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AN ABSTRACT OF THE DISSERTATION OF

David A. Stick for the degree of Doctor of Philosophy in Fisheries Science
presented on December 6, 2011.

Title: Identification of Optimal Broodstock for Pacific Northwest Oysters.

Abstract approved:

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Christopher J. Langdon Mark D. Camara

The United States Pacific Northwest is well known for its shellfish farming. Historically, commercial harvests were dominated by the native Olympia oyster, *Ostrea lurida*, but over-exploitation, habitat degradation, and competition and predation by non-native species has drastically depleted their densities and extirpated many local populations. As a result, shellfish aquaculture production has shifted to the introduced Pacific oyster, *Crassostrea gigas*. An underlying objective of this dissertation is the use of molecular genetics to improve our ability to accurately identifying optimal oyster broodstock for either restoration of Olympia oysters or farming of Pacific oysters.

The ecological benefits provided by oysters as well as the Olympia oyster's historical significance, has motivated numerous restoration/supplementation efforts but these efforts are proceeding without a clear understanding of the genetic structure among extant populations, which could be substantial as a consequence of limited dispersal, local adaptation and/or anthropogenic impacts. To facilitate this understanding, we isolated and characterized 19 polymorphic microsatellites and used 8 of these to study the genetic structure of 2,712 individuals collected from 25 remnant Olympia oyster populations between the northern tip of Vancouver Island BC and Elkhorn Slough CA. Gene flow among geographically separated extant Olympia oyster populations is surprisingly limited for a marine invertebrate species whose free-swimming larvae are capable of planktonic dispersal as long as favorable water conditions exist. We found a significant correlation between geographic and genetic distances supporting the premise that coastal populations are isolated by distance. Genetic structure among remnant populations was not limited to broad geographic regions but was also present at sub-regional scales in both Puget Sound WA and San Francisco Bay CA. Until it can be determined whether genetically differentiated *O. lurida* populations are locally adapted, restoration projects and resource managers should be cautious of random mixing or transplantation of stocks where gene flow is restricted.

As we transition from our Olympia oyster population analysis to our Pacific oyster quantitative analysis, we recognize that traditional quantitative trait locus (QTL) mapping strategies use crosses among inbred lines to create segregating populations. Unfortunately, even low levels of inbreeding in the Pacific oyster (*Crassostrea gigas*) can substantially depress economically important quantitative traits such as yield and survival, potentially complicating subsequent QTL analyses. To circumvent this problem, we constructed an integrated linkage map for Pacific oysters, consisting of 65 microsatellite (18 of which were previously unmapped) and 212 AFLP markers using a full-sib cross between phenotypically differentiated outbred families. We identified 10 linkage groups (LG1-LG10) spanning 710.48 cM, with an average genomic coverage of 91.39% and an average distance between markers of 2.62 cM. Average marker saturation was 27.7 per linkage group, ranging between 19 (LG9) and 36 markers (LG3).

Using this map we identified 12 quantitative trait loci (QTLs) and 5 potential QTLs in the F1 outcross population of 236 full-sib Pacific oysters for four growth-related morphometric measures, including individual wet live weight, shell length, shell width and shell depth measured at four post-fertilization time points: plant-out (average age of 140 days), first year interim (average age of 358 days), second year interim (average age of 644 days) and harvest (average age of 950 days). Mapped QTLs and potential QTLs accounted for an average of 11.2% of the total phenotypic variation and ranged between 2.1 and 33.1%.

Although QTL or potential QTL were mapped to all Pacific oyster linkage groups with the exception of LG2, LG8 and LG9, three groups (LG4, LG10 and LG5) were associated with three or more QTL or potential QTL. We conclude that alleles accounting for a significant proportion of the total phenotypic variation for morphometric measures that influence harvest yield remain segregating within the broodstock of West Coast Pacific oyster selective breeding programs.

# Identification of Optimal Broodstock for Pacific Northwest Oysters

by
David A. Stick

A DISSERTATION

submitted to

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in partial fulfillment of
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degree of

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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David A. Stick, Author

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understanding of the shellfish industry and providing all background knowledge concerning oyster husbandry. Staff from both MBP and the USDA-ARS Shellfish Genetics Lab and fellow graduate students associated with each, graciously donated labor at key periods in the research, when the effort of one person alone would not have been enough to complete the task. I would also thank Liu Xin, owner of the Oregon Oyster Farms, Newport OR and Joth Davis, Breeding and Research Manager at Taylor Shellfish United, Shelton WA for their assistance and use of facilities and growout sites.

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Finally, I must thank my wife and family for all the love and support they have provided through these difficult years. I promise the best is yet to come.

# CONTRIBUTION OF AUTHORS

Michael A. Banks provided workspace, financial support and samples allowing for the completion of Chapter 2, as well as the initial awareness of the issues concerning Olympia oyster restoration.

Chris J. Langdon provided financial support allowing for the completion of Chapters 2 and 3.

Mark D. Camara provided workspace, financial support and contributed in the experimental design and data analysis of Chapters 2-5.

All authors contributed in the editing process.

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DEDICATION

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# Chapter 1
# Identification of Optimal Broodstock for Pacific Northwest Oysters: General Introduction and Goals

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When laymen hear the word “genetics” they commonly envision the realm of the molecular laboratory, a room filled with technicians wearing lab coats, gloves and goggles, working with test tubes, pipettes and wondrous machines whose purpose is to identify differences in the code of life, DNA. However, genetics is not limited to that which exists at the molecular level known as molecular genetics. Genetics is actually a broad characterization that describes many disciplines, each a science among themselves, yet each with their basis firmly linked to the inheritance characters first published by Gregor Mendel in 1866.

The field of Mendelian genetics and its underlying theory based on the study of simple, qualitative characteristics controlled by one or very few genes largely unaffected by the environment, has developed into the fundamental research commonly associated with the Human Genome Project where researchers attempt to identify and ultimately treat diseases and abnormalities caused by point mutations in the DNA code. Population genetics expands Mendelian theory to the scale of the entire population or at least among breeding demes, culminating with the concept of natural selection favoring alleles that ultimately improve the population’s overall fitness. Restoration genetics, conservation genetics and even evolution are all sub-fields within the domain of population genetics. Quantitative genetics also expands Mendelian theory but does so to

include continuous traits controlled by the combined effect many if not hundreds of genes, which are also influenced by the environment. It is with quantitative genetics that the foundation for all selective breeding programs, both plants and animals, originated.

However, it is with our use of tools originally associated with molecular genetics that has blurred the boundaries of these once independent genetic fields, allowing researchers the opportunity to explore their discipline deeper than ever imagined and answering questions that were impossible to address even as recently as five years ago. It is in this underlying context, the use of molecular tools to answer specific questions associated with Pacific Northwest oysters, which would otherwise be unanswerable using standard population or quantitative theory alone, that this dissertation is based.

The Olympia oyster, *Ostrea lurida*, is the only oyster species native to the Pacific Northwest. Over-exploitation, habitat degradation, and competition and predation from non-native species have drastically depleted densities or extirpated many Olympia oyster populations. However, the ecological benefits provided by oyster beds and the species' historical significance has motivated numerous restoration efforts. Unfortunately, these efforts have been proceeding without a clear understanding of existing genetic structure, which may be substantial as a consequence of limited dispersal, local adaptation and/or

anthropogenic impacts. Research summarized in Chapter 2 identifies and characterizes microsatellite markers specific to the Olympia oyster. We then used a subset of those microsatellites to document the genetic population structure among and within major geographical regions of this oyster's contemporary range (Chapter 3). We expect our research to influence how broodstock are selected by Olympia oyster restoration programs.

The Pacific oyster, *Crassostrea gigas,* is the most widely cultivated aquaculture species worldwide and it is this species that dominates the Pacific Northwest shellfish industry with an estimated annual total economic impact exceeding $200 million. The Molluscan Broodstock Program (MBP) is a Pacific Northwest oyster genetic improvement breeding program that has focused on improving the economic trait of harvest yield. The identification of quantitative trait loci (QTL) affecting Pacific oyster growth segregating within MBP broodstock may be of immediate use to the breeding program. Research summarized in Chapter 4 describes the creation of an integrated genetic linkage map, utilizing both microsatellite and AFLP markers, from an outcrossed mapping family. We then used that linkage map to identify and map QTLs specific for multiple growth-related morphometric measures at different oyster ages (Chapter 5). We expect our results to influence the direction of future genomic research projects designed to increase our quantitative understanding of Pacific oyster growth-related traits.

# Chapter 2
# Nineteen novel microsatellite markers for the Olympia oyster, *Ostrea lurida*

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**Abstract**

Understanding the genetic structure of remnant *Ostrea lurida* populations is critical for developing appropriate restoration efforts. Here we report 19 polymorphic microsatellites suitable for analyses of population differentiation, pedigree reconstruction and linkage map construction. We screened clones from four enriched genomic libraries, identified 73 microsatellite-containing sequences and designed PCR primers for 44 of these loci. We successfully optimized PCR conditions for 20 loci, including one monomorphic locus. In a Willapa Bay reference sample, mean observed ($H_O$) and expected heterozygosity ($H_E$) were 0.6729 and 0.8377. Nine loci deviated from Hardy-Weinberg equilibrium. These markers have proven useful for genetic studies of the Olympia oyster.

