 1993 (Federal Register 1993; Feyrer *et al.* 2007). A major threat to delta smelt is water diversion by the Federal and California State Water Projects, which export water from the Delta to central and southern California for agricultural use and urban drinking water. Additional threats include reduced water quality from urban and agricultural runoff, and competition and predation by introduced species (Moyle *et al.* 1992; Feyrer *et al.* 2007). Microsatellite markers characterized for delta smelt will allow us to assess population structure and conduct genetic studies relevant to the conservation of this species.

METHODS

Whole genomic DNA was extracted from fin tissue of delta smelt collected near Decker Island in the lower Sacramento River, CA using QIAGEN's DNeasy Tissue Kit protocol. Eight libraries enriched for tetranucleotide repeat motifs (AAAC)_n, (CAGA)_n, (CATC)_n, (TAGA)_n (at two different annealing temperatures), (AAAG)_n, (TACA)_n, and (TGAC)_n were constructed, screened, and sequenced by Genetic Identification Services (GIS) (Chatsworth, CA, USA) according to Meredith & May (2002). The library with tetranucleotide repeat (CAGA)_n was particularly rich in microsatellites and 584 clones of that library were sequenced.

We analyzed sequences using SEQUENCHER version 4.7 (Gene Codes Corporation) to compare sequences for duplicates and employed MREPS version 2.5 (Kolpakov *et al.* 2003) to identify repeat regions. PRIMER3 (Rozen & Skaletsky 2000) was used to create primer pairs flanking the repeat regions of interest for 163 loci. Primer pairs were initially tested on five delta smelt individuals to determine microsatellite amplification and polymorphism.

Polymerase Chain Reaction (PCR) was performed with the following conditions: 5 ng DNA template, 1x *Taq* DNA polymerase buffer B, 2.0 mM MgCl₂, 0.2 mM of each dNTP, 10 μM of each primer and 0.38 U *Taq* DNA polymerase (all reagents from Promega), for a total reaction volume of 10μL. PCR was performed using a Bio-Rad DNA Engine Dyad thermal cycler under the following conditions: 95 °C for 1 min, 30 cycles at 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min, followed by 60 °C for 10 min, and held at 10 °C. Amplified products were diluted 1:1 with 98% formamide loading buffer, denatured at 95 °C for 2 min, and chilled immediately on ice before electrophoresis. PCR products were separated on a 5% denaturing polyacrylamide gel at 50 W for 70 min, visualized using the SYBR-GreenTM-agarose overlay protocol (Rodzen *et al.* 1998), and scanned with a GE Healthcare FluorImager 595. Product sizes were estimated by comparison with a standard 400 bp ladder (The Gel Company).

RESULTS AND DISCUSSION

Twenty-four of the 163 loci were polymorphic and well resolved in the initial screening (Table 1). Those 24 loci were screened with an additional 25 delta smelt individuals (total n = 30) also collected near Decker Island. We also tested the 24

polymorphic loci for cross-species amplification in six individuals of longfin smelt (*Spirinchus thaleichthys*) and Wakasagi smelt (*Hypomesus nipponensis*).

Multiplex PCR amplifications were performed using the same conditions described above for the initial screening, except the cycle number was increased to 31 and 1 μ M of fluorescently-labeled primer (NED, VIC, and PET from Applied Biosystems (ABI), 6-FAM from Integrated DNA Technologies) was added into a total reaction volume of 15 μ L. One μ L of multiplexed PCR product was run undiluted on an ABI 3130xl Genetic Analyzer with a LIZ600 size standard (ABI). GENEMAPPER version 4.0 (ABI) was used to analyze the electropherograms and allelic sizes were confirmed manually.

Data analysis was performed using GENETIC DATA ANALYSIS (GDA) (Lewis & Zaykin 2001). MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004) was used to estimate the probability of the occurrence of null alleles. Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) significance was evaluated using Fisher's exact test with 10,000 permutations, and missing data discarded. Characteristics of the microsatellite loci amplifying in *H. transpacificus* are presented in Table 1. One locus, *HtrG122*, deviated from HWE expectations ($P < 0.05$) after applying sequential Bonferroni correction (Holm 1979). Heterozygote deficiency at this locus suggests the presence of null alleles ($P < 0.001$). However, 23/24 loci conform to HWE expectations, suggesting the 30 individuals included in the analysis may originate from a single panmictic population. Significant pairwise genotype LD ($P < 0.05$) was found in two pairs of loci after applying a sequential Bonferroni correction and excluding *HtrG122*: *HtrG115/HtrG131* and *HtrG127/HtrG131*.

Of the 24 primer pairs developed for delta smelt and tested for cross-amplification in *H. nipponensis* and *S. thaleichthys*, only one (4%) resulted in no amplification in either species. Fifteen (62.5%) of the 24 primer pairs amplified in *S. thaleichthys*, while twenty-two (91.6%) amplified in *H. nipponensis* (Table 2).

The microsatellite loci discussed here will be used to conduct genetic studies relevant to the conservation of delta smelt and related species.

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Table 1.1 Characterization of 24 microsatellite loci in delta smelt (*Hypomesus transpacificus*) from the San Francisco Bay Estuary, CA, USA. GenBank Accession numbers, primer sequences, number of individuals genotyped, repeat motif, number of alleles, estimated allele size range (bp), observed and expected heterozygosities

Locus	GenBank Accession no.	Primer sequence (5' - 3')	<i>n</i>	Repeat motif	No. of alleles	Estimated allele size range (bp)	H_O	H_E
<i>HtrG103</i>	EU621763	F: GCACGCATCATGTCAGAAATA R: TCAGGCTAAGAGGACCTGGA*	30	(GACA) ₁₀	13	91-150	0.87	0.86
<i>HtrG104</i>	EU621764	F: GTGCTGACAGGTAGGCAGGT R: CCGCATGGTAACAGGAAGTT*	30	(CAGA) ₈ (AG) ₅	6	113-160	0.53	0.60
<i>HtrG105</i>	EU621765	F: CTGGGACAGACACCTCTGGT* R: TCCCTAACCGCTAAACCATCT	5	(CTGT) ₈	4	75-200	0.40	0.64
<i>HtrG106</i>	EU621766	F: TCCCTCAAACCGTTTTTCAC* R: GCTGGTAAGCTCGAGACTGG	24	(GTCT) ₆	2	75-200	0.17	0.16
<i>HtrG107</i>	EU621767	F: TGGACAGACACAGAGAAGCAG§ R: GGACATAGCTGGACCCTCAG	25	(CAGA) ₇	9	100-215	0.68	0.75
<i>HtrG108</i>	EU621768	F: TTGGTACACGGCAACTGAAA§ R: AGCCCTGCCAGAGAGAGAAT	22	(GT) ₉ (TCTA) ₈	12	75-250	0.86	0.87
<i>HtrG109</i>	EU621769	F: GGACAGCACAAAGTCTGGT§ R: GACTCAGACAGTCTCATCG	30	(TCTG) ₁₁ (GTCT) ₄	15	145-218	0.90	0.89
<i>HtrG110</i>	EU621770	F: AAACGTGTCTGGTGGTGCA§ R: CCCACCCAGTCTGTCTGTTT	28	(CAGA) ₁₇	21	100-275	0.96	0.94
<i>HtrG112</i>	EU621771	F: AGTCTTACGGATCCACAGC§ R: ACTGTCTGTCTGCGGCTTTT	29	(CAGG) ₄	2	100-299	0.21	0.19
<i>HtrG113</i>	EU621772	F: GCTGGCTGGCTAGCTGAC‡ R: CGTCTCCACCCTACATGCT	6	(AGAC) ₆	3	100-300	0.50	0.68
<i>HtrG114</i>	EU621773	F: ACCATGGGAGACAAGTCTGG‡ R: TCACTGGCACAACGAGAAG	28	(TCTA) ₅ (TCTG) ₁₁	19	175-272	1.00	0.95
<i>HtrG115</i>	EU621774	F: CTCTCCCTCCGTTTGTCT‡ R: CTGGTCTTGCAACGTGTTTG	29	(CTGT) ₁₈	12	175-240	0.79	0.90
<i>HtrG116</i>	EU621775	F: CGTTTTTAGCGTCTCCAC* R: GCTGGCTGGCTAGCTGAC	18	(TGTC) ₅	3	175-250	0.33	0.37
<i>HtrG117</i>	EU621776	F: CACACTCCAAGAGCAGGA† R: CTGTCTCTCTGCCACCTTC	24	(GACA) ₁₇	12	150-300	0.96	0.91
<i>HtrG118</i>	EU621777	F: GTTGGGGATTCTTAAACCA‡ R: CCCCAAAGAAGCCAGATGTA	30	(ACAG) ₅	4	150-300	0.37	0.32
<i>HtrG119</i>	EU621778	F: AAGCTTCTGTGGACGAGAC† R: ACTCCTACCGAACCGTGATG	29	(ACAG) ₂₁	26	179-272	0.97	0.96
<i>HtrG120</i>	EU621779	F: ACAGCGAAACAACCATCA† R: GCGTGGTCTAGGCTTGAAAA	30	(AGAC) ₆	8	230-279	0.60	0.74
<i>HtrG122</i>	EU621780	F: AACACATTGCAGCAAGGCTA† R: TGACCTACGATTGGTGGAGA	24	(TGTC) ₃₀	8	250-300	0.42	0.86
<i>HtrG123</i>	EU621781	F: TTAGCCAGTCAGTCATGTGGA* R: GATCCCTTTTCATCCTGCAA	30	(GACA) ₂₂	22	240-349	0.93	0.95
<i>HtrG126</i>	EU621782	F: GATCCCTTTTCATCCTGCAA R: TTAGCCAGTCAGTCATGTGGA*	30	(TCTG) ₂₅	21	243-335	0.87	0.95
<i>HtrG127</i>	EU621783	F: GCATTCTTAGCCGTCTGGAG R: CCCATTCCCTCCCTATCT*	30	(AGAC) ₃ (ACAG) ₂₆	24	209-350	0.80	0.95
<i>HtrG128</i>	EU621784	F: CTGCTCTGTCCAATCAGCA* R: GAAGCTGCCTGTCTGTCTAGC	19	(ACAG) ₂₆	12	200-375	0.84	0.84
<i>HtrG129</i>	EU621785	F: ACTGCCTGGAAGAGCACACT§ R: CAAAGTCTGTGCAACTTGGAA	28	(TGTC) ₅ (CTGT) ₇	6	300-360	0.64	0.66
<i>HtrG131</i>	EU621786	F: GAGAGAAGGGATGGGGAGTC§ R: GGCCAAGGGACAGTTCATAA	27	(CAGA) ₂₈	21	281-381	0.78	0.95

Labeled Primers: *6-FAM, †NED, ‡VIC, §PET

Table 1.2 Cross-species amplification results of 24 microsatellite loci for the smelt family Osmeridae, genus *Hypomesus* (*H. nipponensis*) and *Spirinchus* (*S. thaleichthys*). Species, sample size (n); 'U' indicates amplification but unclear; '—' indicates no amplification; number of alleles; (num-num) indicates size range in bp

Locus ID	<i>H. nipponensis</i> (n=6)	<i>S. thaleichthys</i> (n=6)
<i>HtrG103</i>	U	2 (111-115)
<i>HtrG104</i>	2 (112-147)	—
<i>HtrG105</i>	1 (140)	1 (94)
<i>HtrG106</i>	1 (147)	—
<i>HtrG107</i>	3 (122-149)	U
<i>HtrG108</i>	4 (148-198)	U
<i>HtrG109</i>	4 (145-162)	1 (109)
<i>HtrG110</i>	2 (106-115)	1 (118)
<i>HtrG112</i>	1 (285)	U
<i>HtrG113</i>	2 (124-231)	2 (142-237)
<i>HtrG114</i>	1 (204)	1 (195)
<i>HtrG115</i>	U	—
<i>HtrG116</i>	—	—
<i>HtrG117</i>	U	U
<i>HtrG118</i>	7 (238-298)	8 (243-276)
<i>HtrG119</i>	—	U
<i>HtrG120</i>	2 (268-273)	—
<i>HtrG122</i>	2 (283-288)	—
<i>HtrG123</i>	12 (261-343)	—
<i>HtrG126</i>	4 (260-295)	U
<i>HtrG127</i>	3 (220-289)	—
<i>HtrG128</i>	10 (236-367)	U
<i>HtrG129</i>	U	U
<i>HtrG131</i>	5 (328-376)	—
Total no. of amplified loci	22	15

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CHAPTER 2
HATCHERY GENETIC MANAGEMENT TECHNIQUES FOR ENDANGERED
SPECIES USING THE DELTA SMELT CAPTIVE REFUGIAL POPULATION
AS A MODEL

ABSTRACT

Fish hatcheries designed to produce captive fish to supplement wild populations are considered powerful tools for species conservation. Because genetic diversity may deteriorate in captive populations due to accumulation of inbreeding, adaptation to captivity and reduced effective population size, many hatcheries implement genetic management plans in an attempt to preserve the genetic health of the species. This study describes the implementation of a genetic management plan for a captive refugial population of delta smelt (*Hypomesus transpacificus*). In this context, a captive refugial population is defined as a genetically managed captive population that is designed to preserve a wild endangered population by acting as a manmade refugium. Delta smelt are endangered estuarine fish endemic to the San Francisco Estuary, CA. Due to record low abundance indices in recent years, a delta smelt refugial population was established at the University of California, Davis Fish Conservation and Culture Laboratory in Byron, CA in 2008. The goal of the refugial population is to act as a genetic bank in the event of species extinction, but currently there are no plans to reintroduce these fish to the wild. The genetic management plan for the delta smelt refugial population entails tagging fish, molecular data collection, pedigree reconstruction, relatedness estimation and recommending fish crosses annually in an effort to minimize mean kinship and limit

inbreeding in the captive population. This method aims to equalize founder representation in an attempt to maximize the captive effective population size. The results of this study yield insights into the practical application of genetic management plans for captive populations and conservation hatcheries. With the incorporation of these refined techniques, captive breeding may become an increasingly effective tool in species conservation.

INTRODUCTION

Captive breeding is considered a powerful strategy for preserving declining wild fish populations when the causes of species decline cannot be remediated quickly enough to prevent extinction (Caughley 1994). When the goal is species conservation, fish hatcheries are generally managed to maintain the demographic and genetic viability of their populations. However, when the goal is for economic gain, mitigation or population enhancement, as have historically been the main goals of fish hatcheries, genetic management tends to assume a lesser priority, in part, due to the large scale of these programs (Lichatowich 1999). As a result, the detrimental effects of the lack of management in fish hatcheries have been well-documented (Allendorf & Ryman 1987; Berejikian & Ford 2004; Busack & Currens 1995; Flagg *et al.* 2004; Johnson & Jensen 1991; Miller & Kapuscinski 2003; Sharma *et al.* 2006; USFWS 2000; Williamson *et al.* 1996). Even in fish hatcheries aimed at species conservation, management plans are not always feasible or effective, due to a lack of pedigree information and other factors (Rudnick & Lacy 2008).

Detrimental genetic changes common in captive populations include reduced genetic diversity, increased inbreeding, reduced viability and fecundity, and reduced effective population size (Araki *et al.* 2007a; Araki *et al.* 2007b, 2009; Falconer 1981; Frankham 1995; Frankham 2008; Heath *et al.* 2003; Ralls & Ballou 1983; Reisenbichler & McIntyre 1977; Reisenbichler & Rubin 1999; Ryman & Laikre 1991; Waples & Drake 2004). These are often a consequence of founding a captive population with relatively few fish from an already declining population. This makes the captive population susceptible to loss of genetic variability, which will further erode over subsequent generations in captivity (Allendorf & Luikart 2007; Allendorf & Ryman 1987; Nielsen 1995; Waples 1991). However, effects of captive breeding have to be evaluated in the context of what would occur in nature in the absence of a program, as a captive breeding program that accumulates detrimental genetic changes slower than the wild population alone is still beneficial to the wild population. To mitigate the negative effects of captive breeding, management schemes have been developed that maximize gene diversity retention, limit inbreeding, preserve founding gene diversity and maintain the effective population size of captive populations (Ballou & Foose 1996; Ballou & Lacy 1995; Foose & Ballou 1988; Hedrick *et al.* 2000a; Hedrick & Hedgecock 1994; Hedrick *et al.* 1995; Hedrick *et al.* 2000b; Hedrick & Miller 1992; Lacy 1994).

While many of these management schemes have been successful in maintaining gene diversity and effective population size, domestication cannot be completely eliminated, so all programs will accumulate some detrimental genetic changes (Fraser 2008; Hedrick *et al.* 1995; Hedrick *et al.* 2000b; Osborne *et al.* 2006). These accumulated genetic changes in fish hatcheries are especially harmful when wild

populations are supplemented with large numbers of captive fish. As wild populations continue to decline, many fish hatcheries attempt to prevent population extinctions by reintroducing captive animals into the wild (Berejikian & Ford 2004; Flagg *et al.* 2004; Pollard & Flagg 2004). When this occurs, fish hatcheries may do more harm than good, as they negatively affect the wild population that they are trying to preserve (Berejikian & Ford 2004). As a result, there is a need for studies that evaluate methods to improve fish hatchery management and the selection of individuals for reintroduction.