## Introduction

The Olympia oyster, *Ostrea lurida* Carpenter 1864, is the only oyster species native to the United States Pacific Northwest. Although the correct nomenclature of this species is controversial, recent genetic evidence supports Carpenter's original classification of northern populations as *Ostrea lurida* and populations from mainland Mexico as *Ostrea conchaphila* (Polson *et al.* 2009). Historically, this species ranged from southeastern Alaska southward through Mexico in densities capable of supporting both tribal subsistence fisheries and large commercial harvests. Over-exploitation, habitat degradation, competition and predation from non-native species have drastically depleted or extirpated many local populations. Ecological benefits provided by oyster reef habitats and the species' historical significance has fueled numerous restoration and supplementation efforts. Unfortunately, these efforts are proceeding without a clear understanding of existing genetic structure among populations, which could be substantial as a consequence of limited dispersal and/or anthropogenic impacts such as genetic bottlenecks or population admixture. Microsatellites developed for other oyster species, specifically *Crassostrea gigas* and *Ostrea edulis,* failed to amplify in *Ostrea conchaphila/lurida*. Here we report 19 novel microsatellite primer sets designed specifically for this species.

**Methods and Results**

We extracted high molecular weight DNA from the adductor muscle and mantle of a Willapa Bay, WA oyster using the DNAeasy Tissue Kit (Qiagen Inc., Valencia, CA) and further concentrated it as described in Sambrook *et al*. (1989). Genetic Identification Services, (Chatsworth, CA) constructed four genomic DNA libraries enriched for repeated CA, AAT, ATG, TAGA motifs based on methodology described in Jones *et al*. (2002) and sequenced randomly selected clones from all four libraries on an ABI Model 377 DNA sequencer using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ). PCR primers were designed for the regions flanking confirmed microsatellite sequences using DesignerPCR version 1.03 (Research Genetics Inc., USA). We optimized PCR conditions using DNA similarly extracted from four Yaquina Bay, Oregon oysters. Optimized primers were evaluated using a reference population consisting of 100 individuals from Willapa Bay, WA.

We performed 5 µL PCR reactions containing the following components: 1x GoTaq FlexiPCR buffer pH 8.5 (Promega, Madison, WI), 0.15 mM dNTPs (Promega, Madison, WI), 0.2 µM 5'-fluorescently labeled forward (ABI, Foster City, CA) and unlabeled reverse (Integrated DNA Technologies Inc., Coralville, IA) primers (Table 2.1), locus-specific [$MgCl_2$] (Table 2.2) and 0.025 U/ µL GoTaq FlexiDNA Polymerase (Promega, Madison, WI) on a MJ Research PTC225 Tetrad

thermocycler running the following program: 1) denaturing for 5 min at 94°C, 2) amplification using 40 cycles of 30 s at 94°C followed by 30 s at the locus-specific annealing temp (Table 2.2) and 45 s at 72°C, and 3) final extension for 30 min at 72°C. We resolved products using a 3730xl automated DNA sequencer with GeneScan 500 LIZ size standard (ABI, Foster City, CA) and scored them using GeneMapper version 3.7 (ABI, Foster City, CA). For Willapa Bay reference samples, we performed PCR reactions on a GeneAmp PCR 9700 thermocycler (ABI, Foster City, CA) using the same parameters. We also produced five full-sib Olympia oyster families at the Hatfield Marine Science Center and extracted DNA from each parental pair and 94 of their 14-day-old larvae. We verified Mendelian segregation using contingency table analysis against expected allele frequencies according to Mendelian expectations. We further tested observed heterozygosities at all markers for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using GenePop version 4.0.7 (Rousset 2008). We estimated null allele frequency using Micro-Checker version 2.2.3 (Van Oosterhout *et al.* 2004).

We found microsatellite-containing sequences in 73 of 100 clones screened. We designed primers for 44 sequences after excluding those that failed to meet default design parameters (Jones *et al.* 2002). Any marker that resulted in poor or no amplification, non-specific amplification, multiple alleles or excessive null alleles was excluded from further development. We successfully optimized

conditions for twenty primer pairs. The observed number of alleles per locus ranged from 1 to 43, with an average of 21 in the reference population (Table 2.2). The monoallelic *Olur06* has been found to be polymorphic in other populations (Stick, unpublished data). Mean observed ($H_O$) and expected heterozygosity ($H_E$) were 0.6729 (±0.2884) and 0.8377 (±0.1319) (Table 2.3). After sequential Bonferroni correction (Rice 1989), nine of the 19 tests for deviations from HWE (*Olur01, Olur02, Olur03, Olur05, Olur07, Olur08, Olur09, Olur14, Olur16*) (Table 2.3) and 5 out of 171 pairwise tests for LD (*Olur02-Olur03; Olur13-Olur15; Olur10-Olur17; Olur13-Olur19; Olur15-Olur19*) remained significant. Deviations from HWE are common in oyster species as a consequence of null alleles (Hedgecock *et al.* 2004). Null alleles are suspected to be segregating in all loci except *Olur06, Olur10, Olur11, Olur12, Olur13, Olur15, Olur17, Olur19 and Olur20* (Table 2.2).

We confirmed Mendelian segregation for nine loci by comparing observed larval genotypic frequencies to expectations based on parental genotypes (Table 2.2). We observed no cross-amplification in a panel of 6 individuals from each of the following species: *Ostrea edulis, Crassostrea gigas, C. virginica, C. ariakensis, C. sikamea and C. hongkongensis*. We are currently using these loci in studies of population differentiation. They should also be suitable for monitoring the effects of ongoing restoration efforts using parentage analysis and for linkage map construction.

**Table 2.1** Microsatellite primer characterization for Olympia oyster, *Ostrea lurida*. Sequenced DNA, primers and microsatellite motifs have been submitted to GenBank (Accession numbers EU587388-EU587407).

| Locus | Clone ID | GenBank Accession # | Primer Sequences | 5' Label | Repeat Motif of Clone |
|---|---|---|---|---|---|
| *Olur01* | A102 | EU587388 | F: 5'-AAGACACTGTATAGCGGTAAGA-3'<br>R: 5'-AAGACCCGTGATTCTCAC-3' | VIC | $(GT)_{14}$ |
| *Olur02* | A103 | EU587389 | F: 5'-TGCGACAAATGCACGTAC-3'<br>R: 5'-GTCGGAAGACAGAGGCTACA-3' | FAM | $(GA)_{9}$ |
| *Olur03* | A103a | EU587390 | F: 5'-TCTTCCGACAACGTACTATTTC-3'<br>R: 5'-CCGGTTTTTAAGGGTCATATA-3' | VIC | $(CA)_{12}$ |
| *Olur04* | A115 | EU587391 | F: 5'-GTTGGGAATGAGTTTCAAGGT-3'<br>R: 5'-TGCCTAGATTACCTCAAAATCC-3' | PET | $(GT)_{22}$ |
| *Olur05* | C2 | EU587392 | F: 5'-CAGCAGATGACAAGATAAGCTC-3'<br>R: 5'-CAGGTGTCGTCACTATTGTTG-3' | VIC | $(CAT)_{15}$ |
| *Olur06* | C4 | EU587393 | F: 5'-CCATCCTGTGTTTCAAATTCC-3'<br>R: 5'-CAAGGCTTATCTTTCCTGGTG-3' | NED | $(CAT)_{2}CGT(CAT)_{5}$ |
| *Olur07* | C6 | EU587394 | F: 5'-ACATGCTAACAAGATTCAGATC-3'<br>R: 5'-ATCAGATGATGACGATGTATTG-3' | FAM | $(CAT)_{18}$ |
| *Olur08* | C7 | EU587395 | F: 5'-CGAATCGAATCAGTTGAAATAC-3'<br>R: 5'-AAATGATGATGGACACTGGTAG-3' | VIC | $(ATG)_{10}$ |
| *Olur09* | C9 | EU587396 | F: 5'-ATCTCCAGTTAAATCCCCATAC-3'<br>R: 5'-CGTCCTCAGATGATGATTATTC-3' | NED | $(CAT)_{7}$ |
| *Olur10* | C105 | EU587397 | F: 5'-TGCTTCAGTCACTTATCAACAG-3'<br>R: 5'-AGGAGGAGTAGCATTCCTTG-3' | NED | $(CAT)_{11}$ |
| *Olur11* | C122 | EU587398 | F: 5'-CTCGCCATCACTTACACTTC-3'<br>R: 5'-TGGAGAGCAAAACGATTATG-3' | NED | $(CAT)_{14}$ |
| *Olur12* | C123 | EU587399 | F: 5'-CATGCGGACAAAACTTTG-3'<br>R: 5'-CAGAAGCTGGTCAACTGATC-3' | FAM | $(CAT)_{11}$ |
| *Olur13* | D3 | EU587400 | F: 5'-GTGAAACATTCTTTCCTGAGTG-3'<br>R: 5'-CGAGTTCGACATAATGAAGTTC-3' | PET | $(ATCT)_{17}$ |
| *Olur14* | D6 | EU587401 | F: 5'-TGACCAAAAACAGCTACTTCTG-3'<br>R: 5'-ACATGCCGTTACTCCTCTG-3' | VIC | $(GATA)_{18}AATA(GATA)_{4}$ |
| *Olur15* | D8 | EU587402 | F: 5'-CTTTCCATCGAGTTCGACATAA-3'<br>R: 5'-GGTGCGGACTGTGATGTAATAC-3' | PET | $(TAGA)_{12}$ |
| *Olur16* | D12 | EU587403 | F: 5'-AGCATCGAACAAGCACTAAA-3'<br>R: 5'-GGAATTGAAACTCTCAAAGTTG-3' | FAM | $(GATA)_{21}$ |
| *Olur17* | D101 | EU587404 | F: 5'-ATCGAAACTGAACGAGTGTTG-3'<br>R: 5'-TTGGTCACTGATTGCTGAAAC-3' | FAM | $(TCTA)_{24}TC(CATC)_{9}$ |
| *Olur18* | D104 | EU587405 | F: 5'-TGGTGTCCTTTATATCGAGTTC-3'<br>R: 5'-CGCTATTTGTGGGGAGAT-3' | PET | $(TATC)_{21}TGTC(TATC)_{3}$ |
| *Olur19* | D107 | EU587406 | F: 5'-CTTTCCATCGAGTTCGACATAA-3'<br>R: 5'-TTAGCGTGTAGTCAACGGTCTC-3' | PET | $(GATA)_{20}$ |
| *Olur20* | D127 | EU587407 | F: 5'-TCCTTATGTTGGTCACTGATTG-3'<br>R: 5'-ATCGAAACTGAACGAGTGTTG-3' | NED | $(TGGA)_{12}(TAGA)_{17}$ |

**Table 2.2** Microsatellite PCR characterization for Olympia oyster, *Ostrea lurida*. Locus names with * indicate Mendelian segregation tested and verified; θ indicate presence of null alleles as determined by Micro-Checker. Null allele frequencies estimated as the Brookfield 1 (B1) and Brookfield 2 (B2) estimators using Micro-Checker. These loci are highly polymorphic as indicated by the allele size range (bp) and number of alleles observed ($N_A$) from the number of Willapa Bay reference samples (n) successfully amplified.

| Locus | Annealing Temp. (°C) | [$MgCl_2$] (mM) | Allele Size Range (bp) | # Allelles Observed ($N_A$) | # Samples Amplified (n) | Frequency Null Alleles (B1 / B2) |
|---|---|---|---|---|---|---|
| *Olur01* θ | 50 | 2.0 | 220-240 | 6 | 97 | 0.1755 / 0.2416 |
| *Olur02* θ | 56 | 1.5 | 231-273 | 12 | 96 | 0.2005 / 0.2774 |
| *Olur03* θ | 50 | 2.0 | 212-261 | 16 | 85 | 0.3079 / 0.4887 |
| *Olur04* θ | 48 | 1.5 | 187-294 | 43 | 100 | 0.0201 / 0.0201 |
| *Olur05* * θ | 56 | 2.0 | 249-355 | 26 | 100 | 0.0671 / 0.0671 |
| *Olur06* * | 60 | 3.0 | 233 | 1 | 100 | 0.0 / 0.3317 |
| *Olur07* * θ | 56 | 1.5 | 168-258 | 21 | 99 | 0.1162 / 0.1438 |
| *Olur08* * θ | 54 | 3.0 | 199-338 | 24 | 88 | 0.0882 / 0.2820 |
| *Olur09* * θ | 54 | 1.5 | 195-213 | 7 | 99 | 0.1497 / 0.1713 |
| *Olur10* | 56 | 1.5 | 214-312 | 24 | 100 | 0.001 / 0.001 |
| *Olur11* | 56 | 2.5 | 137-180 | 10 | 100 | -0.0122 / 0.0 |
| *Olur12* | 56 | 2.0 | 180-275 | 16 | 100 | -0.0206 / 0.0 |
| *Olur13* * | 52 | 2.0 | 230-314 | 21 | 95 | 0.0051 / 0.0955 |
| *Olur14* * θ | 50 | 2.5 | 230-366 | 34 | 94 | 0.0491 / 0.1462 |
| *Olur15* * | 56 | 1.5 | 150-233 | 22 | 100 | -0.0169 / 0.0 |
| *Olur16* * θ | 50 | 1.5 | 236-451 | 32 | 97 | 0.1777 / 0.2264 |
| *Olur17* | 60 | 3.0 | 196-292 | 27 | 100 | -0.0098 / 0.0 |
| *Olur18* θ | 56 | 2.0 | 211-357 | 29 | 99 | 0.0275 / 0.0513 |
| *Olur19* | 54 | 2.5 | 202-285 | 21 | 100 | -0.0170 / 0.0 |
| *Olur20* | 56 | 2.0 | 204-301 | 28 | 96 | -0.0025 / 0.0752 |

**Table 2.3** Microsatellite characterization for deviation from Hardy-Weinberg equilibrium (HWE) in the Olympia oyster, *Ostrea lurida*. Significant departures of observed heterozygozity ($H_O$) from expectation assuming Hardy-Weinberg equilibrium ($H_E$) after sequential Bonferroni correction are in boldface type (least significant α after correction = 0.00417).

| Locus | Expected Heterozygosity ($H_E$) | Observed Heterozygosity ($H_O$) | P-value HWE test |
|---|---|---|---|
| *Olur01* | 0.5659 | **0.2887** | **<0.0001** |
| *Olur02* | 0.6189 | **0.2917** | **<0.0001** |
| *Olur03* | 0.8238 | **0.2588** | **<0.0001** |
| *Olur04* | 0.9643 | 0.9200 | 0.3404 |
| *Olur05* | 0.9019 | **0.7700** | **0.0041** |
| *Olur06* | 0.8900 | 0.0000 | n/a |
| *Olur07* | 0.6606 | **0.4444** | **<0.0001** |
| *Olur08* | 0.8243 | **0.6591** | **<0.0001** |
| *Olur09* | 0.7738 | **0.5051** | **<0.0001** |
| *Olur10* | 0.9164 | 0.9100 | 0.5450 |
| *Olur11* | 0.6036 | 0.6200 | 0.1059 |
| *Olur12* | 0.7281 | 0.7600 | 0.7793 |
| *Olur13* | 0.9306 | 0.9158 | 0.2792 |
| *Olur14* | 0.9630 | **0.8617** | **<0.0001** |
| *Olur15* | 0.9322 | 0.9600 | 0.5412 |
| *Olur16* | 0.9356 | **0.5876** | **<0.0001** |
| *Olur17* | 0.9258 | 0.9400 | 0.0211 |
| *Olur18* | 0.9367 | 0.8788 | 0.1204 |
| *Olur19* | 0.9320 | 0.9600 | 0.5064 |
| *Olur20* | 0.9271 | 0.9271 | 0.0172 |

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# Chapter 3
# Genetic structure among remnant populations of the native Olympia oyster (*Ostrea lurida* Carpenter 1864) at two spatial scales

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**Abstract**

The Olympia oyster, *Ostrea lurida* Carpenter 1864, is the only oyster species native to the United States Pacific Northwest. The ecological benefits provided by oyster beds as well as the species' historical significance has motivated numerous restoration/supplementation efforts. These efforts, however, are proceeding without a clear understanding of the genetic structure among extant populations, which could be substantial as a consequence of limited dispersal, local adaptation and/or anthropogenic impacts.

We used recently-developed microsatellite DNA markers to study the genetic structure of 25 remnant populations of Olympia oysters between British Columbia and central California. Using analysis of molecular variance (AMOVA), we found evidence for genetic differentiation at both the regional scale (i.e. between geographically separated estuarine systems) and local scale (i.e. within the large, complex estuaries). Partitioning the entire study area and genetic variation into eight broad geographic regions showed that approximately 72% of the molecular genetic variation is among them. Sub-partitioning Puget Sound into four geographical regions also showed that approximately 75% of the genetic variation in Puget Sound was distributed among regions. A sub-analysis of San Francisco Bay sites found genetic differentiation between populations but not between the north and south Bay. Clustering based on Nei's genetic distance

largely corresponded with geographical regions and using a Mantel test we found a significant positive Spearman's Rank correlation between geographic and genetic distances indicating a strong pattern of isolation by distance. Until it can be determined whether this surprisingly high level of molecular genetic structure among populations is adaptive, restoration projects and resource managers should be cautious about mixing and transplanting stocks that currently have restricted gene flow between them.

## Introduction

The Olympia oyster, *Ostrea lurida* Carpenter 1864, is the only oyster species native to the United States Pacific Northwest. Historically, this species existed in densities capable of supporting both tribal subsistence fisheries and large commercial harvests throughout its range. Over-exploitation, habitat degradation, as well as competition and predation from non-native species, however, have drastically depleted these densities and extirpated many local populations. The ecological benefits provided by oyster beds and the species' historical significance have motivated numerous restoration/supplementation efforts. These efforts, however, are proceeding without a clear understanding of existing genetic structure among extant populations, which could be substantial as a consequence of limited dispersal, local adaptation and/or anthropogenic impacts resulting in genetic bottlenecks or population admixture due to historical commercial practices.

Although the taxonomy of the Olympia oyster has been controversial (Baker 1995; Coan *et al.* 2000; Harry 1985), genetic evidence supports Carpenter's original classification with northern populations, including the Pacific coastline of Canada and United States designated as *Ostrea lurida* and southern populations associated with the Pacific coastline of mainland Mexico southward into Panama categorized as *Ostrea conchaphila* (Kirkendale *et al.* 2004; Lapègue *et al.* 2006; Ó

Foighil& Taylor 2000; Polson *et al.* 2009; Shilts *et al.* 2007). All of our samples are from populations well north of any potential hybridization zone (Hertlein 1959) and represent *O. lurida.*

Historically, *O. lurida* ranged from Sitka Harbor in southeastern Alaska southward through Cabo San Lucas, Baja California, Mexico (Dall 1914). However, Gillespie (2009) speculates that the contemporary range of Olympia oyster populations may be as much as 40% smaller than previously reported, starting south of Queen Charlotte Island, British Columbia, and continuing southward through Bahia San Quintin, Baja California, Mexico. Extant populations of *O. lurida* are not continuous throughout this range but are highly fragmented and largely restricted to moderately stable polyhaline bays, tidal channels, estuaries, river inlets and sloughs that experience periodic events of very low salinity (Baker 1995; Hopkins 1937; Quayle 1941).

Archaeological shell middens found near extant Olympia oyster populations highlight *O. lurida's* historical importance for tribal subsistence fisheries (Baker 1995; Baker *et al.* 1999; Barrett 1963; Elsasser& Heizer 1966; Groth& Rumrill 2009; Steele 1957). One of the largest of these, the Emeryville Shellmound near San Francisco CA, exceeded 350 ft in diameter and 45 ft in height and was composed entirely of Olympia oyster shells. The resurgence of Pacific Northwest

Native American ceremonial and cultural traditions has also bolstered interest in *O. lurida* restoration.