This study explores the implementation of a genetic management plan for a captive refugial population of delta smelt (*Hypomesus transpacificus*). We define „refugial population“ as a genetically managed captive population designed to preserve a species in the event of extinction by providing a manmade refugium. Conservation hatchery is a similar term; however, the goal of the refugial population is to provide a genetic bank of delta smelt in the event of species extinction, instead of supplementing a wild population with captive fish to bolster census size. Currently there are no plans to reintroduce these captive fish to the wild. Delta smelt are endangered, annual, estuarine fish endemic to the San Francisco Estuary, CA. Threats to delta smelt include water diversions from the Estuary for urban and agricultural uses, introduction of non-native species, habitat alterations, and contaminants (Moyle 2002; Moyle *et al.* 1992; Nichols *et al.* 1986). The delta smelt refugial population was established in 2008 due to record low delta smelt abundance indices in the wild (Newman 2008). It is located at the University of California, Davis Fish Conservation & Culture Laboratory (FCCL) in Byron, CA, where culture techniques have been developed to maintain the captive population (Lindberg *et al. this volume, pp.*).

The genetic management plan for the delta smelt refugial population is based on the annual tagging and individual genetic identification of thousands of captive fish using 12 microsatellite markers to reconstruct the pedigree (Fisch et al. 2009). Molecular estimates of relatedness were used to ascertain the relationships among the original founders of the refugial population, and standard parentage assignment techniques have been subsequently used each spawning season to accurately reconstruct the pedigree from the microsatellite data. After the pedigree is reconstructed each year, recommendations on which fish to strip spawn in single pair crosses are made based on mean kinships calculated from the pedigree. This method aims to minimize the accumulation of inbreeding and equalize family representation (Ballou & Lacy 1995). Wild fish are incorporated into the captive population annually, as available, to provide gene flow between the wild and captive population to maintain genetic diversity and minimize genetic divergence of the captive fish from the wild population. The results of this study provide insight into the practical application of genetic management plans for captive populations and conservation hatcheries, in an attempt to preserve the genetic integrity of endangered species. With the incorporation of these refined techniques, fish conservation hatcheries may become increasingly effective tools in species conservation.

METHODS

Sample Collection & DNA Preparation

Fin clips were collected from the caudal or adipose fin of captive fish at the FCCL during fish tagging (visible-implant alpha-numeric tag, “VI-alpha tag”; Northwest Marine Technology, Inc.), and preserved in 95% EtOH. Captive population founders and

additional wild fish were collected in December prior to each spawning season in the Lower Sacramento River, CA (Figure 1). Thousands of fish were sampled and genotyped each generation to reconstruct the pedigree and recommend pair crosses ($F_0 = 290$; $F_1: n = 1,400$; $F_2: n = 1,858$). Whole genomic DNA was extracted from all samples using the DNeasy Tissue Kit (QIAGEN) following the manufacturer's directions. All samples yielded high-molecular weight DNA.

Microsatellite Genotyping

Twelve microsatellite markers described by Fisch *et al.* (2009) were amplified by multiplex polymerase chain reaction (PCR) (Table 1). PCR products were visualized using an ABI 3730 DNA Analyzer (Applied Biosystems, Inc.) with LIZ500 internal size standard. Genotyping was conducted using ABI's Genemapper™ 4.0 and verified manually (Applied Biosystems, Inc.). Two control samples with known allele sizes were included in every 96-well PCR plate, allele calls were independently scored by two people and genotypes with questionable allele calls were re-amplified and scored again to reduce genotyping errors.

Founding & Management of the Captive Population

The captive delta smelt population was founded with 290 wild delta smelt collected from the lower Sacramento River, CA in December 2006 (Figure 1). These fish (the F_0 generation) were spawned in captivity during 2008 (January-May) at the FCCL. The fish were crossed randomly in single pair crosses (one male and one female) and no individual fish was used twice. In captivity, delta smelt have not spawned naturally. As

a result, the staff at the FCCL manually expressed the eggs from a single female and combined them with the milt of a single male in a dish to create a single pair cross. The offspring from each full-sibling family were combined in tanks (~8 families/tank) due to space limitations at the FCCL. This made it necessary in later generations to conduct parentage analyses to determine to which full-sibling family each individual fish belonged. In each subsequent generation, ~50 wild fish were captured from the same location in the lower Sacramento River in December prior to spawning season and incorporated into the captive population. Wild fish were preferentially mated with wild fish to produce a new founding pair; however, as the sex ratio of captured wild fish was unequal, wild fish without a wild mate were paired with a captive fish. Wild fish were incorporated into the captive population each generation to build an open system that allows gene flow from the wild into the captive population. Each year, a representative sample of captive delta smelt is transferred to Livingston Stone National Fish Hatchery, Shasta Lake, California (US Fish and Wildlife Service) to protect against catastrophic loss at either facility.

Identifying Founder Relationships

Relatedness coefficients between all pairs of individuals in the F_0 generation ($n = 290$) were calculated to determine if any close relatives (i.e., full-siblings or half-siblings) were present among the captive population founders to increase the accuracy of kinship calculations from the reconstructed pedigree. Relatedness is the probability that two individuals share an allele sampled at random due to identity by descent from a common ancestor. The performance of different relatedness estimators differs based on the

number of alleles, allele frequency distributions and numbers of the microsatellite loci used in the study, and the composition of relationship categories present in a population (Lynch & Ritland 1999; Milligan 2003; Queller & Goodnight 1989; Ritland 1996; Van de Casteele *et al.* 2001; Wang 2002). To account for this, sampling variances for three relatedness estimators were calculated using computer simulations developed by Ivy *et al.* (2009) for four relationship categories (full siblings, half siblings, parent-offspring and unrelated). The relatedness estimators tested were r_{xy} QG (Queller & Goodnight 1989), r_{xy} LR (Lynch & Ritland 1999), and r_{xy} Wang (Wang 2002).

Microsatellite genotypes of 1,000 pairs of individuals were simulated for the four relationship categories. The delta smelt founder allele frequencies were used to simulate unrelated genotypes, which served as the basis for creating pairs of individuals in the remaining relationship categories. Relatedness coefficients for all pairs of individuals in each relationship category were calculated using the software program SPAGeDi (Version 1.3a; (Hardy & Vekemans 2002)). For each of the four relationship categories, the means and variances of the relatedness coefficients were calculated for each of the three estimators (Table 2). Then, the relatedness estimator possessing the smallest variance across relationship categories was used to calculate relatedness among individuals in the founding population and additional wild-caught individuals.

Information gained from identification of first-order relatives among the founders was used to facilitate the pedigree-based mean kinship calculations used to spawn the F₂ generation of captive delta smelt. In pedigree-based management, captive population founders are generally assumed to be unrelated and not inbred (Ballou 1983). Pairs of F₀ individuals identified as close relatives were given hypothetical parents in the captive

delta smelt pedigree to capture those relationships, rather than assuming those fish to be unrelated. Those hypothetical parents were assumed to be unrelated to all F_0 fish, conceptually making them founders in place of their assigned offspring.

Pedigree Reconstruction

Prior to selection of breeding pairs each season, a molecular analysis was needed to identify parentage and reconstruct the delta smelt pedigree because young fish of multiple family groups were mixed together in tanks due to space limitations. A sire and dam were assigned to each individual in the captive population using Cervus 3.0 (Kalinowski et al. 2007). This program provides a statistical means to evaluate the results of parentage assignment. We simulated 10,000 offspring based on the allele frequencies of the founders (F_0 : $n = 290$), and conducted a parent pair (sexes known) parentage analysis with a genotyping error rate of 0.01 for each individual. Confidence levels were fixed at 95% for strict confidence and at 80% for relaxed confidence. Cervus was first run with all candidate parents in the population to assign parentage to a single individual. The analysis was run a second time with a narrowed pool of candidate parents based on which tank the individual came from (each tank was comprised of an average of 8 full-sibling families). As the mated pairs in the previous generation were known, parentage assignments that correctly assigned a known mated pair increased our confidence in the results. The reconstructed pedigree was visualized with PedigreeViewer 6.4b (Kinghorn & Kinghorn 2009). With parentage assigned to each individual, mean kinships could be calculated from the pedigree to apply the mean kinship selection scheme described below for selecting breeding pairs.

Pair Cross Recommendation

Prior to spawning in the F₁ and F₂ generations, 3,258 fish were sampled, genotyped, and the pedigree was reconstructed in order to identify the most genetically valuable individuals for spawning. In both generations, fish were selected to reproduce based on a modified version of the method of mean kinship (MK) selection (Ballou & Lacy 1995; Fernandez & Toro 1999; Sonesson & Meuwissen 2001). Mean kinship selection aims to minimize a population's average kinship by breeding genetically underrepresented individuals with low mean kinships (*mk*). Kinship (*f*) between two individuals is the probability that two alleles at a given locus, one randomly drawn from each individual, are identical by descent from a common ancestor (Falconer 1981). Mean kinship measures an individual's genetic distinctiveness, and is calculated as the average of kinship between that individual and all living individuals in the population, including itself:

$$mk_x = \frac{\sum_{y=1}^N f_{xy}}{N} \quad (1)$$

Traditionally, MK selection is based on pedigree calculations of *mk*, although molecular estimates of relatedness, called mean relatedness (*mr*), have been proposed as suitable substitutes for pedigree-based *mk* estimates (Doyle et al. 2001). For the F₁ generation, breeding recommendations were based on *mr* calculations, due to the practicality of the approach and the shallow nature of the pedigree. Because relatedness estimates are notoriously inaccurate and suffer from large sampling variances (Csillery *et al.* 2006; Lynch & Ritland 1999; Milligan 2003; Queller & Goodnight 1989; Ritland 1996; Van de Castele *et al.* 2001; Wang 2002), starting with the F₂ generation, we

recommend that a pedigree-based, *mk* breeding pair selection strategy be implemented for the delta smelt conservation hatchery, at least until the impacts of using *mr* estimates for genetic management can be better quantified in future studies.

A software program written in the C programming language by J. Ivy was used to calculate *mk* for each individual, rank them in order of preference, and recommend preferred crosses that would minimize mean kinship in the subsequent generation. The process of ranking animals by *mk* was similar to that used by Johnston and Lacy (1995) to identify the optimal animals from which to cryopreserve gametes. As delta smelt possess discrete generations in the wild and typically have an annual lifecycle, generations in captivity were maintained separately and matings were not recommended between generations.

Because many more offspring are produced by the delta smelt conservation hatchery each year than are needed for breeders, the method by which breeding pairs were selected was not constrained by the availability of genetically valuable individuals. Thus, to create the pool of potential breeders for a given generation, an equal number of hypothetical offspring were created from the previous generation's breeding pairs. The number of hypothetical offspring created was suitably large enough to ensure that not all of the hypothetical offspring from a given pair were selected as breeders, which allowed the breeding pair selection method to maximally minimize *mk* within the set of selected breeders without being constrained by the availability of offspring from specific crosses. An inbreeding cutoff was set to avoid close inbreeding in the selected crosses, and was determined to be the average mean kinship among the hypothetical offspring created. Crosses were rejected if their kinship was greater than the inbreeding cutoff.

After the set of potential breeders was created, new crosses were selected by a modified version of the pair selection scheme used by Johnston and Lacy (1995). First, *mks* were calculated for all potential breeders (i.e., the hypothetical offspring). Next, the individual with the highest mean kinship was removed from the pool of potential breeders and placed in List #1. Then, the mean kinships of all unranked individuals remaining in the pool of potential breeders were recalculated. Again, the individual with the highest mean kinship was removed from the pool, placed in List #2, and the mean kinships of all unranked individuals were recalculated. This process was repeated until all possible breeders were ranked into a list. Individuals were placed into a list above the previous individual added to that list, so that lists were populated from the bottom to the top.

Once all potential breeders were ranked, crosses were selected by taking the top ranked individual from List #1 and pairing it with the top ranked individual from List #2. The second cross was made by taking the second ranked individual from List #1 and pairing it with the second ranked individual in List #2. The pairing continued in this fashion until the specified number of crosses was achieved. Any recommended cross that exhibited a kinship greater than the inbreeding cutoff was rejected. If a cross was rejected, the remaining individuals in List #2 were evaluated in ranked order (from top to bottom) to determine if a suitable cross could be made. If a suitable match was not found, the rejected individual from List #1 was removed from the pool of potential breeders and the next individual in List #1 was paired (the rejected individual from List #2 remained in its ranked list).

Once all the crosses were selected, the family from which each hypothetical breeder came was identified. An output file was created listing which families should be selected to make the recommended crosses. As the pedigree is reconstructed each generation for the delta smelt conservation hatchery, only the parents of the potential breeder pool are known prior to mate selection, not the specific breeders. Recommending crosses based on parental information allows for flexibility when making crosses, as siblings can be used interchangeably.

RESULTS

Using relatedness simulations based on allele frequencies in the founding population, the means and variances of the relatedness coefficients for each of four relationship categories were calculated for three different relatedness estimators (Table 2). Sampling variances ranged from 0.002 to 0.025 across relatedness estimators and from 0.002 to 0.019 for r_{xy} Wang. The smallest variances were observed for parent-offspring pairs. r_{xy} Wang possessed the smallest variance across two relationship categories, where the other two estimators had the smallest variance in only one relatedness category each. As a result, the pairwise relatedness coefficients for the 290 wild founders were calculated using Wang's r_{xy} . Given the means and variances observed for the four simulated relationship categories with this estimator (Table 2), only one pairwise relatedness value indicated a close relationship; one pair of founders with an r_{xy} of 0.34 was assumed to be related at the half-sibling relationship category (Figure 2).

After conducting parentage analysis on each unknown tagged fish, parentage allocation at the 95% confidence level was highly successful, with 100% of offspring

assigned to both parents by CERVUS. The probability of exclusion for the first parent ranged from 1.08×10^{-10} to 9.23×10^{-5} and for the parent pair from 9.05×10^{-27} to 1.17×10^{-11} .

Once the pedigree was reconstructed, founder representation was calculated in the F₁ & F₂ generations and was variable among the original 290 founders and the additional wild individuals. Figure 3 shows founder representation of the original 290 founders and the additional 89 wild founders as the proportion each founder is represented relative to all other founders in the given generation. Of the 290 original founders, 38 are no longer represented in the F₂ generation due to mortality. Additionally, 89 founders were added as wild fish into the captive population to make a total of 341 founders.

After recommending pair crosses based on a modified version of the MK selection method, the average mean kinship was calculated in the F₂ generation and was 0.002 (ranging from 0 to 0.005; Figure 4).

DISCUSSION

Founder Relationships

Conservation breeding programs typically assume that wild-caught individuals used to found captive populations are non-inbred and unrelated (Ballou 1983). Research is beginning to emerge that suggests this assumption has little impact on the ability of genetic management methods to conserve gene diversity in a captive population over the long-term (Ivy *et al.* 2009; Rudnick & Lacy 2008). However, the genetic impacts of assumptions about founder relationships have generally been investigated for populations with single founding events, rather than those that use sustained imports of wild-caught

animals to allow continuing gene flow from the wild to the captive population. Because the delta smelt breeding program continues to incorporate wild-caught fish into the captive population each year, close relationships among those wild-caught fish could negatively affect both short and long-term genetic management. After calculating the pairwise relatedness values for all initial captive population founders, we determined that only one pair was related at the half-sibling relationship level (Figure 2). This pair was not used as a mated pair when F_0 fish were randomly mated. As a result, the existence of a single, undetected half-sibling pair among the 290 fish used to found the captive population would be unlikely to significantly impact genetic management (based on results from (Ivy *et al.* 2009; Rudnick & Lacy 2008)). However, as the identity of a half-sibling pair was recognized after molecular analyses, this information was incorporated into subsequent, pedigree-based breeding recommendations. The knowledge that only two out of 290 randomly collected wild delta smelt were found to be closely related suggests that capture techniques and the current size of the wild population are sufficient to support small collections of unrelated fish. Thus, unless those factors change, it seems reasonable to assume that additional collections of wild-caught fish (including the F_1 and F_2 fish) are unrelated for the purposes of the genetic management of the captive population. However, relatedness will be calculated for additional wild founders in an effort to prevent future matings between closely related individuals. No half sibling or greater relationships were detected among the newly incorporated wild fish in the F_1 & F_2 generations.

Pedigree Reconstruction

Pedigree reconstruction and relatedness estimation are ways to incorporate molecular data into hatchery genetic management plans. Once molecular data have been collected, pedigrees can be reconstructed based on parentage analyses, although usually not with any temporal depth beyond the parental generation. The reconstructed pedigrees can then be used to conduct traditional pedigree-based genetic management (Ivy et al. 2009; Jones et al. 2010). However, reconstructing pedigrees requires the ability to identify individuals, which may not be feasible in a hatchery setting.

The delta smelt refugial population presents us with a unique opportunity to manage a large captive fish population similar to a zoo-based captive breeding program using traditional pedigree-based genetic management (Figure 5). Since each fish can be uniquely tagged, individual identification is possible. Coupled with fin clips and genetic analyses, the pedigree is reconstructed for each tagged fish, which allows for calculations of mean kinship and subsequent pair cross recommendations. As pedigree reconstruction generally is most effective for the parental generation, this method requires excellent record keeping in each generation. In addition, the development of software to calculate mean kinship from a reconstructed pedigree in later generations is essential for accurate calculations and record keeping.