*O. lurida* is a small, slow-growing oyster with adults rarely exceeding 6 cm in length. Even under favorable growing conditions, achieving this size can take upwards of 3 years in the northern half of its range (Couch& Hassler 1989) and half that time in the southern part (Coe& Allen 1937). This diminutive size encouraged over-exploitation in many populations since approximately 2,400 adults are required to produce one gallon of shucked meat, the historical unit of commercial production (Brennan 1939; Wallace 1966). Peak landings in 1896 from Willapa Bay, WA alone have been estimated at 150,000 gallons (Galtsoff 1929) and comparable landings were reported throughout Puget Sound, WA (Steele 1964), Yaquina Bay, OR (Fasten 1931) and San Francisco Bay, CA (Packard 1918a, b). Commercial oyster beds in San Francisco Bay were routinely supplemented with oysters from Washington and Oregon (Barrett 1963; Conte 1996; MacKenzie 1996) as their own yields became less profitable. Over-exploited populations were rapidly reduced to numbers too small to recover from additional anthropogenic pressures such as siltation from logging and mining operations (Trimble *et al.* 2009), toxic effluent from paper and pulp mills (Hopkins 1931; Kirby 2004; McKernan *et al.* 1949), environmental degradation (Galtsoff 1929) and competition, predation and diseases caused by introduced species (Bower *et al.* 1997; Buhle& Ruesink 2009; Friedman *et al.* 2005; Hopkins

1937; Loosanoff 1955; Trimble *et al.* 2009; White *et al.* 2009). Today, the Olympia oyster is a candidate species of concern in Washington, Oregon and British Columbia. It is unknown whether population densities became so low that genetic bottlenecks and drift may have influenced the population structure.

The Olympia oyster can be found as high as several meters above mean low tide, especially as juveniles (Baker 1995), but is more abundant in the low intertidal (+0.5m) to shallow subtidal (-2.0m) (Hopkins 1937) and far greater depths having been documented (Baker 1995; Dimick *et al.* 1941; Fasten 1931; Hertlein 1959). Adults cannot tolerate freezing conditions (Davis 1955; Townsend 1896) and many intertidal commercial beds in the Pacific Northwest were surrounded by man-made dikes that retained 15-30cm of water to minimize air exposure and freezing (Hopkins 1936). *O. lurida* requires minimum water temperatures well above those normally observed in nearshore ocean waters to spawn: 12°C in the northern parts of its range (Hopkins 1936, 1937; Santos *et al.* 1992) and 16°C in the southern (Coe 1931). It is unknown whether *O. lurida* larvae can withstand long-term exposure to cold, high salinity nearshore waters typical of the Pacific Northwest (Hopkins 1937).

*O. lurida* is an alternating, protandric hermaphrodite and all degrees of hermaphroditism may be found within a population (Coe 1931; Coe 1932, 1934; Hopkins 1936). As with all *Ostrea* species, the Olympia oyster is a larviparous

brooder. Fertilization occurs within the “female” brachial cavity and the developing larvae are raised on the labial palps and gills for 10-18 days before being released into the water column as veliger larvae (Coe 1931; Hopkins 1936, 1937). Free-swimming veligers (175-185 μm in size) continue to develop in the plankton for a variable amount of time depending upon environmental conditions (Baker 1995; Breese 1953; Hopkins 1937; Imai *et al.* 1954). Once they reach the pediveliger stage (300μm in size) larvae are ready to set on any suitable substrate. Only anecdotal information is available on Olympia oyster larval dispersal patterns (Pineda 2000; Seale& Zacherl 2009) and how this may affect population structure is unknown.

Oysters provide several important ecological services that help stabilize estuarine ecosystems. As efficient particulate filter feeders, they reduce water turbidity by removing not only excess phytoplankton but also large quantities of suspended materials from the water column and deposit it on the bottom as pseudofeces (Shpigel& Blaylock 1991). The recent collapse of the Chesapeake Bay ecosystem has been attributed largely to the demise of the Eastern oyster, *Crassostrea virginica*, which resulted in dramatic increases in turbidity and phytoplankton concentration (Wetz *et al.* 2002) that destabilized benthic-pelagic coupling (Newell 2004), increased the frequency of harmful algal blooms and promoted eutrophication (Boesch *et al.* 2001; Cooper& Brush 1993) that culminated in catastrophic anoxic conditions (Kennedy 1996).

Bivalves also act as "bio-sinks," concentrating and retaining bio-toxins such as pesticides (Dumbauld *et al.* 2001), herbicides (Smith *et al.* 2003), PCBs (Chu *et al.* 2000), chemical wastes and by-products (Alzieu 1998; Nice *et al.* 2000), heavy metals (Aune *et al.* 1998; Boening 1999; Haynes& Toohey 1998; Kawaguchi *et al.* 1999; Meyer *et al.* 1998) and coliform bacteria from agricultural runoff (Scott *et al.* 1998). Researchers have long proposed utilizing this bioaccumulation ability, which is largely the result of an oyster's filtration capacity, to monitor overall ecosystem health over long periods of time (Boening 1999; Haynes& Toohey 1998; Meyer *et al.* 1998).

Finally, oysters are habitat builders that create favorable environmental conditions and enhance biodiversity and nekton density. Oysters promote native eelgrass (*Zostera marina*) growth in Pacific Northwest estuaries, an essential habitat for many juvenile fish species including endangered salmonids, not only by reducing water turbidity (Boening 1999; Hosack 2003; Tallis *et al.* 2004) but also through direct oyster and eelgrass bed interactions (Tallis *et al.* 2009). Oyster shells can accumulate to create structurally complex beds capable of providing refuge for numerous invertebrate and fish species (Breitburg *et al.* 2000; Burrell 1986; Coen& Luckenbach 2000; Dumbauld *et al.* 2000; Gregory& Volety 2005; Lenihan *et al.* 2001; Posey *et al.* 1998; Zimmerman *et al.* 1989). Wells (1961) reported over 300 different animal species in association with a *C.*

*virginica* oyster bed in North Carolina. These same beds stabilize shorelines by reducing wave-induced erosion, effectively maintaining habitat well above the tidal height associated with the bed (Meyer *et al.* 1997; Piazza *et al.* 2005).

Continued loss of native oysters from the Pacific Northwest will only intensify present environmental problems in most bays and estuaries (Nichols *et al.* 1986), potentially destabilizing entire ecosystems (Jackson *et al.* 2001; Raj 2008). Beck *et al*. (2009) recently rated the current condition and distribution of all *O. lurida* beds as either poor (90-99% lost) or functionally extinct (>99% lost). Because of the ecological benefits provided by oyster bed habitats and the species' historical significance, it has been widely suggested that restoring or supplementing extant Olympia oyster populations using hatchery produced seed may be a viable option for the improvement of Pacific Northwest bay and estuarine environments (Jackson *et al.* 2001; Ruesink *et al.* 2005). Numerous site-specific restoration/supplementation efforts are presently either under consideration or have been initiated in Washington, Oregon and California. However, these efforts are proceeding without a clear understanding of existing genetic structure among extant populations. Population structure, however, may be substantial as a consequence of both the physiology and history of *O. lurida* (Camara& Vadopalas 2009).

We have recently developed a number of microsatellite DNA markers in *O.lurida* (Stick et al. 2009) and have used these markers to document genetic structure among and within major geographical regions of this oyster's contemporary range. In this paper, we provide evidence of surprisingly high levels of genetic structure in the Olympia oyster. This information will benefit decision-makers overseeing restoration efforts.

## Materials and Methods

### *Sample collection*

We sampled 2,712 individuals from 25 extant Olympia oyster populations between the northern tip of Vancouver Island BC and Elkhorn Slough CA. Some populations were sampled more than once but in all cases any repeated surveys represented either different year classes or locations separated by 10 km or more. A total of 40 different sample groups were collected. Sampled oysters ranged from first-year set to adults and the tissue type collected varied from whole bodies for small juveniles to small pieces of gill, mantle, or labial palp for adults. All samples were preserved in 95% ethanol with the following exceptions: Samples provided by University of California Davis (UCD) were freeze dried at -80°C and samples provided by Coastal Oregon Marine Experiment Station (COMES) were stored in tissue storage buffer (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA pH 8.0). Some COMES samples were too degraded to be usable. Details of sampling localities and sample sizes are provided in Table 3.1 and Figures 3.1 and 3.2.

**Table 3.1** Sample group identification code, general population location and collection details of surveyed Olympia oyster populations. We sampled 2,712 individuals surveying 40 different locations comprising 25 extant Olympia oyster (*O. lurida*) populations. Some geographical areas were sampled multiple times. Approximate latitude and longitude are given in decimal degrees; (*N*) number of samples surveyed from site collection event.