Although tagging, fin clipping and genetically analyzing thousands of fish each generation to reconstruct the pedigree is very costly and time consuming, we must rely on this method for the refugial population of delta smelt due to facility constraints that require us to put multiple families in each tank. Ideally, each family would be housed in a separate tank, eliminating the need for tagging, genetic analysis and pedigree

reconstruction. Using this method, the pedigree would be tracked for each family/tank, and calculations of mean kinship could be conducted without the use of molecular analyses. Using the modified method of MK selection described in this paper, full-siblings are interchangeable with one another. If each family was housed separately, the kinship value of the fish in each tank and mean kinship of each tank to all other tanks could be calculated at the start of the spawning season. Crosses could then be recommended based on the method described here, and when a fish was ready for spawning, it could be taken from one tank and strip spawned with a mate from the recommended tank. This method would be ideal, as the costs of genetic analyses, tagging, and labor would be eliminated while implementing an effective genetic management method that minimizes mean kinship and limits inbreeding. However, the goal of the delta smelt refugial population is to maintain a breeding population of ~500 individuals in ~250 single pair crosses, which would require 250 individual family tanks in each life stage. This is prohibitive in all but the largest hatchery facilities, but due to the cost effectiveness of genetic management without tagging and genetic analysis, the differences in costs may balance out. Otherwise, using molecular data to reconstruct the pedigree each generation is an efficient method to manage large populations with a pedigree-based MK selection scheme.

Founder Representation

Once a pedigree is reconstructed in each generation, the founder representation should be monitored (Figure 5). An important goal in captive breeding programs is to maintain the effective population size to minimize genetic drift, which can be

accomplished by equalizing founder representation (Allendorf 1993). The effective population size is related to differences in reproductive success among individuals, such that in a population of constant size,

$$N_e = \frac{4N - 2}{2 + V_k} \quad (2)$$

where N_e is the variance effective population size, N is the census size of the population, and V_k is the variance in progeny number (Crow & Denniston 1988). By attempting to equalize founder representation, the effective population size in captivity will increase, and may actually surpass the census size of a population if the reproductive variance between founders is zero (Allendorf 1993). The genetic and fitness benefits conferred by equalizing founder representation include reducing inbreeding, genetic drift, and domestication selection (Allendorf 1993; Fraser 2008).

In the delta smelt refugial population, we observe variable founder representation in each generation (Figure 3). If the MK selection method could be optimally applied, founder representation would eventually be equalized in future generations. Currently, the variable founder representation results, in part, from an inability to make 100% of recommended breeding pairs. From some families, an insufficient number of offspring are sometimes recovered in the next generation to spawn the optimal number of breeders from those families. Even though equalizing founder representation is beneficial, it may not prevent within-family selection, allowing for new mutations in the captive populations to become fixed from domestication selection and relaxed natural selection in captivity (Bryant & Reed 1999; Rodriguez-Ramilo *et al.* 2006).

Pair Cross Recommendations

Using a modified MK selection method to recommend pair crosses for the delta smelt refugial population has minimized mean kinship through the F₂ generation (Figure 4). If mating were at random, the average mean kinship of the captive population would be the expected mean inbreeding coefficient of all of the offspring, which is equal to the proportional loss of gene diversity in the next generation (Ballou & Lacy 1995).

Incorporating wild individuals into the captive population each generation will work to minimize the average mean kinship, as each wild individual is considered a new founder, unless molecular relatedness estimates suggest otherwise. Thus, the greater the number of wild individuals incorporated into the captive population, the lower the average mean kinship, and the lower the loss of gene diversity in the next generation. However, when wild individuals are not available, the MK selection method should still effectively minimize kinship in the captive population.

The goal of hatchery genetic management plans and captive breeding is to retain genetic variation while limiting inbreeding (Ballou & Lacy 1995; Foose & Ballou 1988; Hedrick & Miller 1992; Lacy 1994). Computer simulations and empirical studies have demonstrated that the best strategy to meet this goal is one that minimizes overall kinship (i.e., relationship) in a population (Ballou & Lacy 1995; Fernandez & Toro 1999; Montgomery *et al.* 1997; Toro *et al.* 1999). However, the calculation of kinship values depends on a population's pedigree. When a pedigree is unknown, such as in hatcheries where parentage is not tracked, the incorporation of molecular data into genetic management may be a viable alternative to traditional genetic management methods. With recent advances in molecular techniques, including microsatellite and single

nucleotide polymorphism (SNP) genotyping and next generation sequencing, molecular data are readily available and easily obtainable, providing that sufficient samples are available for a robust study. This makes molecular data useful tools to incorporate into fish hatcheries where pedigrees are unknown or poorly resolved. Furthermore, molecular markers allow us to track the ancestries of genes rather than individuals, which may potentially allow us to optimize genetic management beyond what is attainable through pedigree-based management (Wang 2004).

In addition to using molecular data to reconstruct pedigrees, another approach used to resolve founder relationships is to manage captive populations using molecular relatedness estimator values as proxies for pedigree-based kinships. Doyle et al. (2001) incorporated molecular data into hatchery genetic management by using molecular estimates of relatedness as a proxy for pedigree-based kinships and Toro et al. (1999) investigated the benefits of using molecular markers to minimize the homozygosity by descent in captive populations to minimize the average group coancestry (Doyle *et al.* 2001; Toro *et al.* 1999). These two studies introduced novel ways to incorporate molecular data into captive population management. However, neither study compared the utility of their methods with traditional pedigree-based mean kinship methods or with other methods of incorporating molecular data into genetic management plans. Future studies need to address the relative utility of these methods to improve genetic management plans.

CONCLUSION

The genetic management plan for the captive delta smelt refugial population aims to minimize mean kinship in the captive population, equalize founder representation, limit inbreeding and maximize effective population size. The goal of the refugial population is to create a genetic bank for this species in the event of extinction in the wild. The novel adaptation of the MK selection method for hatchery applications results in more efficient genetic management techniques for large populations. The flexibility of this method also makes genetic management of hatcheries more widely available to a variety of hatchery managers, as this method may be employed for hatcheries with the ability to hold families in individual tanks and for those where visual identification of individuals from each unique family through tagging early life stages is possible. The results of this study provide insight into the practical application of captive population genetic management plans for endangered species. With the incorporation of these refined techniques, captive breeding may become an increasingly effective tool in species conservation.

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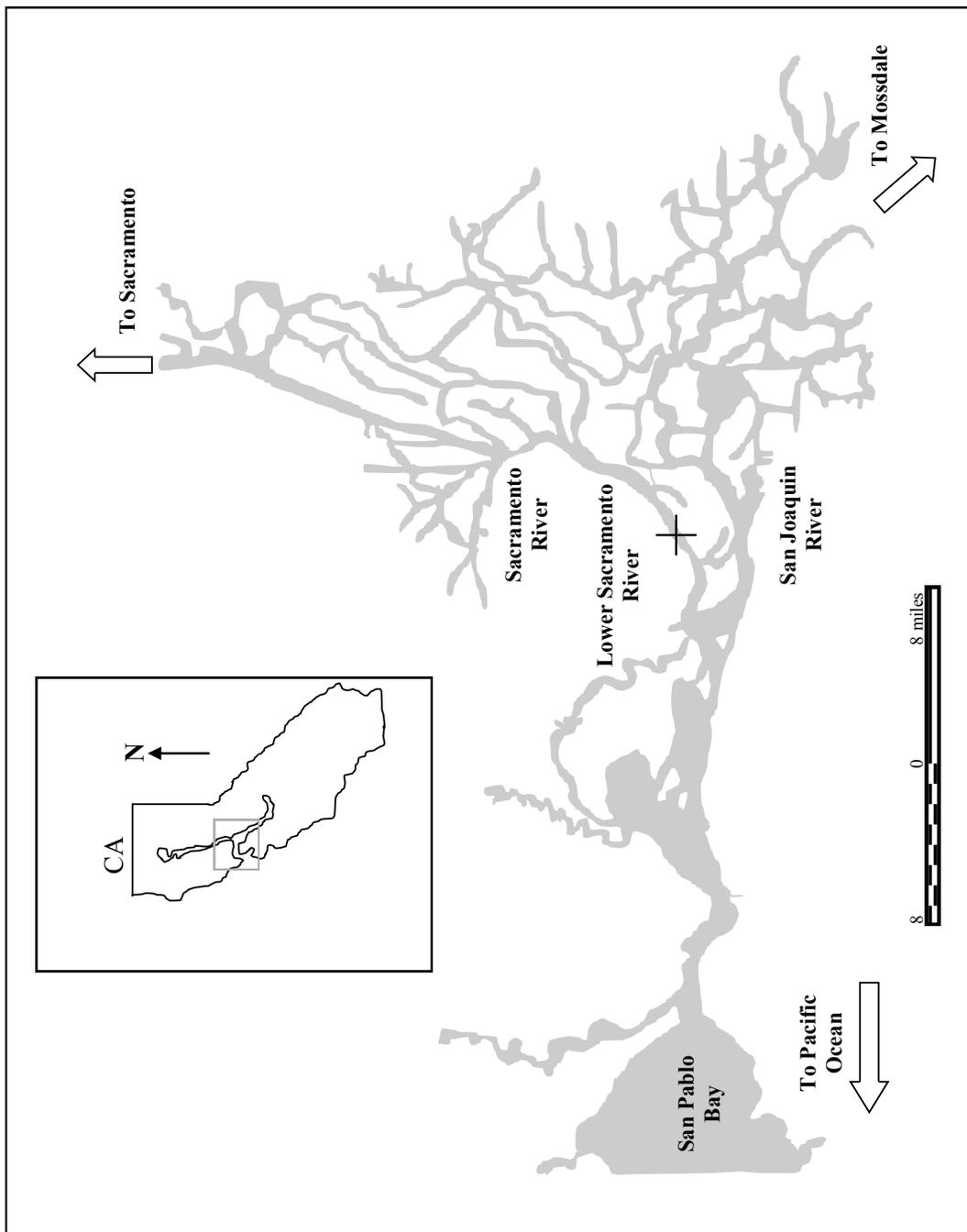


Figure 2.1 Map of the San Francisco Estuary, CA. The delta smelt refugial population founders were collected in the Lower Sacramento River.

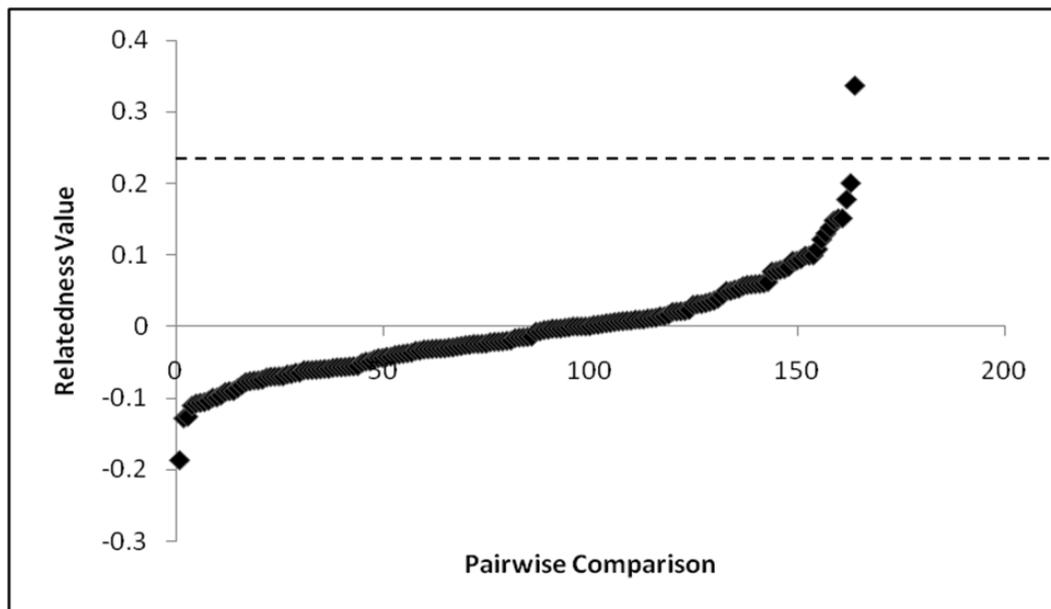


Figure 2.2 Pairwise relatedness coefficients estimated for the 290 founders, graphed in order of increasing value. The dashed horizontal line represents the cutoff between related individuals (half siblings) at 0.24 for Wang's r_{xy} . One value was considered to be related based on this cutoff value.

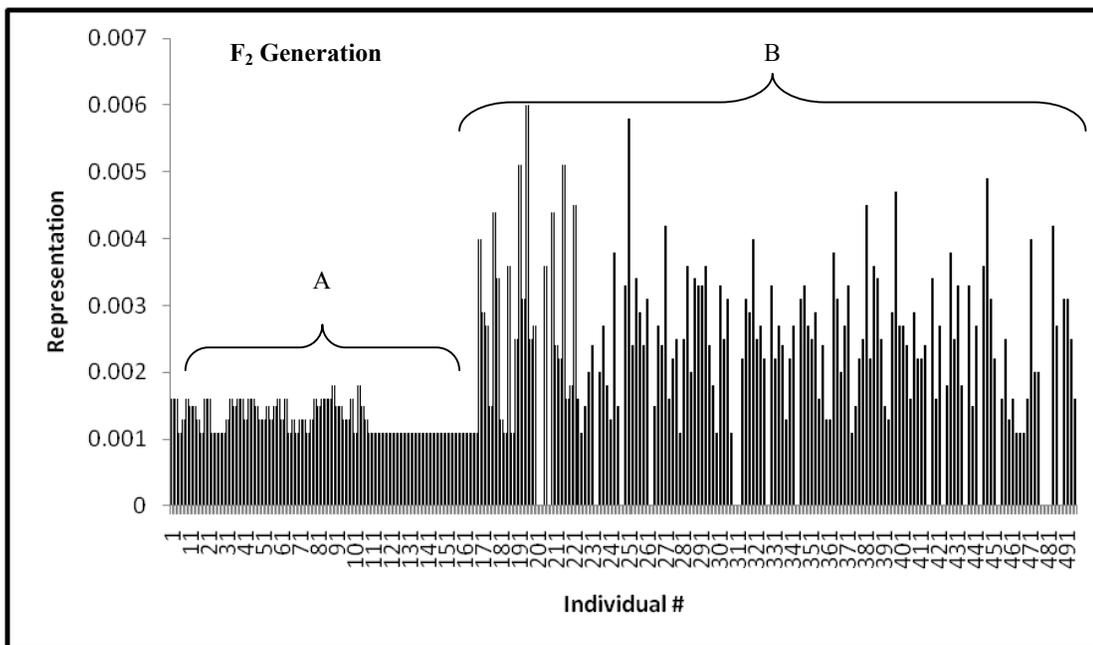
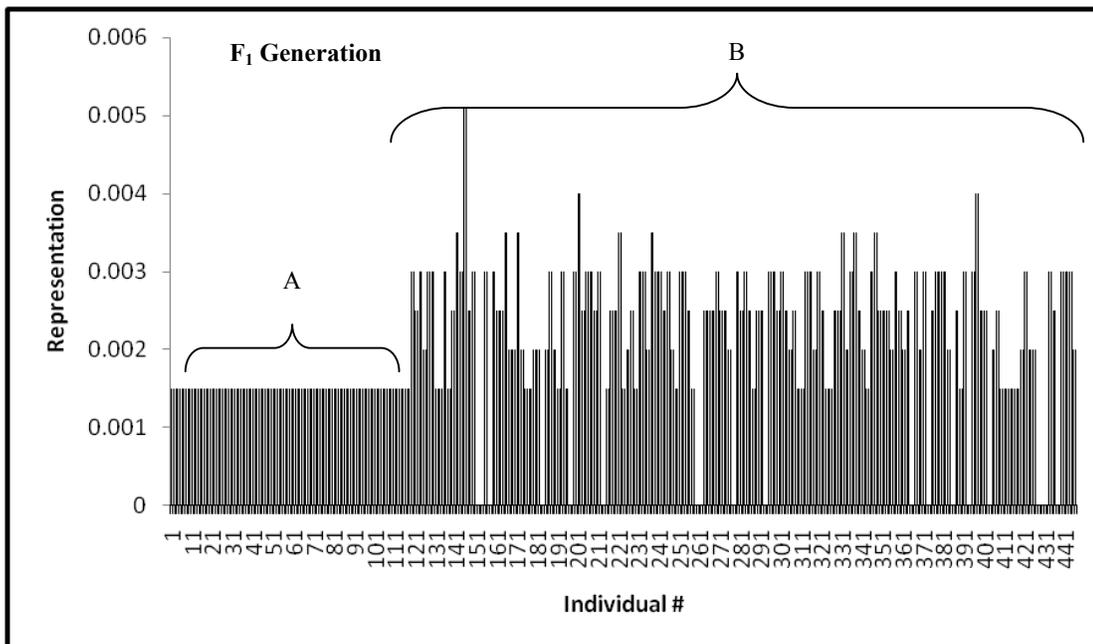


Figure 2.3 Founder representation in the F₁ and F₂ generations, which includes the original 290 individuals in the founding population (B) and 89 additional wild individuals incorporated each generation (A). Founder representation, the proportion each founder is represented in subsequent generations, is displayed on the y-axis. Founder ID numbers are displayed on the x-axis. Gaps represent founders no longer represented in the current generation.