| Sample Group | General Population Location | Latitude | Longitude | *N* | Collection Date | Tissue Type | Preservation | Collaborators |
|---|---|---|---|---|---|---|---|---|
| Oc01 | Klaskino Inlet, Vancouver Island, BC (near Port Alice) | 50.298667 | -127.723633 | 53 | 7/7/2002 | body | 95% EtOH | UV |
| Oc02 | Amai Inlet, Vancouver Island, BC | 50.022667 | -127.101667 | 50 | 7/10/2002 | body | 95% EtOH | UV |
| Oc03 | Port Eliza, Vancouver Island, BC | 49.932333 | -127.045500 | 50 | 7/11/2002 | body | 95% EtOH | UV |
| Oc04 | Toquart Bay, Vancouver Island, BC (Lucky Creek, Barkley Sound) | 49.026500 | -125.306167 | 50 | 7/12/2002 | body | 95% EtOH | UV |
| Oc05 | Shoal Bay, Lopez Island, WA (North Puget Sound) | 48.553791 | -122.873174 | 20 | 6/4/2002 | body | 95% EtOH | PSRF |
| Oc06 | Shoal Bay, Lopez Island, WA (North Puget Sound) | 48.553493 | -122.876672 | 52 | 4/10/2004 | gill, mantle | 95% EtOH | WDFW |
| Oc10 | Discovery Bay, WA (Striaght of Juan de Fuca) | 48.013263 | -122.834331 | 100 | 7/20/2005 | body | 95% EtOH | WDFW |
| Oc12 | Clam Bay, Rich Passage, WA (Puget Sound Central Basin) | 47.564532 | -122.548627 | 100 | 5/26/2004 | gill, mantle | 95% EtOH | WDFW |
| Oc13 | Clam Bay, Rich Passage, WA (Puget Sound Central Basin) | 47.572561 | -122.549427 | 76 | 8/6/2005 | gill, mantle | 95% EtOH | WDFW |
| Oc14 | Triton Cove, WA (Hood Canal) | 47.605134 | -122.986777 | 100 | 9/24/2004 | gill, mantle | 95% EtOH | WDFW |
| Oc15 | Triton Cove, WA (Hood Canal) | 47.605698 | -122.983523 | 50 | 4/18/2002 | body | 95% EtOH | PSRF |
| Oc16 | Seal Rock, WA (Hood Canal) | 47.714151 | -122.885001 | 71 | 8/16/2005 | gill, mantle | 95% EtOH | WDFW |
| Oc17 | North Bay, Case Inlet, WA (South Puget Sound) | 47.367625 | -122.784096 | 100 | 4/10/2004 | gill, mantle | 95% EtOH | WDFW |
| Oc18 | Oakland Bay, Hammersley Inlet, WA (South Puget Sound) | 47.227291 | -123.040588 | 100 | 5/8/2004 | gill, mantle | 95% EtOH | WDFW |
| Oc19 | Oyster Bay, Totten Inlet, WA (South Puget Sound) | 47.147496 | -122.967953 | 50 | 6/21/2002 | body | 95% EtOH | PSRF |
| Oc20 | Willapa Bay, Nahcotta, WA | 46.492702 | -124.020645 | 50 | 2/27/2002 | body | 95% EtOH | PSRF |
| Oc21 | Willapa Bay, Nahcotta, WA | 46.491556 | -124.027173 | 100 | 4/23/2004 | gill, mantle | 95% EtOH | WDFW |
| Oc24 | Yaquina Bay, Oregon Oyster, OR | 44.579539 | -123.995746 | 192 | 9/16/2003 | body | 95% EtOH | OSU |
| Oc25 | Yaquina Bay, Oregon Oyster, OR | 44.579454 | -123.994758 | 182 | 6/20/2004 | body | 95% EtOH | OSU |
| Oc26 | Coos Bay, North Bend, OR | 43.406398 | -124.220809 | 54 | 11/15/2005 | mantle | 95% EtOH | SSNERR |
| Oc27 | Coos Bay, Citrus Dock, OR | 43.381471 | -124.219257 | 59 | 11/15/2005 | body | 95% EtOH | SSNERR |
| Oc28 | Coos Bay, Blossom Creek Boardwalk, OR | 43.365581 | -124.212094 | 68 | 11/15/2005 | body | 95% EtOH | SSNERR |
| Oc29 | Coos Bay, Shinglehouse Slough, OR | 43.325775 | -124.206159 | 60 | 11/15/2005 | mantle | 95% EtOH | SSNERR |
| Oc30 | Coos Bay, North Bend, OR | 43.408087 | -124.220765 | 30 | 1/20/2002 | body | 95% EtOH | SSNERR |

BC, British Columbia; WA, Washington; OR, Oregon; CA, California.

UV, University of Vancouver BC; PSRF, Puget Sound Restoration Fund; WDFW, Washington Department of Fish and Wildlife; OSU, Oregon State University; SSNERR, South Slough National Estuarine Research Reserve; COMES, Coastal Oregon Marine Experimental Station; MU, Malaspina University BC; MACTEC Engineering and Consulting CA; UCD, University of California Davis.

**Table 3.1** Continued.

| Sample Group | General Population Location | Latitude | Longitude | *N* | Collection Date | Tissue Type | Preservation | Collaborators |
|---|---|---|---|---|---|---|---|---|
| Oc31 | Tomales Bay, CA (East Bay) | 38.115873 | -122.855906 | 30 | 4/5/2001 | body | tissue buffer | COMES |
| Oc32 | Tomales Bay, CA (West Bay) | 38.117549 | -122.874497 | 30 | 4/6/2001 | body | tissue buffer | COMES |
| Oc34 | San Francisco Bay, Redwood Port, CA | 37.501449 | -122.222504 | 20 | 2/2/2001 | body | tissue buffer | COMES |
| Oc36 | Toquaht River, Vancouver Island, BC | 49.036110 | -125.349140 | 100 | 2/9/2006 | gill, mantle | 95% EtOH | MU |
| Oc37 | San Francisco Bay, Sailing Lake, CA | 37.451609 | -122.035358 | 109 | 10/6/2007 | mantle | 95% EtOH | MACTEC |
| Oc38 | Henderson Inlet, WA (South Puget Sound) | 47.116058 | -122.832702 | 68 | 10/22/2007 | mantle | 95% EtOH | PSRF |
| Oc39 | Eld Inlet, WA (South Puget Sound) | 47.063365 | -123.009806 | 57 | 10/22/2007 | mantle | 95% EtOH | PSRF |
| Oc40 | Liberty Bay, WA (Puget Sound Central Basin) | 47.720616 | -122.655823 | 39 | 10/26/2007 | mantle | 95% EtOH | PSRF |
| Oc41 | Liberty Bay, WA (Puget Sound Central Basin) | 47.720717 | -122.654472 | 60 | 10/26/2007 | mantle | 95% EtOH | PSRF |
| Oc42 | Humbolt Bay, Mad River Estuary, CA | 40.703882 | -124.217313 | 58 | 2/16/2004 | labial palp | dried | UCD |
| Oc43 | Tomales Bay, CA (South Bay) | 38.112667 | -122.852954 | 53 | 4/20/2004 | labial palp | dried | UCD |
| Oc44 | Tomales Bay, CA (North Bay) | 38.207370 | -122.940185 | 54 | 8/31/2004 | labial palp | dried | UCD |
| Oc45 | Drakes Bay, CA | 38.048352 | -122.952593 | 60 | 7/8/2004 | labial palp | dried | UCD |
| Oc46 | Elkhorn Slough, CA | 36.815792 | -121.767181 | 53 | 5/26/2004 | labial palp | dried | UCD |
| Oc47 | San Francisco Bay, Point Orient, CA | 37.955067 | -122.421800 | 53 | 8/23/2006 | labial palp | dried | UCD |
| Oc49 | San Francisico Bay, Candlestick Park, CA | 37.708665 | -122.377607 | 51 | 1/21/2005 | labial palp | dried | UCD |

BC, British Columbia; WA, Washington; OR, Oregon; CA, California.

UV, University of Vancouver BC; PSRF, Puget Sound Restoration Fund; WDFW, Washington Department of Fish and Wildlife; OSU, Oregon State University; SSNERR, South Slough National Estuarine Research Reserve; COMES, Coastal Oregon Marine Experimental Station; MU, Malaspina University BC; MACTEC Engineering and Consulting CA; UCD, University of California Davis.

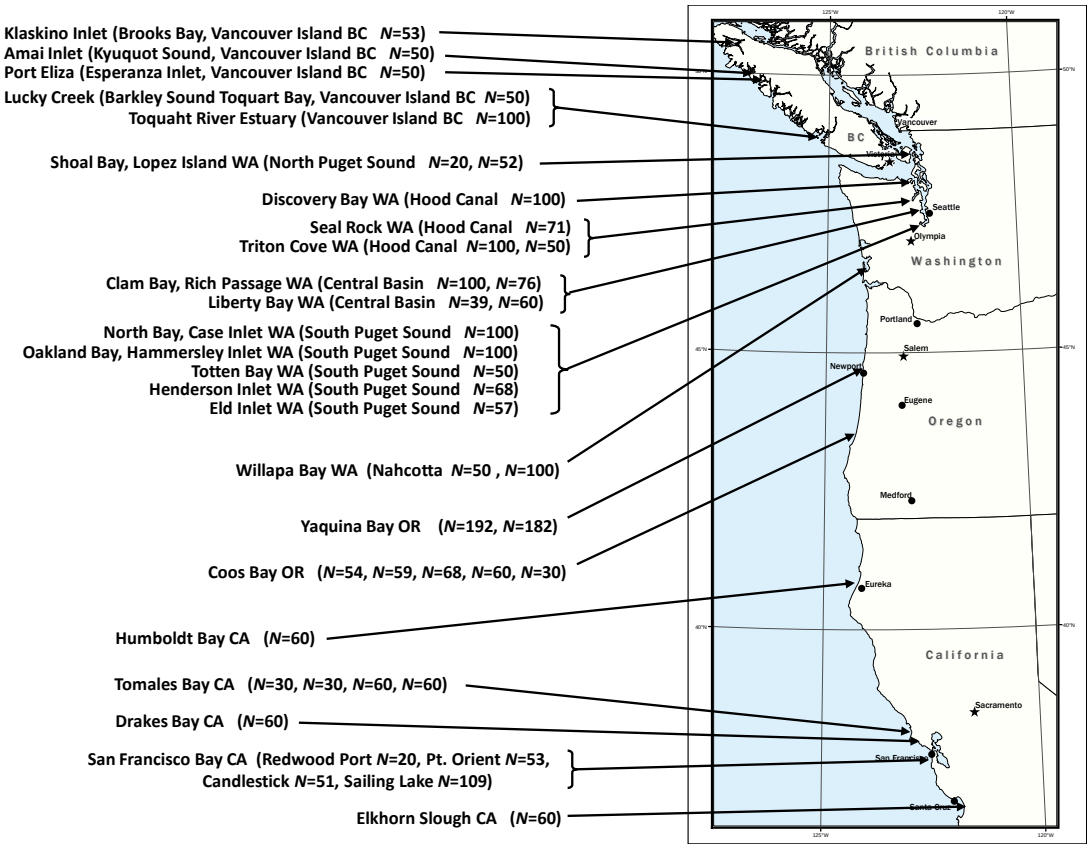


**Figure 3.1** General location of surveyed Olympia oyster (*O. lurida*) populations. Sites ranged from the northern tip of Vancouver Island BC southward to Elkhorn Slough CA. Some geographical areas were sampled multiple times. (*N*) number of individuals surveyed from each collection event.