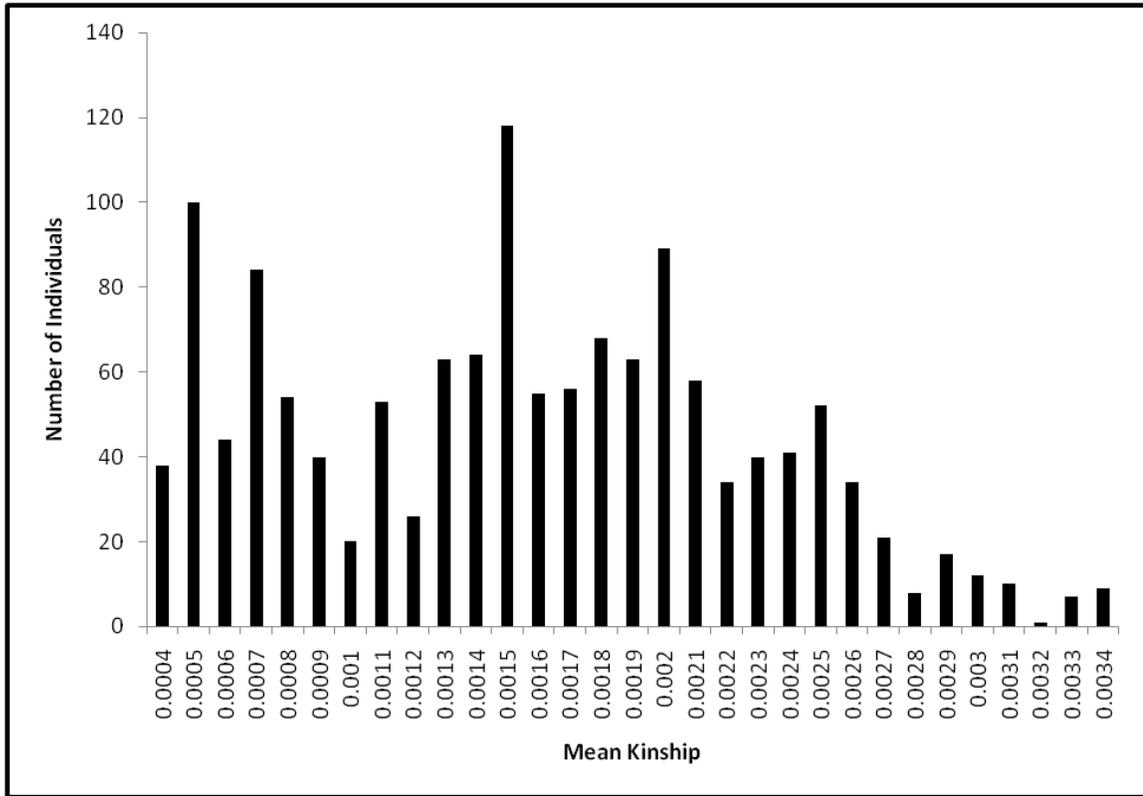


Figure 2.4 Histogram of mean kinship in the F₂ generation of the delta smelt captive population.

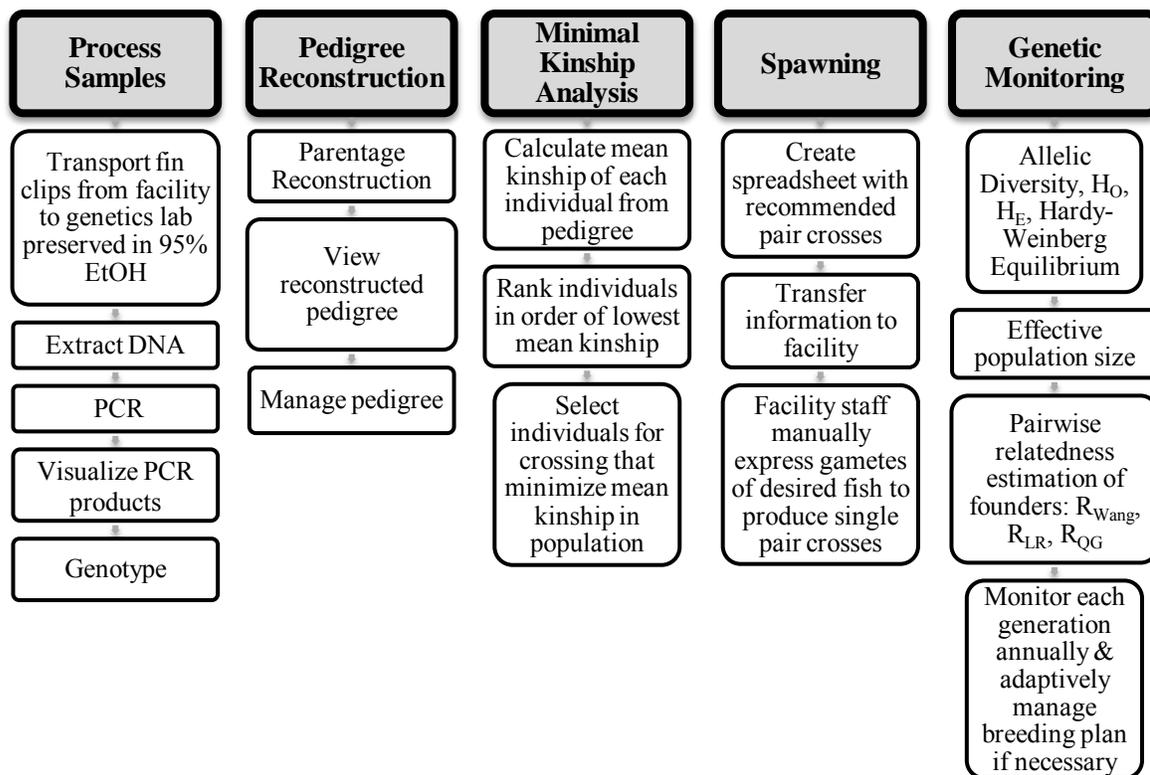


Figure 2.5 Standard operating procedure for the genetic management of the captive delta smelt population.

Table 2.1 Microsatellite loci used for the genetic management of the delta smelt captive refugial population (Fisch et al. 2009)

Marker	Multiplex ^a	A ^b	H ^c	Allelic Range ^d
HtrG103	C	21	0.88	77-181
HtrG104	A	8	0.53	115-183
HtrG109	C	19	0.89	137-225
HtrG114	B	29	0.94	160-316
HtrG115	A	26	0.93	164-300
HtrG116	B	9	0.59	195-267
HtrG117	C	25	0.92	158-298
HtrG119	B	33	0.95	164-324
HtrG120	A	18	0.81	233-333
HtrG126	C	33	0.94	211-359
HtrG127	A	33	0.96	211-367
HtrG131	B	29	0.95	276-412

^a Letter in the multiplex column indicates marker included in one of three multiplex PCR reactions (A-C).

^{b,c} Number of alleles per locus (A) and expected heterozygosity (H) estimated from the founding population (F₀) of the delta

^d Allelic range is the size range of alleles for each marker.

Table 2.2 Mean relatedness coefficients and variances for three relatedness estimators, based on allele frequencies from the captive delta smelt population

	Relationship Category			
	Unrelated	Half-Sibling	Full-Sibling	Parent-Offspring
$r_{xy \text{ QG}}$	-0.012 (0.010)	0.234 (0.013)	0.487 (0.018)*	0.490 (0.004)
$r_{xy \text{ LR}}$	-0.011 (0.004)*	0.201 (0.014)	0.439 (0.025)	0.445 (0.011)
$r_{xy \text{ Wang}}$	-0.001 (0.008)	0.243 (0.012)*	0.492 (0.019)	0.498 (0.002)*

Values were based on 1000 simulated pairs from each of the relationship categories.

*Smallest variance per relationship category.

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CHAPTER 3

**FISH HATCHERY OR ZOO POPULATION? GENETIC ANALYSIS OF THE
DELTA SMELT CAPTIVE BREEDING PROGRAM**

ABSTRACT

Delta smelt (*Hypomesus transpacificus*), an endangered fish species endemic to the San Francisco Estuary, California, USA, was recently brought into captivity for species preservation. Once one of the most abundant fishes in the Estuary, delta smelt have declined dramatically in abundance over the last several decades due to anthropogenic stressors. The delta smelt captive breeding program aims to genetically manage this population as rigorously as a zoo-based conservation breeding program, rather than operate as a fish hatchery, to serve as a genetic bank in the event this species faces extinction in the wild. In this study, we assessed the outcomes of managing the delta smelt captive breeding population as a managed zoo population, rather than as a fish hatchery. Employing 16 microsatellite DNA markers, temporal genetic diversity was examined for each generation to determine the effects of intensive genetic management on the population and to quantify the amount of wild genetic diversity present within each captive generation. The results indicate that managing a fish population as a conservation breeding program preserves the genetic integrity of the captive population.

INTRODUCTION

Delta smelt, *Hypomesus transpacificus* (Osmeriformes, Osmeridae), are pelagic planktivorous fish threatened with extinction due to extreme anthropogenic alterations to their ecosystem. The species is endemic to the upper San Francisco Estuary (SFE), California, USA, which has become highly urbanized and affected by introduction of non-native species, water diversions, contaminants, and the conversion of complex tidal habitats to leveed channels (Moyle et al. 1992; Nichols et al. 1986). Delta smelt are particularly sensitive to these environmental alterations due to their annual lifecycle, low fecundity, poor swimming abilities, and restricted distribution (Bennett 2005; Moyle 2002; Moyle *et al.* 1992). Delta smelt were relatively abundant prior to 1980, with populations declining dramatically after this time (Newman 2008). This decline was due, in part, to increased water exports from the Estuary for urban and agricultural uses and ecosystem alterations caused by non-native species and humans (Baxter *et al.* 2008; Feyrer *et al.* 2007; Sommer *et al.* 2007). The species was listed as threatened by both federal and state governments in 1993, and was listed as endangered under the California Endangered Species Act in 2009 (CDFG 2009; Miller *et al.* 2006; USFWS 1993). In response to this decline, captive breeding efforts have been initiated to establish a captive population of delta smelt at the University of California, Davis Fish Conservation & Culture Laboratory (FCCL) located near Byron, CA. The goal of the delta smelt captive breeding program is to create a genetically and demographically robust captive population that will act as a genetic bank in the event this species becomes extinct in the wild, as well as potentially serve as a source for supporting wild populations if such a need arises (Fisch *et al.* 2010; Fisch *et al.* *In Review.*; Fisch *et al.* 2009b).

It has been suggested that captive breeding programs should maintain 90% of the founding population's gene diversity for 100 years, which has been proposed as the time required for habitat recovery (Ballou *et al.* 2006; Soulé *et al.* 1986). To achieve this objective, the genetic health of the population must be maintained by the implementation of a breeding scheme that aims to maximize gene diversity while limiting inbreeding in the captive population (Ballou & Foose 1996; Ballou & Lacy 1995; Foose & Ballou 1988). Zoo-based conservation breeding programs manage captive populations through recurring breeding recommendations specifically tailored to each species (Ivy *et al.* 2009). For example, the Whooping Crane and St Vincent Parrot captive populations are managed using comprehensive genetic pedigrees to identify genetically important individuals for breeding ((Jones *et al.* 2002; Russello & Amato 2004). As fish populations become increasingly over-exploited, hatcheries have become widely-used tools in an attempt to recover fish populations. However, they frequently operate without the rigorous genetic management of conservation breeding programs (Berejikian & Ford 2004; Fraser 2008; O'Reilly & Doyle 2007).

The traditional goal of fish hatcheries is to boost the wild adult census size by supplementing wild stocks with captive-reared fish in order to maintain fisheries (Hedrick *et al.* 2000a; Naish *et al.* 2008; Palm *et al.* 2003; Waples *et al.* 2007). More recently, however, hatchery objectives have diversified, and current goals range from supplementation of wild populations to preservation of the genetic integrity of endangered populations (Fraser 2008; Hedrick *et al.* 2000b). Supplementation hatcheries, by definition, are hatcheries designed to integrate hatchery and wild populations to mitigate declines in wild populations due to anthropogenic or

environmental causes (Fraser 2008; Naish *et al.* 2008; Waples *et al.* 2007). Because hatchery fish are released by the billions into the wild every year, hatchery practices have the potential to be severely detrimental to wild fish populations if appropriate captive management and reintroduction plans are not implemented (Augerot & Foley 2005; Heard 1995). In contrast, conservation hatcheries, or fish captive breeding programs, aim to preserve populations that are unable to persist in the wild in order to prevent imminent extinction of the declining species or population (Utter & Epifanio 2002). The ultimate goal of these captive breeding programs is similar to zoo-based conservation breeding programs: to maintain genetic variability and fitness within captive populations until they can be reintroduced to the wild as self-sustaining populations (Fraser 2008; Pollard & Flagg 2004; Utter & Epifanio 2002).

Although genetic management guidelines have been established to maintain the genetic health of hatchery populations, their implementation in fish hatcheries is limited and various detrimental effects of hatcheries on both captive and wild supplemented populations have been documented (Allendorf & Ryman 1987; Berejikian & Ford 2004; Busack & Currens 1995). The detrimental effects of hatchery populations may be caused by founding these populations with small numbers of fish from already declining populations. This makes captive populations susceptible to loss of genetic variability, as the founding genetic diversity is already reduced, and it may further erode over subsequent generations in captivity (Allendorf & Luikart 2007; Allendorf & Ryman 1987; Nielsen 1995; Waples 1991). The most common negative genetic changes in fish hatcheries include high levels of inbreeding, adaptation to captivity, reduced viability and fecundity, and reduced effective population size, all of which may result in decreased

fitness of supplemented wild populations (Araki *et al.* 2007; Frankham 2008; Ryman & Laikre 1991). For example, Araki *et al.* (2007) compared the lifetime performance between supplemental hatchery-wild crosses and wild crosses of steelhead trout and observed reduced reproductive success in hatchery-wild fish compared to wild fish. In addition, Ryman & Laikre (1991) demonstrated that supplementing wild populations with captive fish may reduce the wild effective population size far below what it would be without any supplementation. This reduction in wild effective population size results in a more rapid loss of gene diversity, causing a genetic bottleneck that increases genetic stochasticity in already demographically depressed populations (Ryman & Laikre 1991).

With intensive genetic management of both supplementation and conservation hatcheries, many of the negative genetic changes to wild fish populations may be mitigated. By implementing a genetic management plan designed to maximize gene diversity and limit inbreeding, the founding gene diversity of the captive population can be preserved, consequently maintaining the effective population size (Ballou & Lacy 1995; Lacy 1994). The winter-run Chinook salmon conservation hatchery provides a good example of a successful genetic management plan. By attempting to equalize founder contribution, the supplementation of winter-run Chinook salmon into the wild did not appear to decrease the overall wild effective population size (Hedrick & Hedgecock 1994; Hedrick *et al.* 2000b). However, conservation hatchery populations have also been shown to accumulate negative genetic changes (Fraser 2008; Hedrick *et al.* 2000a; Osborne *et al.* 2006). For example, Hedrick *et al.* (2000a) evaluated the bonytail chub captive broodstock and discovered low genetic diversity due to a small number of founders. In addition, analysis of the Rio Grande silvery minnow propagation

program revealed that it maintained allelic diversity but still resulted in higher inbreeding in captive versus wild fish stocks (Osborne *et al.* 2006). These results highlight the need for rigorous genetic management of captive populations to preserve their genetic integrity.

The delta smelt captive breeding program aims to operate not as a conservation fish hatchery, but as something more like a zoo conservation breeding program. Traditional conservation hatchery genetic management plans rely on random matings or natural mate choice, which with the limitations imposed in a hatchery facility, may lead to inbreeding, loss of genetic variation and unequal family sizes (Fraser 2008; Wedekind 2002). It is for this reason we recommend the conservation breeding program model, where mate selection is controlled in an effort to minimize mean kinship, thus maintaining gene diversity and limiting inbreeding (Ballou & Lacy 1995). Under this model, hatcheries develop genetic management plans for their hatchery and supplementation programs, in an effort to decrease the demographic and genetic consequences of supplementing the wild population with hatchery fish as discussed above (Allendorf & Ryman 1987; Araki *et al.* 2007; Waples & Drake 2004).

The delta smelt genetic management plan is based on a modified method of minimal kinship selection (Ballou & Lacy 1995). Each year prior to the spawning season, captive fish are tagged, fin clipped and genetically analyzed using 12 microsatellite markers. The pedigree of the captive population is reconstructed every generation with parentage analysis (Fisch *et al. In Review.*). Prior to pedigree reconstruction, the identity of individual fish are unknown, as fish are housed in tanks comprised of many families due to facility space limitations. Genetically important

individuals are identified from the pedigree and manually strip-spawned in single pair crosses to minimize mean kinship and limit inbreeding in the captive population. Wild individuals are incorporated into the captive population annually to maximize genetic diversity and minimize genetic divergence from the wild population (Fisch *et al. In Review.*).

In this paper, we assess the utility of the delta smelt captive population genetic management plan in preserving the genetic diversity of the species relative to that of the wild population. To determine if the delta smelt captive breeding program is meeting the genetic management program goals of retaining 90% gene diversity for 100 years, we assess the progress and current status of the captive population by genetically analyzing wild fish and captive fish from three generations with 16 microsatellite DNA markers. We then recommend future management strategies for the captive population and suggest that the delta smelt captive breeding program may serve as a management model for other fish hatchery programs.