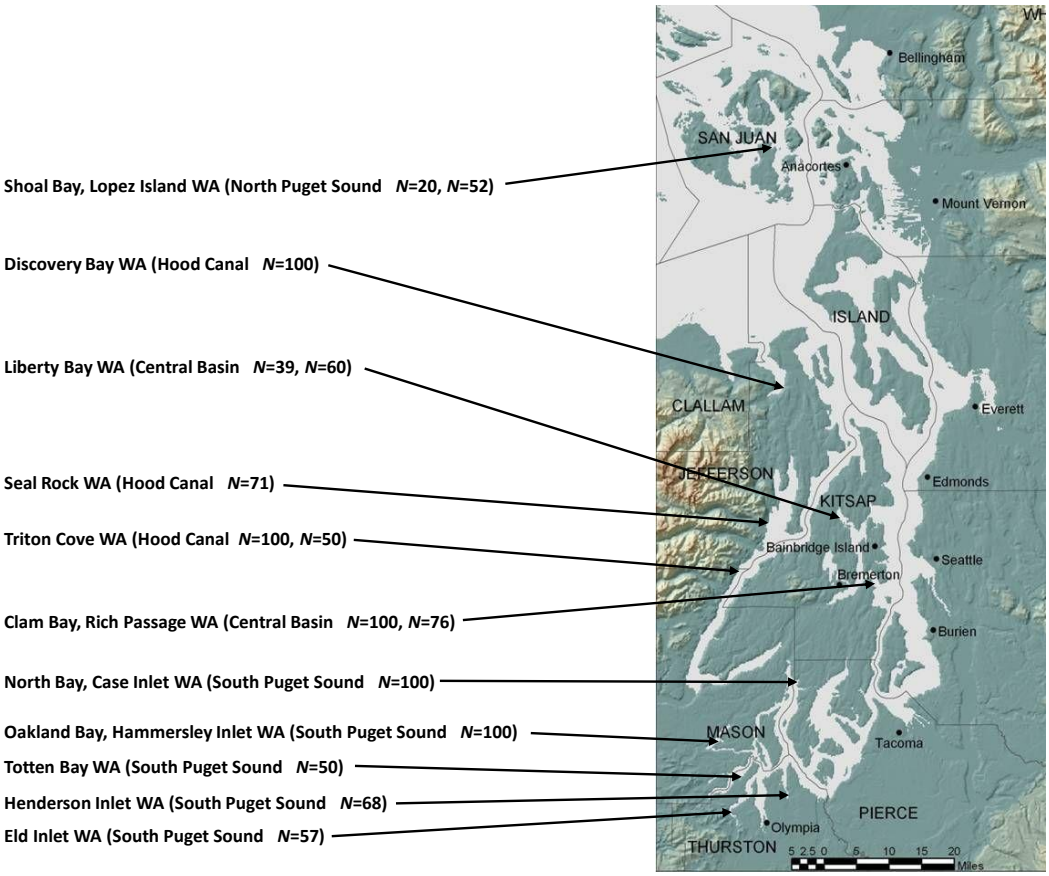


**Figure 3.2** General location of surveyed Olympia oyster (*O. lurida*) populations within four geographical regions of the Puget Sound WA region (North Puget Sound, Hood Canal, Central Basin and South Puget Sound). Some geographical areas were sampled multiple times. (*N*) number of individuals surveyed from each collection event.

*DNA extraction and microsatellite genotyping*

We extracted genomic DNA from preserved samples using DNeasy® 96 Blood and Tissue Digestion Kits (Qiagen Inc., Valencia CA) following a slightly modified protocol. Modifications included overnight (16-18 hr) sample lysis at 56°C, rinsing samples twice with AW2 buffer to reduce salt carryover and incubating the silica-gel columns uncovered at 70°C for 10 min prior to elution to evaporate residual ethanol. We quantified DNA concentrations using Quant-iT® PicoGreen dsDNA Assay Kits (Invitrogen Probes Inc., Eugene OR) on a Perkin Elmer Wallac Victor$^3$V 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Shelton CT) and standardized all samples to working concentrations of 10 ng/μL using a Beckman Coulter Biomek FX liquid handling system (Beckman Coulter Inc., Fullerton CA). We confirmed the DNA concentration of random standardized samples from each extraction plate using a NanoDrop ® ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE).

We previously developed nineteen microsatellites for the Olympia oyster (Stick *et al.* 2009) and used ten of these after re-optimization (Table 3.2) to genotype all samples. We amplified loci individually in 10 μL PCR reactions containing the following components: locus-specific [$MgCl_2$] (Table 3.2), 1x GoTaq® FlexiPCR buffer, pH 8.5 (Promega Inc., Madison WI), 0.15 mM dNTPs (Promega Inc., Madison WI), 0.2 μM 5'-fluorescently labeled forward primer (ABI, Foster City

CA) and unlabeled reverse primer (Integrated DNA Technologies Inc., Coralville IA) (Table 3.2) and 0.025 U/µL GoTaq® FlexiDNA Polymerase (Promega Inc., Madison WI) on a GeneAmp® PCR 9700 thermocycler (ABI, Foster City CA) running the following program: 1) initial denaturing for 5 min at 94°C, 2) amplification using 40 cycles of 30 sec at 94°C followed by 30 sec at the locus-specific annealing temperature (Table 3.2) and 45 sec at 72°C, 3) final extension for 15 min at 72°C and 4) a final hold at 10°C. We resolved products using an ABI 3730xl automated DNA sequencer with GeneScan® 500 LIZ size standard (ABI, Foster City, CA) and scored them using GeneMapper® version 3.7 (ABI, Foster City, CA). We confirmed our genotypic calls by independently amplifying and genotyping most samples multiple times.

**Table 3.2** Microsatellite primer and PCR characterization for Olympia oyster, *O. lurida*. *Marker excluded from analyses due to high incidence of null alleles.

| Locus | Clone ID | GenBank Accession # | Primer Sequence | 5' Fluorescent Label | Repeat Motif of Clone | Annealing Temp. (°C) | [$MgCl_2$] (mM) |
|---|---|---|---|---|---|---|---|
| *Olur07** | C6 | EU587394 | F: 5'-ACATGCTAACAAGATTCAGATC-3' | FAM | $(CAT)_{18}$ | 56 | 1.5 |
| | | | R: 5'-ATCAGATGATGACGATGTATTG-3' | | | | |
| *Olur09** | C9 | EU587396 | F: 5'-ATCTCCAGTTAAATCCCCATAC-3' | NED | $(CAT)_7$ | 54 | 1.5 |
| | | | R: 5'-CGTCCTCAGATGATGATTATTC-3' | | | | |
| *Olur10* | C105 | EU587397 | F: 5'-TGCTTCAGTCACTTATCAACAG-3' | NED | $(CAT)_{11}$ | 56 | 1.5 |
| | | | R: 5'-AGGAGGAGTAGCATTCCTTG-3' | | | | |
| *Olur11* | C122 | EU587398 | F: 5'-CTCGCCATCACTTACACTTC-3' | NED | $(CAT)_{14}$ | 56 | 2.5 |
| | | | R: 5'-TGGAGAGCAAAACGATTATG-3' | | | | |
| *Olur12* | C123 | EU587399 | F: 5'-CATGCGGACAAAACTTTG-3' | FAM | $(CAT)_{11}$ | 56 | 2.0 |
| | | | R: 5'-CAGAAGCTGGTCAACTGATC-3' | | | | |
| *Olur13* | D3 | EU587400 | F: 5'-GTGAAACATTCTTTCCTGAGTG-3' | PET | $(ATCT)_{17}$ | 52 | 2.0 |
| | | | R: 5'-CGAGTTCGACATAATGAAGTTC-3' | | | | |
| *Olur15* | D8 | EU587402 | F: 5'-CTTTCCATCGAGTTCGACATAA-3' | PET | $(TAGA)_{12}$ | 56 | 1.5 |
| | | | R: 5'-GGTGCGGACTGTGATGTAATAC-3' | | | | |
| *Olur17* | D101 | EU587404 | F: 5'-ATCGAAACTGAACGAGTGTTG-3' | FAM | $(TCTA)_{24}TC(CATC)_9$ | 56 | 3.0 |
| | | | R: 5'-TTGGTCACTGATTGCTGAAAC-3' | | | | |
| *Olur18* | D104 | EU587405 | F: 5'-TGGTGTCCTTTATATCGAGTTC-3' | PET | $(TATC)_{21}TGTC(TATC)_3$ | 56 | 2.0 |
| | | | R: 5'-CGCTATTTGTGGGGAGAT-3' | | | | |
| *Olur19* | D107 | EU587406 | F: 5'-CTTTCCATCGAGTTCGACATAA-3' | PET | $(GATA)_{20}$ | 54 | 2.5 |
| | | | R: 5'-TTAGCGTGTAGTCAACGGTCTC-3' | | | | |

*Statistical analysis*

We evaluated all microsatellite loci for evidence of null alleles using MICROCHECKER version 2.2.3 (Van Oosterhout *et al.* 2004). Basic descriptive population statistics for each sample group, including number of alleles ($N_A$), observed heterozygosity (*Ho*), expected heterozygosity (*He*) under Hardy-Weinberg equilibrium (HWE) and a measure of genotypic deviation from HWE ($F_{IS}$) were estimated using the program GENETIC DATA ANALYSIS (GDA) version 1.1 (Lewis& Zaykin 2001). We used FSTAT version 2.9.3.2 (Goudet 2001) to estimate allelic richness corrected for sample size ($R_S$) for loci within each sample group. We tested for significant departures from HWE within all sample groups for each locus and globally across loci and assessed linkage disequilibrium among locus pairs using GENEPOP version 4.0.9 (Rousset 2008) using the program's default Markov Chain parameters. We evaluated all sample groups for recent reductions in effective population size using a Wilcoxen sign-rank test calculated by BOTTLENECK (Cornuet& Luikart 1996) assuming a two-phase mutation model and using default parameters. We estimated Wright's pairwise $F_{ST}$ values for all sample group comparisons and tested for genetic differentiation without assuming HWE using FSTAT version 2.9.3.2 (Goudet 2001) at a 5% Bonferroni corrected significance criterion based on 15,600 permutations of the data.

We estimated the correlation between genetic distances and geographic distances with a Mantel test using the ISOLDE program found in the web-based GENEPOP version 4.0.10 (http://genepop.curtin.edu.au) using default parameters and 10,000 permutations of the data. Geographic distances were calculated as the shortest linear route over water that followed coastal shapes and hydrology using the ruler tool in Google Earth (http://earth.google.com) and were natural log transformed prior to analysis. We pooled all samples collected from any population surveyed multiple times, either temporally or spatially, and repeated the analysis to account for potential non-independence among sample groups collected from the same location.

We ran a hierarchical Analysis of Molecular Variance (AMOVA) using the program GDA version 1.1 (Lewis& Zaykin 2001) and analyzed the molecular variation among individuals within and among defined geographical regions. For simplicity, we will reference the AMOVA-based measure of population differentiation ($\theta_{ST}$) as the more familiar and analogous Wright's $F_{ST}$ (Weir& Cockerham 1984). Our model initially partitioned the total genetic variation into components attributable to eight broad geographical regions created without any genetic preconceptions (Vancouver Island BC, Puget Sound WA, Willapa Bay WA, Yaquina Bay OR, Coos Bay OR, Tomales Bay CA, San Francisco Bay CA and coastal California estuaries that surround San Francisco Bay, which included Humboldt Bay, Drakes Bay and Elkhorn Slough) (Figure 3.3). We then sub-partitioned our

data and ran another AMOVA analysis looking only at California populations (Figure 3.4). We evaluated potential substructure within the two largest and most complex hydrological estuarine systems (Puget Sound WA and San Francisco Bay CA) by sub-partitioning the total genetic variation within those regions and reanalyzed using AMOVA. Puget Sound samples were assigned to four geographical regions (North Puget Sound, Hood Canal, Central Basin and South Puget Sound) (Figure 3.5) and within the San Francisco Bay, the total genetic variation was assigned to either North or South San Francisco Bay (Figure 3.6). We assessed the significance of *F*-statistics by bootstrapping over all loci using 10,000 replicates and a 0.01 significance threshold.

We created arbitrarily rooted neighbor-joining trees based on Nei's (1978) genetic distance and visualized these using TREEVIEW version 1.6.6 from within GDA version 1.1 (Lewis& Zaykin 2001). We assessed the reliability of our trees by bootstrapping the genetic distances using 10,000 replicates with the program PHYLIP version 3.69 (Felsenstein 2005). Sample groups of interest were directly compared using factorial correspondence analysis within the program GENETIX (Belkhir *et al.* 2001).

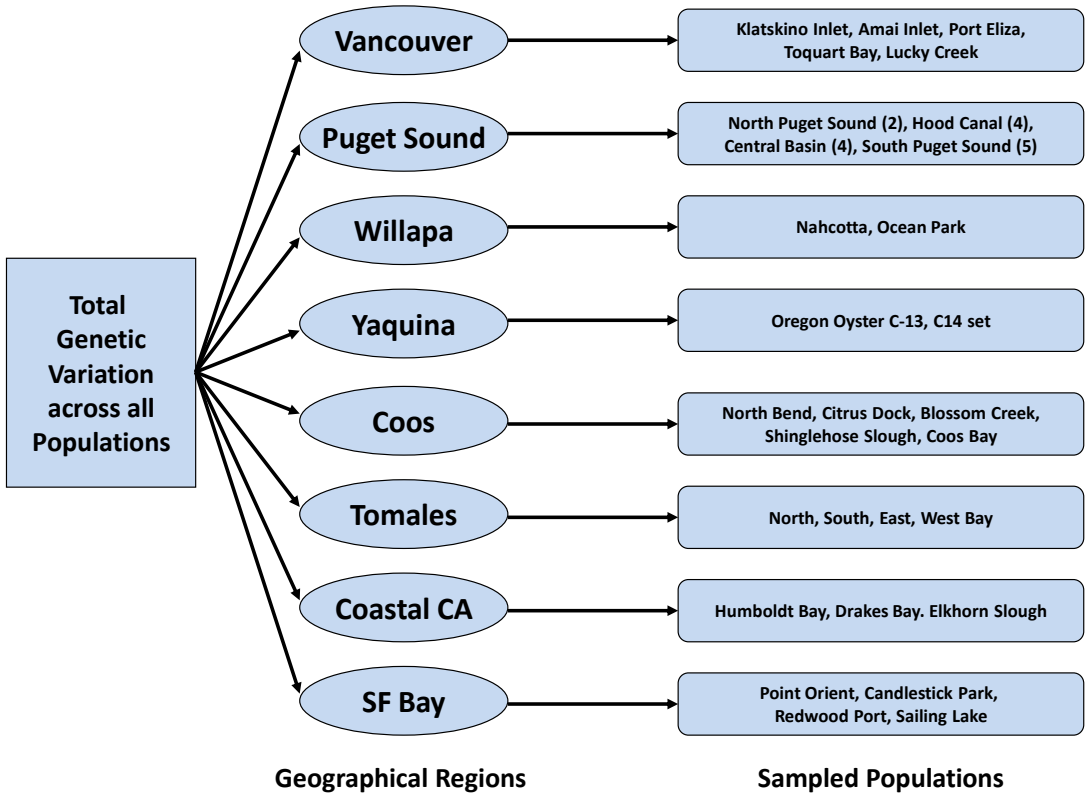


**Figure 3.3** Schematic for regional-scale hierarchical Analysis of Molecular Variance (AMOVA). We partitioned the total genetic variation into components attributable to eight broad geographical regions created without any genetic preconceptions (Vancouver Island BC, Puget Sound WA, Willapa Bay WA, Yaquina Bay OR, Coos Bay OR, Tomales Bay CA, San Francisco Bay CA and coastal California estuaries that surround San Francisco Bay, which included Humboldt Bay, Drakes Bay and Elkhorn Slough).

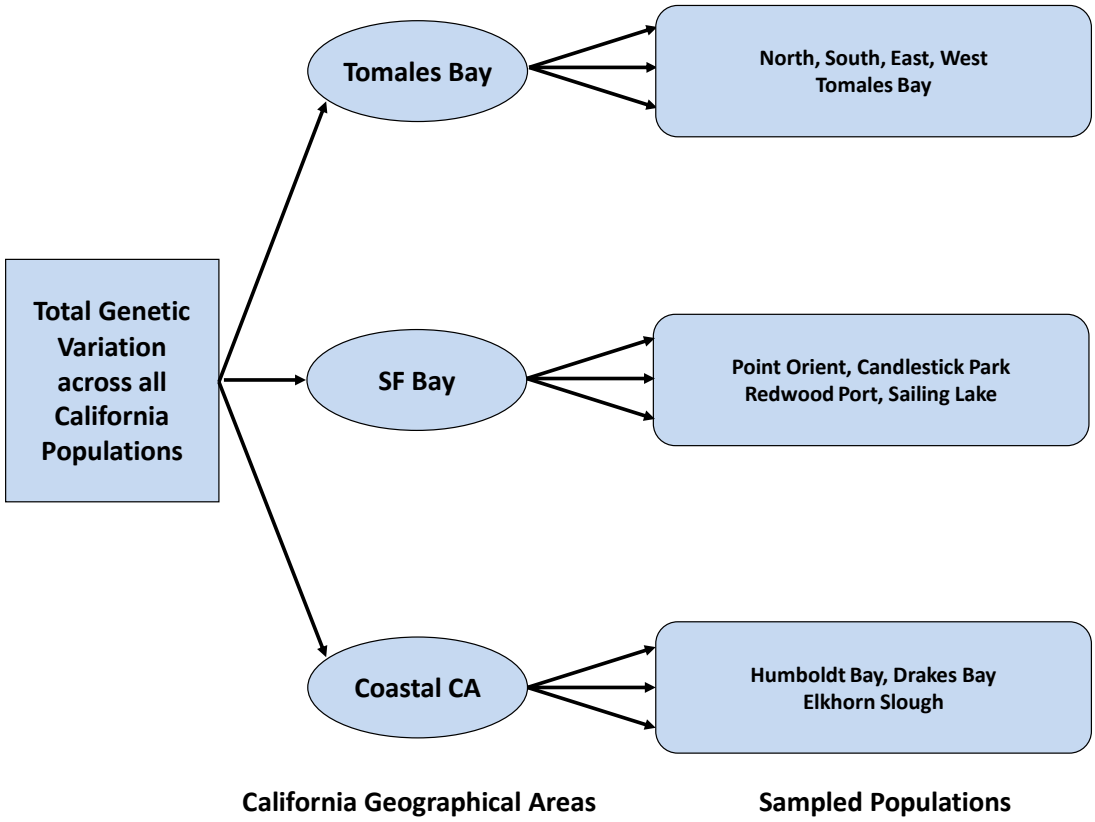


**Figure 3.4** Schematic for California-scale hierarchical Analysis of Molecular Variance (AMOVA). We partitioned the total genetic variation into components attributable to three geographical regions created without any genetic preconceptions (Tomales Bay, San Francisco Bay and coastal California estuaries that surround San Francisco Bay, which included Humboldt Bay, Drakes Bay and Elkhorn Slough).