METHODS

Sample Collection & DNA Preparation

Tissue samples were taken from the caudal or adipose fin of captive fish at the FCCL and preserved in 95% EtOH. The captive population founders and additional wild fish were collected in December prior to spawning season in the Lower Sacramento River (Figure 1). Within the captive population, thousands of fish were sampled each generation to identify the most genetically valuable individuals for breeding using a modified version of minimal kinship selection (F_1 : $n = 1400$; F_2 : $n = 1858$) (Ballou &

Lacy 1995). The fish ultimately selected as breeders in each of three consecutive, discrete generations were used for all subsequent genetic analyses (F_0 : $n = 290$; F_1 : $n = 440$; F_1 wild: $n = 54$; F_2 : $n = 439$; F_2 wild: $n = 35$). Wild samples (SKT2007 sample set) were obtained from muscle tissue of wild fish collected by the California Department of Fish and Game during the 2007 Spring Kodiak Trawl Survey (sampling January-May, 39 geographic sampling stations grouped into 5 regions) and were preserved in 95% EtOH ($n = 372$; Figure 1). Wild fish were collected during the spawning season from their entire range and were assumed to be representative of the species in the wild during the time that the captive population was founded. Genomic DNA was extracted from all samples using the DNeasy Tissue Kit (QIAGEN) following the manufacturer's directions, with all samples yielding high-molecular weight DNA.

Microsatellite Genotyping

A total of 16 microsatellite loci described by Fisch *et al.* (2009a) were amplified by polymerase chain reaction (PCR) for both captive and wild samples (Table 1). PCR products were visualized using an ABI 3730 DNA Analyzer (Applied Biosystems, Inc.) with the LIZ500 internal size standard. Alleles were scored using ABI's Genemapper™ 4.0 and verified manually (Applied Biosystems, Inc.). To reduce genotyping errors, two control samples with known allele sizes were included in every 96-well PCR plate, allele calls were independently scored by two people and genotypes with questionable allele calls were re-amplified and scored again.

Diversity Statistics

To assess the utility of the genetic management plan for preserving the genetic integrity of the captive population relative to the wild population, genetic diversity was estimated for all three captive generations and the wild population as the number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and polymorphic information content (PIC) using Cervus 3.0 (Kalinowski *et al.* 2007). To compare populations with different sample sizes, allelic richness (A_R) was calculated as a measure of the number of alleles independent of sample size using FSTAT 2.9.3 (Goudet 2001). Statistical significance was determined using the Wilcoxon signed-rank test. Presence of null alleles was determined using Micro-Checker (Van Oosterhout *et al.* 2004). Tests of Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted using GenePop 3.4 (Raymond & Rousset 1995) based on the Markov chain method using 1000 dememorization steps, 100 batches and 1000 iterations per batch. Significance was determined by applying sequential Bonferroni correction (Rice 1989). Pairwise comparisons of R_{ST} between sample sets were calculated and tested for statistical significance with 16,000 permutations in Arlequin 3.1 (Excoffier *et al.* 2005).

Effective Population Size

Pedigree-based Estimates of N_e : The reconstructed pedigree was used to calculate the inbreeding effective population size (N_e) using family size as a proxy for lifetime reproductive success. As there are an equal number of female and male parents, N_e was estimated as: $N_e = (Nk - 2) / [k + (V_k/k) - 1]$ with N = total number of parents, k = average

family size and V_k = variance of family size (Crow & Kimura 1970; Herbinge *et al.* 2006).

Genetic Estimate of N_e : The inbreeding N_e of the parental generation was estimated based on linkage disequilibrium among the offspring of this generation according to the methods of Hill (1981) and Bartley *et al.* (1992) and implemented in N_e Estimator (Peel *et al.* 2004). To avoid potential bias in N_e estimates when using full siblings in this method, two offspring from each family were randomly selected and pooled to estimate N_e in each generation.

Cluster analysis

A Bayesian clustering method was implemented in Structure 2.3.3 to estimate the number of groups (generations) represented by the sampled individuals (k) (Pritchard *et al.* 2000). Twenty-five independent runs assuming $k = 1$ to 10 were performed with 1×10^6 Markov chain Monte Carlo (MCMC) repetitions and a burn-in period of 5×10^5 using no prior information, assuming admixture and correlated allele frequencies. The steepest increase of the probability of k was measured by plotting the probability of the data [$P(D)$] and the *ad hoc* statistic Δk to determine the most likely value for k (Evanno *et al.* 2005).

We constructed a neighbor-joining tree from allele frequency data by implementing the neighbor-joining method and bootstrap tests with 10,000 replicates in Poptree2 (Felsenstein 1985; Saitou & Nei 1987; Takezaki *et al.* 2010). Nei's standard genetic distance without sample size bias correction was used for phylogeny construction (Nei 1972).

RESULTS

A total of 389 alleles were identified for the 16 microsatellite loci in the 1631 samples that were genotyped (Table 1). Eleven new alleles were discovered in the F₁ generation and an additional 2 alleles were incorporated into the F₂ generation due to the incorporation of wild individuals. Two alleles in the F₁ generation and two in the F₂ generation were gained possibly due to mutation. A total of 14 alleles were lost in the F₁ generation and 16 were lost from the F₁ to the F₂ generation. The new alleles gained nearly offset the alleles lost, which highlights the importance of bringing wild individuals into captivity to maintain genetic diversity. The number of alleles per locus ranged from 9 at locus *HtrG118* to a maximum of 37 at locus *HtrG127* (Table 1).

Allelic richness (A_R) ranged from 4.0 to 32.7 alleles at each locus across a combined set of all samples based on a minimum sample size of 156 diploid individuals. When each sample set was analyzed individually, the SKT2007 sample set represented the highest allelic diversity ($A_R=22.5$), followed by the F₀ generation ($A_R=20.1$). The allelic diversity of all of the generations in captivity pooled, including the incorporated wild fish, was $A_R=19.0$, which was not significantly lower than that present in the founding generation F₀ ($A_R=20.1$). When compared across all loci, the difference in A_R between the founding captive and wild populations was not significant ($P>0.05$). However, the F₁ and F₂ generations had significantly lower A_R than the SKT2007 sample set ($P<0.02$ for both generations). This is most likely due to the loss of alleles between the F₀ and F₂ generations. On average, between the F₀ and F₁ generations, 1.6 alleles per locus were gained and 1.8 alleles per locus were lost. In the F₂ generation, an average of 1.6 alleles per locus were lost from the F₁ generation and 1 allele per locus was gained.

High levels of heterozygosity were observed in both the wild and captive populations. The mean expected heterozygosity (H_E) in each of the three generations of the captive population (F_0 , F_1 , F_2) was 0.814 (ranging from 0.355 to 0.958), 0.812 (ranging from 0.380 to 0.959), and 0.817 (ranging from 0.288 to 0.958), respectively. The mean expected heterozygosity of the wild sample set SKT2007 was 0.795 (ranging from 0.306 to 0.959) (Table 2).

HWE tests revealed that some of the loci deviated from HWE in the captive and wild populations after sequential Bonferroni correction. There was no evidence of null alleles in the data set according to Micro-Checker, as the frequency of null alleles at each locus was less than five percent. Full siblings were identified and only one individual was included from each family (Table 2). In the F_0 generation, 4 loci (*HtrG104*, *HtrG118*, *HtrG119* and *HtrG126*) deviated from HWE. In the F_1 generation, three loci deviated from HWE (*HtrG114*, *HtrG118* and *HtrG119*). In the F_2 generation, all loci conformed to HWE. In the SKT 2007 wild population, 4 loci (*HtrG107*, *HtrG116*, *HtrG118* & *HtrG129*) deviated from HWE ($P < 0.05$; Table 2). The significant heterozygote deficit ($P < 0.05$) in the wild population may explain the departure from HWE. In addition, F_{IS} statistics revealed much higher F_{IS} values for the loci that did not conform to HWE. All F_{IS} values are positive for SKT 2007 and the F_1 generation, but there was a significantly negative F_{IS} value at one locus in the F_0 generation, indicating heterozygote excess at this locus (Table 2). Linkage disequilibrium analysis was significant only for loci out of HWE (*HtrG104*, *HtrG107*, *HtrG114*, *HtrG116*, *HtrG118*, *HtrG119*, *HtrG126*, *HtrG129*).

R_{ST} values for the captive generations and the wild population are presented in Table 3. The R_{ST} values between the wild population and the three captive generations

indicate little to no differentiation, and the R_{ST} values between generations were also negligible. None of the R_{ST} values were significant.

Effective Population Size

The pedigree-based estimate of effective population size (N_e^\dagger) for the total captive population was 926, 81% of the total number of broodfish over three generations (Table 4). The total N_e^\dagger was estimated assuming equal family contribution in the F_2 generation, as these offspring have not yet contributed to the next generation (F_3). Variance of family contribution was moderate in the F_0 generation ($V_k = 3.16$) and low in the F_1 generation ($V_k = 1.34$). The linkage disequilibrium $N_e(LD)$ estimates for two generations ($F_1: N_e = 352$ and $F_2: N_e = 296$) were in relatively close agreement with N_e^\dagger . In contrast, in the F_0 generation, $N_e(LD)$ was over seven times greater than N_e^\dagger (Table 4).

Genetic Demes

Assignment tests using the Bayesian clustering method in Structure indicated the highest Ln probability occurred when $K = 6$, $\log P[K/X] = -69,881$, which was also supported using the Evanno method. However, the clusters were not associated with the sample set from which they derived, and as the pairwise R_{ST} values between the sample sets were not significant, we concluded that there was only one genetic cluster. As a result, these sample sets are not considered genetically distinct. However, a neighbor joining tree based on Nei's genetic distance matrix showed that the four sample sets were clustered

into two distinct groups (wild and captive) and two distinct subgroups with the captive sample set (Figure 2).

DISCUSSION

Genetic Management Maintains Genetic Integrity of Captive Delta Smelt Population

The combined results regarding the genetic diversity of the captive delta smelt population indicate that the population was initially founded with and continues to retain high levels of allelic diversity and heterozygosity (Table 1). Neither allelic diversity nor heterozygosity are significantly different between the current captive population (F_2) and the wild population, suggesting the genetic management plan utilized in the captive delta smelt population is effective at maintaining genetic diversity, at least over the short-term. The initial founding generation (F_0) was captured from the wild in the fall of 2006 as sub-adults and, as delta smelt are an annual fish, the wild adult fish collected by the CDFG in 2007 are part of the same cohort. As a result, the lack of significant difference in allelic richness and the high percentage of shared alleles between the F_0 generation and the wild population was not unexpected and indicate that the F_0 fish were genetically representative of the wild population. The proportion of the expected heterozygosity of a wild population that is predicted to be captured by the founders of a captive population is equal to

$$H_f = H_w * \left[1 - \frac{1}{1 - 2N} \right], \quad (2)$$

where H_f and H_w are the mean expected heterozygosities in the N founders and the wild population, respectively, from which the founders were sampled (Crow and Kimura 1970). Thus, the 290 initial founders of the captive delta smelt population were predicted

to capture 99.8% of the wild population's heterozygosity. The expected heterozygosities in the SKT2007 and F_0 sample sets were 0.795 and 0.814, respectively, indicating that the initial captive population founders captured comparable expected heterozygosity to that of the wild population (SKT2007 sample set).

R_{ST} values also suggest that the current captive population is an adequate genetic representation of the wild population. Results suggest that there is only low population differentiation among the four sample sets, as R_{ST} values were all negative and none were statistically significant (Table 3). Negative R_{ST} values result from the imprecision of the algorithm used to estimate this value and indicate a value close to zero (Weir & Cockerham 1984). This suggests that the current captive population has not significantly diverged from the wild population.

The predicted loss of gene diversity over time in a captive population arising from the founder effect and subsequent small size effects can be calculated using the equation:

$$\frac{H_t}{H_0} = \left[1 - \frac{1}{2N_{f0}} \right] * \left[1 - \frac{1}{2N * \left(\frac{N_e}{N} \right)} \right]^{t-1}, \quad (3)$$

where N_{f0} = number of effective founders, t = time in generations, H_0 = founder heterozygosity, N = current captive population size, and H_t = expected heterozygosity of captive population at time t (Frankham *et al.* 2002). Given the founding population of 290 individuals with $H_0=0.814$ and a stable population size of 500 individuals, in 100 generations, this population will retain 90.3% of its initial gene diversity, which meets the requirement of a successful captive breeding program (Ballou *et al.* 2006; Soulé *et al.* 1986).

The SKT2007 wild population and founding captive generation each have 4 loci deviating from HWE, and the F₁ generation has 3 loci deviating from HWE (Table 2). In the captive generations, the F₁ generation possesses three loci that deviate from HWE, suggesting that whatever the cause of HWE deviation was in the founding generation, the population management may be bringing the population back into HWE, since in the F₂ generation all loci are in HWE. This observation may arise as one generation of random mating is expected to bring a population back into HWE (Hardy 1908; Weinberg 1908). The most likely assumption that is violated in these populations is that of random mating, but other causes of the deviation from HWE may include subpopulation structure, high variation in effective population size, contemporary dramatic decline in spawning populations, oscillations in population sizes and unequal sex ratios in the wild (Brown *et al.* 2007; Wahlund 1928).

The effective population size of each generation in captivity was relatively consistent with the census size (Table 4). The N_e/N ratio is relatively high for each generation based on both methods, so we can conclude that N_e is being maintained in the captive population. Even when population sizes remain large, N_e can decline due to variance in reproductive success, unequal sex ratios and non-random mating (Luikart *et al.* 2010; Ryman *et al.* 1995). Reductions in N_e will then lead to increased genetic drift, rapid loss of genetic diversity, and higher rates of inbreeding, making populations more susceptible to extinction (Saccheri *et al.* 1998; Waples 2002). The methods employed to manage the delta smelt captive breeding program aim to reduce variance in reproductive success and equalize sex ratios to maximize N_e in order to maximize genetic diversity in the captive population. It is essential to monitor N_e in the captive population each

generation, as N_e is an important tool for monitoring genetic variation (Schwartz et al. 2007).

The Bayesian cluster analysis results support the R_{ST} values by suggesting that the populations are not genetically divergent. However, it did distinguish the number of groups entered into the program, as was revealed by the number of clusters ($k = 6$) that possessed the highest Ln probability. The existence of divergent populations would be more likely if the populations clustered to their populations of origin. As this was not the case, we concluded that the populations were not genetically divergent. The neighbor-joining tree revealed two distinct groups (wild and captive) and two subgroups within the captive group (Figure 2). This grouping reveals patterns of genetic distance that possibly indicate increased genetic distance with increasing generations in captivity. The captive and wild population grouping is expected, as the F_0 generation is a small subset of the total wild population. It will be important to monitor the captive population annually to adapt genetic management strategies to minimize genetic divergence between the wild and captive populations.

Recommendation to Fish Hatcheries to Incorporate Genetic Management Plans

This study demonstrates the utility of using a genetic management plan that minimizes mean kinship to retain genetic variation in a captive fish population. As a result, we recommend that fish hatcheries adopt genetic management plans to manage their broodstock, regardless of the cost. Conservation breeding programs have their origins in zoos, in part, because these institutions manage much smaller populations that are easier to manage than the hundreds to thousands of fish that hatcheries traditionally

manage each generation. The implementation of a genetic management plan might be more difficult and cumbersome for fish hatcheries; however, these institutions generally release large numbers of fish back into wild populations and have been documented to negatively impact the genetic diversity and effective population size of wild populations. Thus, it is essential that fish hatcheries implement genetic management plans to prevent detrimental changes to supplemented wild populations.

Since the adoption and implementation of a genetic management plan in a large fish hatchery is costly and labor intensive, genetic management plans can be tailored specifically to each hatchery. Efforts to maintain genetic diversity and prevent genetic response to captive breeding are essential to success for both conservation and population supplementation purposes, so hatchery managers should determine what is cost effective and physically possible to accomplish with a genetic management plan. A genetic management plan should include pedigree analysis to attempt to equalize family contribution and allow for breeding schemes that minimize kinship in the population. This can be accomplished by keeping family groups in separate tanks or by tagging individuals. This relatively inexpensive procedure still allows for the implementation of a simpler genetic management plan for those hatcheries where genetically reconstructing the pedigree is not feasible. In addition, genetic adaptation in captivity is of particular concern in the delta smelt captive population, since the only natural conditions they are exposed to come from the open flow through water system. To reduce genetic adaptation to captivity in other hatcheries, fish may be exposed to simulated natural conditions in tanks or use an open flow through water system that exposes captive fish to conditions in

their native habitat. Regardless of the complexity or limitations of the genetic management plan, the following guidelines should be followed:

1. Pedigree analysis of the captive population should be conducted to allow equalization of family sizes and allow for a breeding scheme that minimizes kinship in the population.
2. Wild individuals should be periodically incorporated into the captive population to allow gene flow between the wild and captive populations in order to minimize genetic drift in the captive population, if possible.
3. Genetic adaptation to captivity should be minimized through exposure to naturalistic conditions.

CONCLUSION

The results of this study suggest that fish hatcheries utilizing genetic management plans based on a modified method of minimal kinship selection can preserve genetic diversity in captive populations. Incorporation of wild fish into each generation is also an important component to the success of these programs; however, this is not feasible in highly endangered or extirpated populations, indicating the increased need for careful genetic management. Continued genetic management of the delta smelt captive breeding program will preserve this species should it become extinct in the wild, and will serve as a model for fish hatcheries adopting fish hatchery genetic management plans. In an era where we are witnessing an increasing need to bring species into captivity to preserve them from extinction, managing fish hatcheries with genetic management plans similar to

those used in zoo-based conservation breeding programs is an ecologically and socially defensible conservation strategy for preserving the world's biodiversity.

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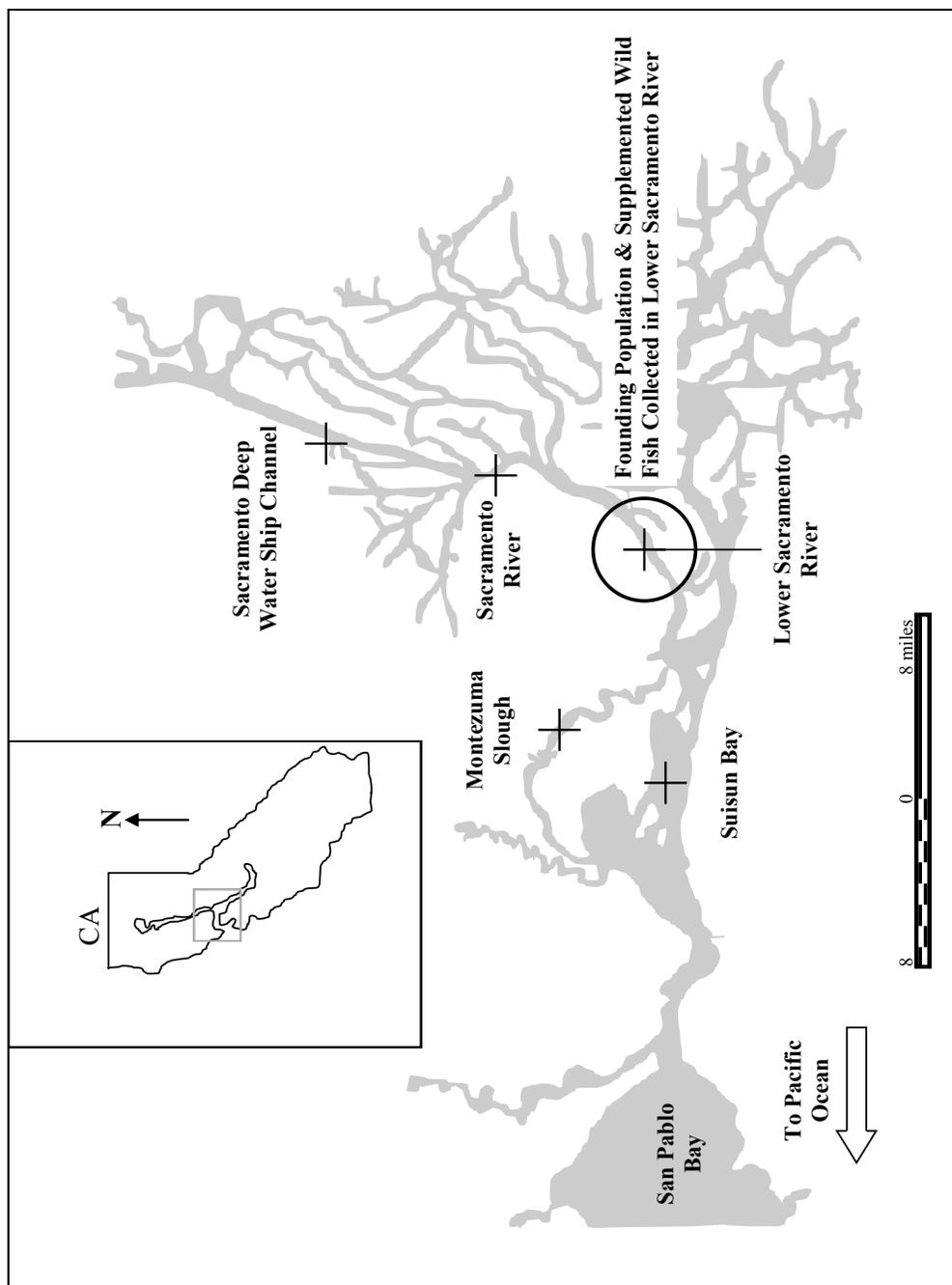


Figure 50 Map of California Department of Fish and Game 2007 Spring Kodiak Trawl Survey sampling locations and collection location of the captive population founders and supplemented wild fish in the San Francisco Estuary, CA, USA.

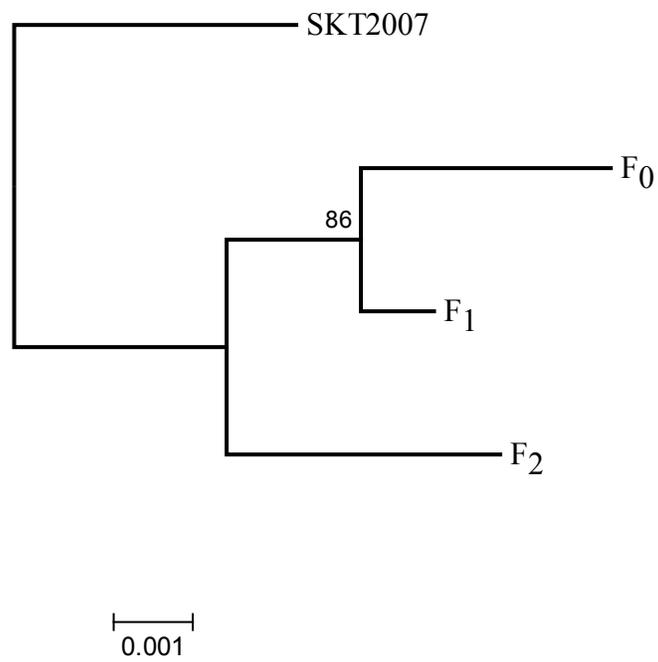


Figure 3.2 Neighbor-joining tree of Nei's genetic distance among the captive and wild sample sets of delta smelt.

Table 3.1 Allelic diversity of delta smelt at 16 microsatellite loci, including locus name, number of alleles per locus (N_a), number of individuals genotyped at each locus (N), number of alleles in each population (A), allelic richness* (A_R) for each population, and number of alleles gained (G_{x-x}) and lost (L_{x-x}) in each population relative to each other population

Locus	Captive Population											Wild Population					
	F ₀			F ₁			F ₂			SKT2007							
	N _a	N	A	A _R	N	A	A _R	G ₀₋₁	L ₀₋₁	N	A	A _R	G ₁₋₂	L ₁₋₂	N	A	A _R
<i>HtrGI03</i>	23	226	21	19.9	487	19	18.0	---	2	467	18	17.5	---	1	364	22	19.8
<i>HtrGI04</i>	11	288	7	6.4	420	8	6.7	1	---	467	7	6.1	---	1	354	9	7.9
<i>HtrGI07</i>	26	192	24	23.3	388	23	22.3	---	1	370	23	22.1	---	---	339	25	23.6
<i>HtrGI09</i>	19	192	19	18.2	451	19	16.6	---	---	465	19	16.9	---	---	364	17	16.7
<i>HtrGI14</i>	31	285	29	27.0	431	30	26.4	1+	---	458	29	26.5	---	1	307	29	27.2
<i>HtrGI15</i>	29	286	26	24.1	458	25	22.5	---	1	464	25	23.2	1+	1	349	27	23.8
<i>HtrGI16</i>	12	239	9	8.1	409	9	7.6	---	---	461	7	6.5	---	2	301	9	8.5
<i>HtrGI17</i>	27	233	25	22.7	480	22	18.9	1+	4	462	22	18.2	---	---	357	21	20.3
<i>HtrGI18</i>	9	240	7	6.5	404	4	4.0	---	3	378	5	4.7	1	---	337	7	6.4
<i>HtrGI19</i>	35	278	32	30.6	385	32	30.8	---	---	459	32	30.0	---	---	304	35	32.1
<i>HtrGI20</i>	21	276	18	17.2	473	19	16.9	1	---	466	17	16.1	---	2	350	21	19.1
<i>HtrGI26</i>	35	275	33	30.2	488	33	29.4	1+	1	465	31	28.2	---	2	365	34	30.5
<i>HtrGI27</i>	37	287	33	31.3	462	36	32.7	3+	---	466	32	29.7	---	4	347	35	31.7
<i>HtrGI28</i>	27	221	22	20.3	387	25	20.7	3+	---	373	25	21.1	1+	1	340	25	22.2
<i>HtrGI29</i>	15	156	8	8.0	331	7	6.3	---	1	285	8	6.9	1	---	324	14	12.1
<i>HtrGI31</i>	32	234	29	27.9	354	30	27.2	2+	1	452	29	27.1	---	1	293	30	27.2
Total	389	---	342	---	---	341	---	13	14	---	329	---	4	16	---	360	---
Average	---	---	---	20.1	---	---	19.2	1.6	1.8	---	---	18.8	1.0	1.6	---	---	22.5

*Allelic richness (A_R) based on a minimum sample size of 156 diploid individuals

+New allele from wild incorporation

Table 3.2 Heterozygosity of delta smelt, including locus name, observed heterozygosity (H_O), expected heterozygosity (H_E), HW E p-values (P) and F -statistics within population (F_{IS})

Locus	Captive Population						Wild Population									
	F_0			F_1			F_2			SKT2007						
	H_O	H_E	P	F_{IS}	H_O	H_E	P	F_{IS}	H_O	H_E	P	F_{IS}				
<i>HtrG103</i>	0.80	0.88	0.02	0.09	0.91	0.89	0.57	-0.03	0.92	0.89	0.98	-0.05	0.90	0.89	0.93	-0.01
<i>HtrG104</i>	0.60	0.53	0.001*	-0.12	0.53	0.52	0.98	0.00	0.54	0.52	0.53	-0.02	0.57	0.55	0.10	-0.04
<i>HtrG107</i>	0.81	0.80	0.59	-0.02	0.81	0.80	0.29	0.03	0.75	0.82	0.08	0.08	0.77	0.85	0.001*	0.09
<i>HtrG109</i>	0.86	0.89	0.83	0.03	0.88	0.89	0.56	0.02	0.91	0.89	0.76	-0.02	0.86	0.89	0.09	0.03
<i>HtrG114</i>	0.92	0.94	0.43	0.02	0.85	0.94	0.001*	0.07	0.91	0.94	0.17	0.04	0.91	0.94	0.38	0.03
<i>HtrG115</i>	0.93	0.93	0.56	0.00	0.88	0.93	0.01	0.05	0.92	0.93	0.06	0.01	0.94	0.93	0.91	-0.01
<i>HtrG116</i>	0.61	0.59	0.14	-0.04	0.57	0.54	0.99	-0.06	0.58	0.55	0.72	-0.06	0.43	0.54	0.001*	0.20
<i>HtrG117</i>	0.92	0.92	0.49	-0.01	0.92	0.91	0.03	-0.01	0.93	0.91	0.90	-0.03	0.92	0.92	0.15	0.00
<i>HtrG118</i>	0.30	0.36	0.001*	0.16	0.28	0.38	0.001*	0.16	0.29	0.29	0.12	0.01	0.28	0.31	0.001*	0.07
<i>HtrG119</i>	0.91	0.95	0.001*	0.05	0.88	0.96	0.001*	0.10	0.91	0.95	0.55	0.04	0.93	0.95	0.34	0.03
<i>HtrG120</i>	0.84	0.81	0.09	-0.04	0.84	0.82	0.45	-0.04	0.78	0.95	0.12	0.03	0.83	0.82	0.40	-0.02
<i>HtrG126</i>	0.87	0.94	0.001*	0.08	0.92	0.95	0.01	0.05	0.95	0.95	0.18	0.07	0.92	0.94	0.49	0.03
<i>HtrG127</i>	0.92	0.96	0.01	0.04	0.94	0.96	0.10	0.05	0.93	0.96	0.33	0.03	0.94	0.96	0.64	0.02
<i>HtrG128</i>	0.91	0.89	0.48	-0.02	0.87	0.89	0.43	0.02	0.90	0.90	0.43	-0.02	0.88	0.90	0.46	0.02
<i>HtrG129</i>	0.58	0.69	0.06	0.16	0.59	0.67	0.01	0.10	0.72	0.70	0.93	0.02	0.72	0.73	0.001*	0.01
<i>HtrG131</i>	0.91	0.95	0.01	0.04	0.94	0.95	0.05	0.01	0.95	0.95	0.04	-0.02	0.91	0.95	0.07	0.04
Average	0.79	0.81	0.31	0.03	0.79	0.81	0.34	0.03	0.81	0.82	0.43	0.01	0.79	0.79	0.82	0.41

*Statistically significant at $P < 0.001$ after bonferroni correction

Table 3.3 R_{ST} values for the four sample sets of delta smelt (below diagonal)

	Captive Population			Wild Population
	F_0	F_1	F_2	SKT2007
F_0	0	NS	NS	NS
F_1	-0.006	0	NS	NS
F_2	-0.003	-0.006	0	NS
SKT2007	-0.012	-0.004	-0.006	0

* Significant ($P < 0.05$) differentiation is indicated with * (upper diagonal). NS = not significant.

Table 3.4 Pedigree-based and genetic estimates of N_e in captive delta smelt over three years

Generation	N	k	V_k	$N_e \dagger$	Genetic Estimate (95% CI) $N_e (LD)$
F₀	290	1.7	3.2	188	1400.7 (1121.6 -1853.9)
F₁	494	1.5	1.3	524	351.8 (336.7 - 367.9)
F₂	364	1.0	1.0	362	296.1 (283.5 - 309.6)
Total Broodfish	1148	1.4	1.8	926	682.9

Number of broodfish (N), average family size (k), variance of family size (V_k), N_e estimate accounting for variance in family size ($N_e \dagger$), and linkage disequilibrium method ($N_e(LD)$). 95% confidence intervals were not calculated for the $N_e \dagger$ method.

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CHAPTER 4

**PRIORITIZING CONSERVATION MANAGEMENT WITH POPULATION
GENETICS: A CASE STUDY OF THE ENDANGERED DELTA SMELT**

ABSTRACT

The increasing number of endangered species results in the need to prioritize conservation resources. Over the last two decades, the collapse of the endangered delta smelt (*Hypomesus transpacificus*) in the San Francisco Bay-Delta has resulted in politically charged conservation decisions, including the rationing of valuable Delta water for use in California agriculture and urban centers. A fundamental question remaining in delta smelt conservation is whether current management strategies have been appropriately designed to protect the remaining genetic variation in delta smelt populations, rather than merely mitigating the decline of the species. We used microsatellite markers to characterize genetic variation within and among sampling regions on geographic and temporal scales, to estimate changes in effective population size over time, and to determine if a genetic bottleneck exists. A genetic bottleneck was detected in each of the four sampling years, and a significant decline in effective population size was observed between sampling years 2003 and 2007. We also detected a weak geographic signal in any given sampling year that was unsupported by temporal consistency of this signal. We assessed two strategies for defining conservation units, which produced contrasting results. However, we concluded that continuing to manage the species as a single, panmictic population throughout its range is the most feasible management strategy. This study demonstrates the utility of using population genetics to

define conservation units. Conservation managers may apply these methods to define conservation units of a variety of endangered species, in an effort to efficiently allocate conservation resources.

INTRODUCTION

The number of endangered species of plants and animals continues to rise due to the increasing anthropogenic demand for natural resources (Millennium Ecosystem Assessment 2005). This results in an increasing need to prioritize conservation efforts, as funding and logistical constraints often preclude preservation of a species in its entire range (Faith 1992; Moritz 2002). Conservation prioritization focuses resources on critical populations or habitats to protect existing genetic diversity in order to preserve the ecological and evolutionary processes necessary for species persistence (Crandall et al. 2000; Mace and Purvis 2008; Moritz 2002).

Population genetics provides many of the tools necessary for prioritizing conservation that preserves evolutionary processes. It allows researchers and managers to assess the amount of intraspecific genetic variation, loss of genetic diversity, levels of inbreeding, effective population size, presence of bottlenecks and temporal and geographic structure within a population (Frankham et al. 2002; Moritz 2002; Petit et al. 1998). These parameters can be used to assess the current evolutionary status of a species, as well as to predict the evolutionary potential of different populations (Mace and Purvis 2008).

Various methods have been proposed for defining conservation units that are used to prioritize management goals (Mace and Purvis 2008). Traditionally, conservation has

been prioritized based on maintaining ecological and evolutionary patterns of diversity (Myers et al. 2000; Smith et al. 1993). More recently, it has been recommended that conservation prioritization should focus on maintaining and restoring evolutionary processes and ecosystem services rather than distinct intraspecific phenotypes (Erwin 1991; Moritz 1995; Rouget et al. 2006). Palsbøll et al. (2007) recommend defining management units based on the amount of genetic divergence at which populations become demographically independent instead of focusing solely on the rejection of panmixia. Another method involves characterization of ecological and evolutionary patterns of diversity to determine what features need to be conserved in order to maintain evolutionary processes (Moritz 2002). The maintenance of evolutionary processes can be accomplished by maintaining connectedness of populations, ensuring adequate genetic diversity, avoiding inbreeding, and preserving species across a range of native habitats and significant boundary zones (Mace and Purvis 2008).

However, in practice, applying the above principles to endangered populations often proves difficult (Ashley et al. 2003). For example, the identification of conservation units within a species may be complicated by geographic variation in phenotypes and molecular characters that may or may not coincide with one another (O'Brien and Mayr 1991). Conservation actions also tend to focus on the preservation of the *status quo* instead of a more dynamic management approach that encourages diversity and evolutionary change (Smith et al. 1993). In addition, a common obstacle for prioritizing conservation management is over-emphasis of differences among population subunits, which may lead to inefficient management when subunits are independently managed (Mace and Purvis 2008). Given the limitations discussed above, prioritization of

conservation actions is imperative to ensure species survival in the short term and genetic diversity in the long term, in order to maintain the evolutionary potential of the species.

This study explores the population genetics of the endangered delta smelt (*Hypomesus transpacificus*), an estuarine fish species endemic to the San Francisco Bay-Delta, CA that is at the center of California's water crisis. Conservation managers and scientists are faced with a difficult decision concerning the delta smelt, which has declined because of massive anthropogenic changes to the Delta ecosystem. To protect delta smelt and other fishes, the timing and amount of water exports from the Delta have been altered, which is perceived by some farmers as a threat to their livelihood because of reduced amounts available for irrigation at times (Lund et al. 2010). For this reason, conservation prioritization for this species is essential, as a balance between human needs for water and the needs of this endangered species and its ecosystem must be reached. Our main objective is to utilize population genetics to prioritize management of delta smelt by defining conservation units. In the present study, we assess the management implications of various prioritization schemes. The results will be used to inform management of this and other endangered species.

METHODS

Study species

Delta smelt are pelagic planktivorous fish endemic to the San Francisco Bay-Delta, California, USA. They are threatened with extinction due to anthropogenic alterations to their ecosystem, including urbanization, non-native species, water diversions, contaminants and the conversion of complex tidal habitats to leveed channels

(Moyle 2008; Nichols et al. 1986). Historically, delta smelt were relatively abundant in the Delta, with populations declining dramatically in the 1980s (Newman 2008). They were listed as threatened by both federal and state governments in 1993, and sustained record-low abundance indices prompted their listing as endangered under the California Endangered Species Act in 2010 (CDFG 2010b; USFWS 1993). A major, and very politically contentious, contributor to their decline has been increased water exports from the Delta for urban and agricultural uses (Bennett 2005). Large water pumps at the southern end of the Delta export large volumes of freshwater to supply California's significant agriculture and urban water demands, resulting in altered hydrodynamics of the Delta that degrade delta smelt habitat quality, as well as cause direct mortality of delta smelt through entrainment at the pumps (Bennett 2005).

Because of these extreme anthropogenic alterations to the San Francisco Bay-Delta, the distribution of delta smelt has contracted significantly over the last several decades. Historically, delta smelt were distributed from San Pablo Bay upstream to Sacramento on the Sacramento River and Mossdale on the San Joaquin River, which varied seasonally and with freshwater outflow (Moyle 2002; Moyle et al. 1992; Radtke 1966). Today, large areas of historic delta smelt habitat and designated critical habitat have become unsuitable for some life history stages of the species, even though key environmental characteristics (e.g. temperature, salinity, water depth) of these areas have not changed (CDFG 2003; Miller et al. 2006). Delta smelt disappeared from the southern portion of their historic habitat in the late 1970s, which coincides with substantial increases in the amounts of water exported from the Delta. It is likely that water export

operations have a great effect on the distribution and abundance of delta smelt (Bennett 2005; Miller et al. 2006; Simi and Ruhl 2005).

Population sampling and genotyping

Delta smelt were collected by the California Department of Fish and Game during the 2003, 2005, 2007 and 2009 Spring Kodiak Trawl Surveys, which were conducted from January to May of each year at 39 geographic sampling stations in five regions of the Delta (2003: $n = 176$; 2005: $n = 316$; 2007: $n = 336$; 2009: $n = 365$; Fig. 1). We grouped sampling stations into five regions of the Delta by their proximity to one another to facilitate geographic genetic analyses. Fish muscle tissue was sampled from delta smelt heads preserved in 95% EtOH. Genomic DNA was extracted using the DNeasy Tissue Kit (QIAGEN) following the manufacturer's directions, with all samples yielding high-molecular weight DNA. We genotyped all samples for sixteen microsatellite loci using the procedures described in Fisch et al. (2009).

Genetic analyses

Genetic diversity was estimated as the number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) using Cervus 3.0 (Kalinowski et al. 2007). Estimations were conducted for all four sampling years independently, sampling sites within years, and sampling sites across years. Allelic richness (A_R) was calculated as a measure of the number of alleles adjusted for sample size using FSTAT 2.9.3 (Goudet 2001) to compare sample sets with different sample sizes. The Wilcoxon signed-rank test was used to determine statistical significance.

The presence of null alleles was determined using Micro-Checker (Van Oosterhout et al. 2004). Exact tests of Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted using GenePop 3.4 (Raymond and Rousset 1995) based on the Markov chain method using 1,000 dememorization steps, 100 batches and 1,000 iterations per batch. Pairwise comparisons of R_{ST} between sample sets were calculated and tested for statistical significance with 16,000 permutations in Arlequin 3.1 (Excoffier et al. 2005). Significance was determined after applying sequential Bonferroni correction (Rice 1989).

We implemented a Bayesian clustering method in Structure 2.3.3 (Pritchard et al. 2000) to estimate the number of genetic clusters (K) and the proportion of membership of those clusters. Assuming admixture and correlated allele frequencies, we performed 25 independent runs at each K value, assuming $K = 1$ to 10 with 1×10^6 Markov chain Monte Carlo (MCMC) repetitions and a burn-in period of 5×10^5 using no prior information. The steepest increase of the probability of K was measured by plotting the probability of the data [$P(D)$] and the *ad hoc* statistic ΔK to determine the most likely value for K (Evanno et al. 2005).

We conducted four sets of analyses in Structure with the aforementioned parameters. First, we pooled all sample sets over years and sampling sites to determine if temporal and geographic samples were in fact genetically distinct. We pooled samples within each sampling year to assess temporal genetic variation. In addition, we pooled samples across years for each geographic sampling site to assess geographic genetic variation. Finally, we analyzed each year by sampling site to determine within year geographic genetic variability.

We used a two-sample method based on temporally separated samples and a one-sample method, based on estimates of linkage disequilibrium, to obtain genetic estimates of the effective population size of the delta smelt population. We used sample sets from every other year, which represents two generations, as delta smelt are an annual fish. The temporal method ($N_e[TM]$) operates based on the logic that the difference in gene frequencies between two temporally collected samples from the same population are inversely proportional to the effective population size in the absence of migration and mutation (Scribner et al. 1997; Waples 1989). The linkage disequilibrium method ($N_e[LD]$) measures the associations between alleles across several loci allowing for the estimation of inbreeding, as a loss of variation is compounded by an increase in linkage disequilibrium, which reduces the frequency of novel gene combinations (Hill 1981; Peel et al. 2004). Both of these methods were implemented in N_e Estimator 1.3 (Peel et al. 2004).

We used an analysis developed by Cornuet & Luikart (1996) to test for recent population bottlenecks in each sampling year and site. This method tests whether there has been a recent reduction in allelic variation in a single population sample based on the loss of rare alleles. We used the program BOTTLENECK 1.2.02 (Piry et al. 1999) to implement this analysis with the following parameters: stepwise mutation model (SMM) and two-phase mutation model (TPM) tested over a range of 0-15% multi-step mutations, as these are the most appropriate for microsatellites (Di Rienzo et al. 1994; Garza and Williamson 2001). We used the Wilcoxon signed-rank test to determine the significance of heterozygosity excess. We calculated combined P-values using Fisher's method and

the Z-transform method to test the overall significance of bottlenecks across regions for each mutation model (Whitlock 2005).

RESULTS

A total of 411 alleles were detected in the 16 microsatellite loci analyzed, which ranged in number of alleles from 7 to 36 alleles per locus. The average expected heterozygosity for all loci was 0.82. For all years combined, we observed significant departures from HWE for *HtrG116* in 3 of the 5 regions; for *HtrG107*, *HtrG118*, and *HtrG126* in 2 of the 5 regions; and for *HtrG114*, *HtrG115*, *HtrG119*, and *HtrG129* in 1 of the 5 regions (Table 1). There was not a significant probability of null alleles at any of the loci according to Micro-Checker, as the frequency of null alleles at each locus was less than five percent. GenePop indicated no linkage disequilibrium between any of the loci over all sampling regions. The average allelic richness (A_R) for the four sampling years was 20.8 (SD 0.5), and did not significantly differ between years or regions ($P > 0.05$).

Genetic differentiation

Population divergence, measured as R_{ST} , revealed a weak geographic differentiation signal across sampling years and inconsistent temporal genetic differentiation. Significant levels of differentiation were observed between years after Bonferroni correction (2003 & 2007; 2005 & 2007) and between regions in years 2005 and 2009 (but only before Bonferroni correction). After Bonferroni correction, R_{ST} values were statistically significant when comparing geographic samples from Montezuma

Slough and Suisun Bay in 2005, and Sacramento Deep Water Ship Channel and the Lower Sacramento River in 2009. Among years, there was not a consistent pattern of significant R_{ST} values between regions after Bonferroni correction (Table 2).

We performed Structure analyses pooling all regions and years. This analysis revealed that 3 genetic demes were present among the 5 regions sampled over 4 years ($K = 3$; $L(K) = -82,000$; $\Delta K = 3.5$). All of the genetic clusters included individuals from all regions and years, indicating lack of consistent geographic or temporal structuring. Given these results, we performed Structure analyses for each year independently to determine the existence of independent genetic demes (K) within each year. The analysis revealed that one genetic deme was present in 2003 ($K = 1$; $L(K) = -13,100$; $\Delta K = 8$), 3 genetic demes were present in 2005 ($K = 3$; $L(K) = -22,750$; $\Delta K = 3.8$), one deme was present in 2007 ($K = 1$; $L(K) = -25,900$; $\Delta K = 2.7$), and 5 demes were present in 2009 ($K = 5$; $L(K) = -28,000$; $\Delta K = 6.5$). Similar to the results from the Structure analysis with all years and regions pooled, all of the genetic clusters included individuals from all regions within a year and the majority of individuals were of mixed ancestry, indicating a lack of consistent geographical structuring and high levels of admixture between regions.

Effective population size & bottleneck detection

The moments-based temporal method yielded an N_e of 1,430 (95% CI: 970 – 2328) when all of the samples were pooled over regions and years. The linkage disequilibrium N_e of each year independently was 7,744 (95% CI: 2,736-10,000) in 2003; an N_e of 2,408 (95% CI: 1,821 - 3524) in 2005; an N_e of 1,111 (95% CI: 969 - 1296) in 2007; and an N_e of 2,435 (95% CI: 1881 - 3428) in 2009 (Fig. 2). The linkage

disequilibrium N_e of Suisun Bay was 353 (95% CI: 271-503); Montezuma Slough was 236 (95% CI: 202-282); Lower Sacramento River was 1,663 (95% CI: 1113-3227); Cache Slough was 409 (95% CI: 309-599); and the Sacramento Deep Water Ship Channel was 806 (95% CI: 588– 1,267). The moments-based temporal method and linkage disequilibrium methods yielded similar results when the N_e of each of the regions were calculated independently.

Significant excess heterozygosity, indicating a recent bottleneck, was observed in Suisun Bay and the Lower Sacramento River in 2003 (P-values = 0.03 and 0.04, respectively), in Montezuma Slough and the Deep Water Ship Channel in 2005 (P-values = 0.02 and 0.03, respectively), in the Deep Water Ship Channel in 2007 (P-value = 0.02), and in no regions in 2009 (Table 3). Mean H_{eq} , calculated as the unweighted mean of locus-specific estimates of equilibrium heterozygosity, was 0.82 in 2003, 0.83 in 2005, 0.83 in 2007 and 0.84 in 2009 (Table 3). Using Fisher's method to calculate combined P-values to test the overall significance of bottlenecks across regions, we found significant excess heterozygosity in years 2003, 2005 and 2007 (2003: P = 0.006; 2005: P = 0.001; 2007: P = 0.002; 2009: P = 0.191), indicating a bottleneck in these years. Using the Z-transform method, we detected significant excess heterozygosity in all four sampling years, indicating an ongoing population bottleneck (2003: P = 0.002; 2005: P < 0.001; 2007: P < 0.001; 2009: P = 0.030) (Table 3).

DISCUSSION

Genetic differentiation

Our results demonstrate that genetic diversity has been maintained over the four sampling years and between sampling locations within years, as there was no significant difference in allelic richness between years or sampling locations within years. It is reasonable to see genetic diversity maintained over such a short time period, even in a population that has recently undergone a bottleneck, when population abundance has stabilized and the population remains outbred (Fig. 2).

Overall, the genetic data indicate a weak geographic signal among sampling regions, unsupported by temporal consistency in this signal, indicating the existence of a single, panmictic population. In all cases of geographic and temporal genetic differentiation, the R_{ST} values were very low ($R_{ST} < 0.05$ for all pairwise comparisons; Table 2). While these values are statistically significant given the large sample sizes, the magnitude of the difference is very small, suggesting a lack of biological relevance.

Bayesian assignment of individuals to genetic demes within years revealed a similar pattern to that of the population differentiation results, as multiple genetic demes were inferred in years 2005 and 2009, but only one deme was inferred in both 2003 and 2007. However, these genetic clusters include individuals from all regions within a year and do not correspond to geographically separated sampling regions within the Delta.

We conclude that the delta smelt population is panmictic, which is logical, as the San Francisco Bay-Delta is a highly connected ecosystem and it appears the majority of delta smelt spawning occurs in the same location (Bennett 2005; Moyle et al. 1992). The ephemeral nature of the population differentiation may be a result of sampling fish during

the spawning season, where they are actively migrating from brackish to fresh water to spawn (Moyle et al. 1992). It may also indicate the existence of different migration patterns of subsets within the population, such as resident fish in the Sacramento Deep Water Ship Channel, natal fidelity or high variance in reproductive success. This existence of a single, panmictic population is also supported by a previous population genetics study of delta smelt using allozyme markers (Trenham et al. 1998). However, samples from the San Joaquin River region were not included in this study, as delta smelt were not present at these sampling stations during the survey period due to a reduction in their historic range. The weak geographic differentiation signal may be attributed to anthropogenic homogenization of the species due to this reduced historic range, as a large subset of the genetic diversity in the species may have been eliminated. Future studies examining historic delta smelt samples from the San Joaquin River region are needed to further clarify the historic population structure and patterns of genetic diversity in delta smelt.

Reduced effective population size

The effective population size decreased significantly from 2003 to 2007, indicating a decrease in genetic diversity between these years, even though this pattern was not similarly observed as a decline in allelic richness. The decrease in effective population size is closely linked to the decrease in the abundance index between 2003 and 2007, calculated based on the methods in Stevens & Miller (1983) by the California Department of Fish and Game during the Fall Midwater Trawl (CDFG 2010a) (Fig. 2). As a population declines, genetic variation is lost, which can be seen as a reduction in the

effective population size. The N_e decline detected without a similarly observed decrease in allelic richness may be due to the short sampling period, or as a result of these samples coming from an already declining population with potentially previously reduced allelic richness. Effective population size is an important tool for monitoring genetic variation in threatened populations. Thus, it will be imperative to monitor N_e as an indicator of the success of management strategies for delta smelt (Schwartz et al. 2007).

Detection of ongoing bottleneck

The presence of a genetic bottleneck was also detected in all sampling years, indicating that the delta smelt population is currently losing genetic diversity as it declines. This can also be observed as a decrease in census size in the Fall Midwater Trawl abundance index (Fig. 2). The Cornuet and Luikart method for detecting bottlenecks does not provide an estimate of the timing of the decline (Cornuet and Luikart 1996). However, the genetic signal of the decline, corroborated by the observed census size declines, support the hypothesis that decreases in N_e have likely occurred over the last few decades. This method has been cited as being the most effective at detecting recent changes in N_e (Garza and Williamson 2001; Williamson-Natesan 2005).

Statistical tests for bottlenecks assume random mating and no gene flow, and as a result, nonrandom mating or population substructure can produce genealogies that resemble bottlenecks, whereas gene flow may resemble recent expansions (Busch et al. 2007; Cornuet and Luikart 1996; Goossens et al. 2006). Consequently, the bottlenecks detected for delta smelt may be artifacts of nonrandom mating or gene flow, as there was evidence of statistically significant but low magnitude R_{ST} values between regions. As

the R_{ST} values were relatively low, gene flow among sampling regions is likely. Gene flow can mimic recent expansion in a population. Since a consistent bottleneck signature was found in spite of the presence of gene flow, we are provided with even stronger evidence for recent reductions in N_e . This may result in the existence of bottlenecks that are more severe than they appear in the analyses (see Funk et al. (2010)). As a result of the observed bottlenecks, delta smelt may become increasingly threatened by reductions in N_e , by experiencing inbreeding depression and the loss of adaptive genetic variation. This may increase the rate of decline through a process known as an extinction vortex (Soule and Mills 1998).

Defining conservation units

These results can be used to define conservation units in two possible prioritization strategies: 1. managing the species as a single panmictic population throughout its range, or 2. managing populations in different parts of the Delta as multiple distinct conservation units. Each prioritization strategy is detailed as follows.

Managing delta smelt as a single panmictic population throughout its range will not alter the conservation management of this species, as this is how it is currently managed (Miller et al. 2006). Resources will continue to be allocated to protect the entire population, and no geographic localities will be favored. This strategy may result in local extinction of some distinct subpopulations, as limited resources or tradeoffs in management decisions may make management of the species throughout the Delta less effective (Taylor et al. 2000). However, if the effective population size is maintained,

this strategy will result in the maintenance of the overall genetic diversity, providing the species with the potential to adapt to future environmental challenges.

On the other hand, subpopulations of delta smelt could be managed independently, based on their utilization of different parts of the Delta. By managing these subpopulations independently, local extinction may be avoided, but it may require a significant increase in resources and might be unfeasible (Mace and Purvis 2008). To assess the need for managing the delta smelt population as multiple conservation units, we used the criteria recommended by Palsbøll et al. (2007) that based the delineation of management units on the amount of population differentiation at which populations become demographically independent instead of simply rejecting panmixia. This approach emphasizes the dispersal rate of individuals as the parameter of interest to conservationists instead of the historical amount of gene flow (Palsbøll et al. 2007).

Using these criteria to assess the management of geographically-defined subpopulations of delta smelt, we calculated the amount of genetic divergence among regions as a function of the number of migrants per generation estimated as mN_e , where m is the probability that an individual is a migrant and N_e is the effective population size, assuming selective neutrality and equilibrium conditions (Palsbøll et al. 2007). Based on these calculations, using an average N_e of 1,500 over all sampling years and a criterion of at least 10% exchange between sites, regions would be demographically isolated if they exchanged less than ~150 adults (Hastings 1993). This corresponds to an R_{ST} value of 0.016 under a Wright-Fisher island population model. From this, we could conclude that regions constitute separate management units if their genetic divergence exceeds $R_{ST} = 0.016$ (Palsbøll et al. 2007).

Only one of the statistically significant R_{ST} values between regions in 2005 was greater than 0.016 (Suisun Bay & Montezuma Slough $R_{ST} = 0.02$), therefore, according to the criteria above, they should be considered independent management units. However, the pattern of statistically significant genetic divergence among regions was not consistent between years; the genetic structure of the species is not temporally stable. Prioritizing conservation management based on these temporally unstable geographic delineations would result in drastic increases in conservation resources required to manage each management unit independently, potentially jeopardizing the preservation of the species. As a result, we recommend that delta smelt continue to be managed as a single, panmictic population in order to focus efforts on maintaining the effective population size as opposed to maintaining populations throughout the Delta. Future conservation plans for delta smelt should integrate data on the distribution of genetic diversity with historical and current ecological data. The survival of this species depends upon a balance between water management and anthropogenic water uses, which can only be reached through conservation management and habitat remediation.

CONCLUSION

The increasing need for conservation prioritization makes it essential to evaluate strategies for defining management units of endangered species. Many different strategies have been proposed; however, their practical application is often nebulous. The results of this study demonstrate the utility of applying a straightforward strategy for defining conservation units that is based on traditional population genetic methods, but uses more stringent criteria for designating conservation units. Our results indicate that

simply rejecting panmixia may overestimate the number of management units, leading to improper allocation of conservation resources. Using population genetics to assess patterns of intraspecific genetic diversity in order to define management units may provide a biologically relevant and tangible definition of conservation units that is particularly relevant to conservation managers. Preserving intraspecific genetic diversity is vital to the overall goal of species conservation, as it provides a good indicator of success of protecting the ecological and evolutionary processes necessary for species persistence. Conservation managers and researchers may use the application of these methods to define conservation units of a variety of endangered species, in an effort to efficiently allocate conservation resources.

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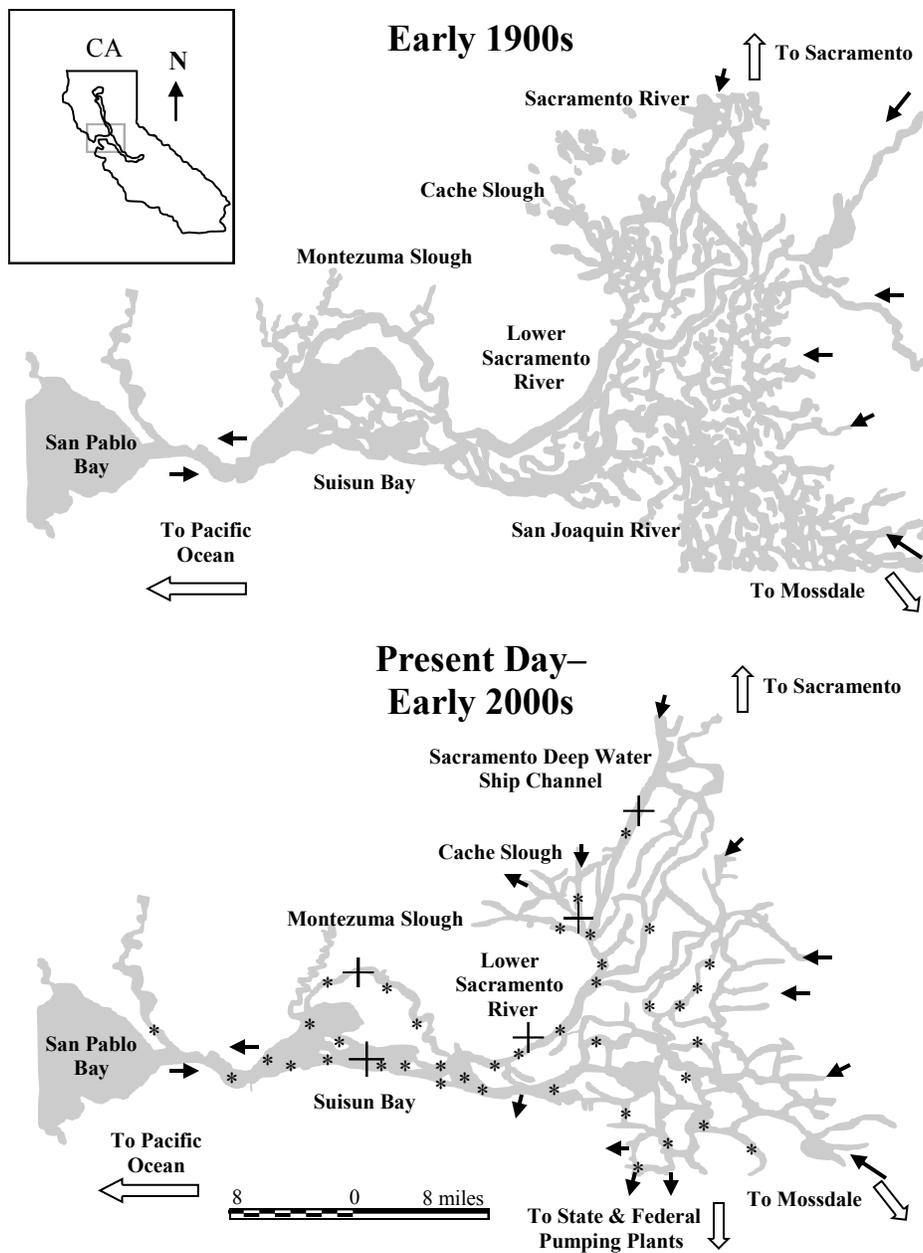
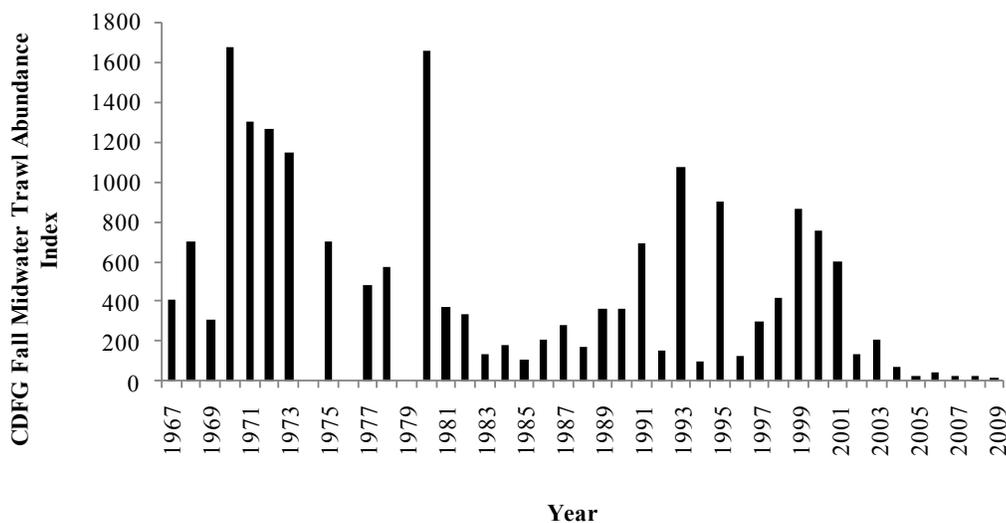
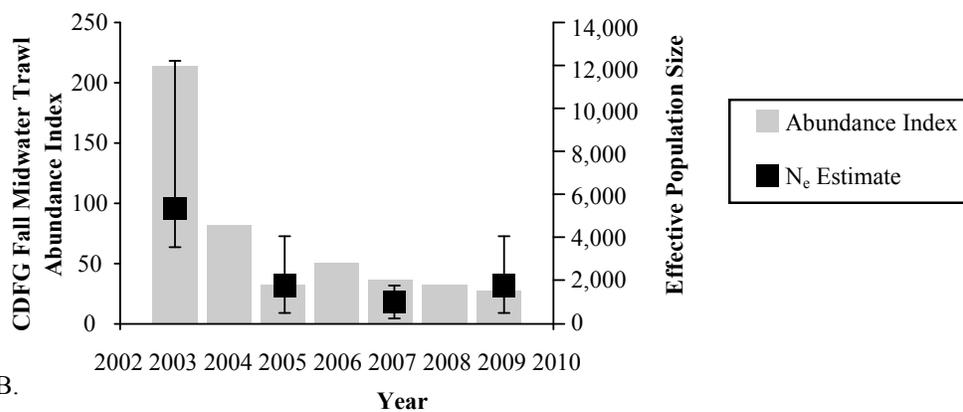


Figure 4.1 Map of the San Francisco Bay-Delta, CA. California Department of Fish and Game Spring Kodiak Trawl Survey sampling locations indicated by *, sampling regions indicated by + and hydrodynamic flows indicated by black arrows.



A.



B.

Figure 4.2 Delta smelt abundance index and effective population size. A. California Department of Fish and Game Midwater Trawl Abundance Index from 1967-2009 (CDFG 2010a). B. Estimates of delta smelt effective population size in years 2003, 2005, 2007 and 2009.

Table 4.1 Sample size, number of alleles, allelic richness, levels of heterozygosity and genetic diversity of all delta smelt sampled in five regions throughout their range in the San Francisco Bay-Delta over four sampling years*

Region	2003						2005						2007						2009					
	N	A	A _R	H _O	H _E	HW	N	A	A _R	H _O	H _E	HW	N	A	A _R	H _O	H _E	HW	N	A	A _R	H _O	H _E	HW
Suisun Bay	11	9	12	0.80	0.79	0	31	14	12	0.78	0.80	1	---	---	---	---	---	---	15	6	12	0.7	0.8	0
Montezuma Slough	15	10	12	0.82	0.82	0	114	18	13	0.81	0.83	1	91	19	13	0.79	0.78	1	91	19	13	0.8	0.8	1
Lower Sacramento River	93	18	12	0.80	0.81	1	42	16	12	0.80	0.82	1	42	16	13	0.77	0.81	0	151	21	13	0.8	0.8	2
Cache Slough Complex	57	16	12	0.80	0.81	1	87	16	13	0.79	0.82	0	60	17	13	0.81	0.81	1	---	---	---	---	---	---
Deep Water Ship Channel	---	---	---	---	---	---	42	15	13	0.77	0.83	4	143	20	13	0.80	0.83	3	108	18	12	0.8	0.8	0
All populations pooled	20	20	20	0.80	0.81	2	22	20	20	0.79	0.82	7	22	21	21	0.79	0.81	5	23	21	21	0.80	0.81	3
Total	176	317				2	316	351				7	336	359				5	365	361				3

*Key: N, number of individuals; A, number of alleles; A_R, allelic richness^a; H_O, observed heterozygosity; H_E, expected heterozygosity; HW, number of loci with significant Hardy-Weinberg disequilibrium^b.

^a Allelic richness (A_R) based on a minimum sample size of 156 diploid individuals for the pooled value compared between years and on 20 diploid individuals when compared within and between years for each region.

^b Statistically significant at $P < 0.05$ after Bonferroni correction

Table 4.2 Pairwise population R_{ST} values for sampled delta smelt in each study region in the San Francisco Bay-Delta collected in four sampling years (lower diagonal) and p-values (upper diagonal)

Region & Year	2003				2005				2007				2009			
	SB	MS	LS	CS	SB	MS	LS	CS	DW	MS	LS	CS	DW	MS	LS	DW
2003																
SB		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
MS	0.002		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
LS	0.002	0.000		NS	0.002**	NS	NS	0.02*	NS	NS	NS	NS	NS	NS	NS	0.04*
CS	0.001	-0.003	-0.002		NS	0.01**	NS	0.04*	NS	NS	NS	NS	NS	NS	0.013*	NS
2005																
SB	-0.001	-0.001	-0.001	-0.003												
MS	0.001	0.001	0.010	0.010	0.008**	NS	NS	0.04*	NS	NS	NS	NS	NS	NS	NS	NS
LS	-0.006	-0.006	0.005	0.000	0.020		NS	0.04*	NS	NS	NS	NS	NS	0.011*	0.001**	0.001**
CS	-0.008	-0.007	-0.006	-0.005	-0.001	0.006		NS	NS	NS	NS	NS	NS	NS	NS	NS
DW	-0.005	-0.005	0.010	0.010	-0.002	0.002	-0.001		0.05*	NS	NS	NS	NS	NS	NS	NS
2007																
MS	-0.009	-0.009	-0.007	-0.008	-0.010	0.002	-0.007	0.005	0.030					NS	NS	NS
LS	0.005	0.006	-0.003	-0.001	0.000	0.007	-0.003	-0.002	0.014	-0.006				NS	NS	NS
CS	-0.003	-0.003	-0.001	0.000	-0.004	0.007	-0.001	-0.003	0.010	-0.007	-0.01			NS	NS	NS
DW	-0.005	-0.004	0.000	-0.004	-0.008	0.003	-0.003	0.005	0.020	-0.001	-0	-0		NS	NS	NS
2009																
MS	0.000	0.000	-0.008	-0.001	0.001	0.010	0.003	-0.011	0.020	-0.014	-0.002	-0.018	-0.013		NS	0.03*
LS	0.003	0.002	0.009	0.010	0.002	0.020	-0.001	0.002	0.000	-0.002	0.002	-0.004	-0.002	-0.005		0.001**
DW	0.006	0.005	0.010	0.005	0.006	0.010	0.004	-0.003	0.030	-0.013	-0.004	-0.006	-0.001	0.010	0.010	

Key: SB = Suisun Bay, MS = Montezuma Slough, LS = Lower Sacramento, CS = Cache Slough, DW = Deep Water Ship Channel

Zero values indicate R_{ST} value < 0.001

* Significant ($P < 0.05$) differentiation. ** Significant ($P < 0.01$) differentiation after Bonferroni corrections. NS = not significant.

Table 4.3 Results and P-values from bottleneck tests implemented in the program Bottleneck for each region and over regions combined within years

Region & Year	# Loci ^a	H _{eq} ^b	P-value ^c	Fisher's Method ^d	Z-Transform Method ^e
2003		0.82		0.006*	0.002*
<i>Suisun Bay</i>	13		0.03*		
<i>Lower Sacramento River</i>	11		0.04*		
<i>Cache Slough</i>	11		0.09		
2005		0.83		0.001*	< 0.001*
<i>Suisun Bay</i>	9		0.30		
<i>Montezuma Slough</i>	12		0.02*		
<i>Lower Sacramento River</i>	12		0.10		
<i>Cache Slough</i>	13		0.06		
<i>Deep Water Ship Channel</i>	11		0.03*		
2007		0.83		0.002*	< 0.001*
<i>Montezuma Slough</i>	10		0.30		
<i>Lower Sacramento River</i>	10		0.16		
<i>Cache Slough</i>	10		0.19		
<i>Deep Water Ship Channel</i>	11		0.02*		
2009		0.84		0.191	0.030*
<i>Montezuma Slough</i>	9		0.35		
<i>Lower Sacramento River</i>	10		0.23		
<i>Deep Water Ship Channel</i>	11		0.16		

*Statistically significant P-values at $P < 0.05$.

^aNumber of microsatellite loci with with significant excess observed heterozygosity for regions within years.

^bThe unweighted mean of locus-specific estimates of equilibrium heterozygosity in each year.

^cP-values of bottleneck tests using the Wilcoxon signed-rank test for each region.

^dCombined P-values to test the overall significance of bottlenecks across regions using Fisher's method.

^eCombined P-values of bottlenecks across regions using the Z-transform method.

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