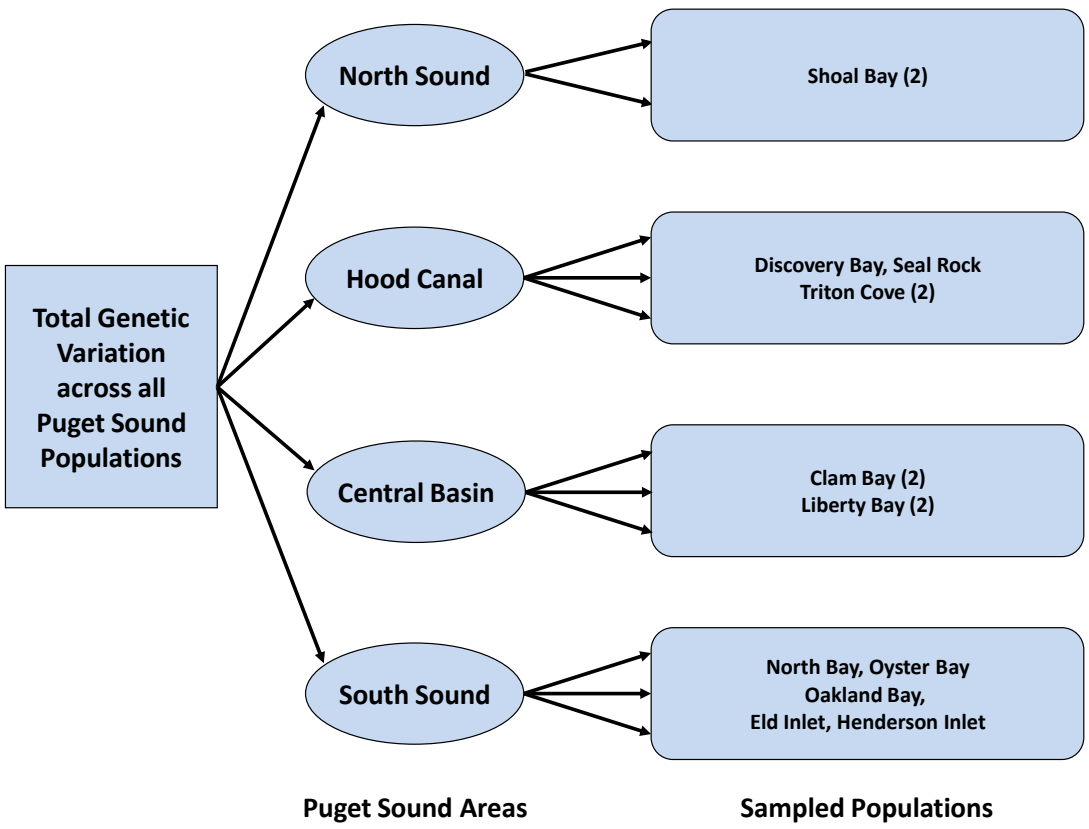


**Figure 3.5** Schematic for Puget Sound WA-scale hierarchical Analysis of Molecular Variance (AMOVA). We partitioned the total genetic variation into components corresponding to four geographical regions created without any genetic preconceptions (North Puget Sound, Hood Canal, Central Basin and South Puget Sound).

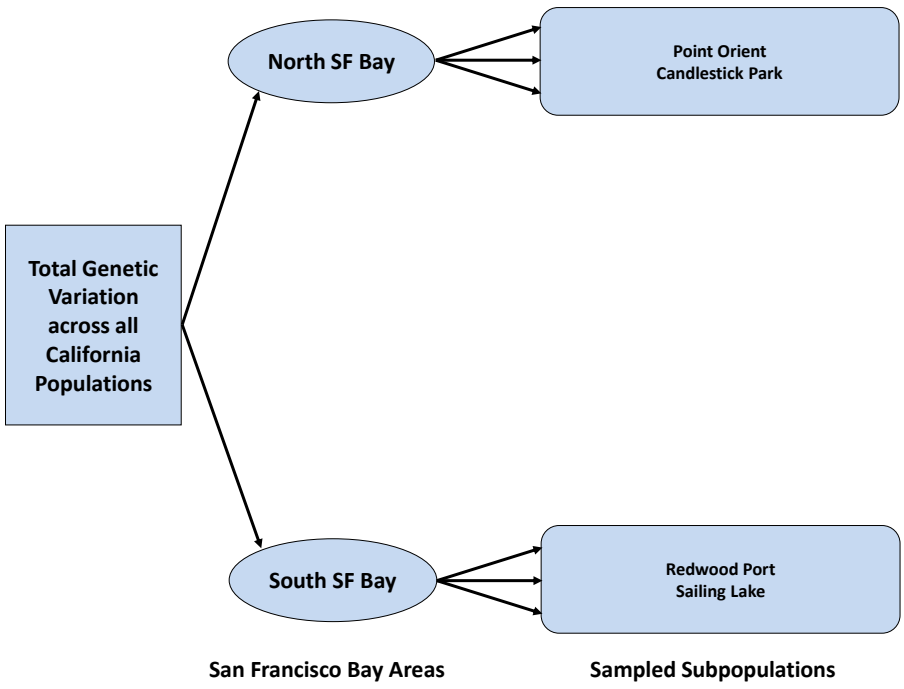


**Figure 3.6** Schematic for San Francisco Bay CA-scale hierarchical Analysis of Molecular Variance (AMOVA). We partitioned the total genetic variation into components corresponding to two geographical regions created without any genetic preconceptions.

## Results

*Data quality and descriptive statistics*

We genotyped 2712 individuals from 40 different sample groups or locations, which surveyed 25 extant *O. lurida* populations covering much of this species' contemporary range at 10 microsatellite loci. For clarity and continuity with accepted terminology of the analysis programs, we will commonly refer to these sample groups or locations as 'populations' even though they may not fulfill the definition of population as an independent breeding deme or localized population found within each of the 25 geographically distinct sampled bays and estuaries. We screened over 80% of these samples multiple times and used a common control on all runs to ensure reliability and to minimize genotyping errors. Overall, 96% of all samples were fully genotyped for at least 8 loci.

Using Fisher's exact test, consistent deviations from Hardy-Weinberg Equilibrium (HWE) were observed at two loci (*Olur07* and *Olur09*) across all populations. We confirmed a high incidence of null alleles at these loci and despite not influencing our expectation to resolve population differentiation (Carlsson 2008; Chapuis& Estoup 2007) and having no overall affects on our conclusions, we excluded these two markers from further analyses. Of the eight remaining loci, 62 of 320 (19%) population-locus combinations deviated from HWE ($P \leq 0.05$) and 34

combinations (10%) were significant at $P \leq 0.01$. Only 18 combinations (5%) remained significant after Bonferroni correction (Table 3.3). We detected significant heterozygote deficiencies relative to HWE ($P \leq 0.05$) for all loci across populations but only six loci remained significant after Bonferroni correction (Table 3.4).

All loci were tested for linkage disequilibrium (LD), but none of the pairwise combinations were significant ($P \leq 0.05$) in more than 8% of the populations with the exception of *Olur13* and *Olur15*, which showed significant LD in 65% of the populations. Global LD tests across populations were significant for 28 locus-pairs ($P \leq 0.05$). However, none of these tests, either paired or global, were significant after Bonferroni correction.

The total number of microsatellite alleles per locus averaged 47.5 across populations but varied greatly among loci, ranging from 20 (*Olur11*) to 107 (*Olur18*) (Table 3.4). However, within populations both $N_A$ and $R_S$ were remarkably consistent, even for highly polymorphic loci (*Olur17* and Olur18), with no individual population being either allele-rich or -poor (Table 3.3). Given that the oyster genome is one of the most variable among animal species (Hedgecock *et al.* 2005) it is not surprising that *O. lurida* microsatellites are also highly polymorphic (Stick *et al.* 2009).

We saw no evidence for recent genetic bottlenecks within populations. Three Puget Sound populations: Shoal Bay 1 ($P$ = 0.0059), Clam Bay 2 ($P$ = 0.0371), Liberty Bay 1 ($P$ = 0.0195) and two California subpopulations: Tomales Bay West ($P$ = 0.0098), Redwood Port ($P$ = 0.0039), had significant Wilcoxon sign-rank tests for excess heterozygous genotypes but none of these remained significant after Bonferroni correction.

We observed a surprising amount of genetic differentiation between *O. lurida* populations (Table 3.5). Pairwise $F_{ST}$ values ranged from -0.0013 to 0.0939. Overall, 703 of 780 (90%) of the pairwise $F_{ST}$ values differed significantly from zero with 95% confidence. The proportion of significant $F_{ST}$ values changed little with more conservative significance thresholds; 671 (86%) at $P \leq 0.01$, 624 (80%) at $P \leq 0.001$ and 543 (70%) after Bonferroni correction ($P \leq 0.000064$).

**Table 3.3** Genetic diversity parameter summary for Olympia oyster microsatellite loci. Table 3.1 Sample group code used to identify individual sampling events. ($N_A$) number of alleles; ($R_S$) allelic richness based on a minimum sample size of 6 individuals; ($H_E$) expected heterozygosity; ($H_O$) observed heterozygosity; ($F_{IS}$) fixation index or the deviation between expected and observed heterozygote proportions. Significant deviations from HWE using Fisher's exact test after Bonferroni correction for population-locus combinations, populations and loci fixation indices are highlighted bold.

**Table 3.3**

| Locus | | Sample Group Oc01 | Oc02 | Oc03 | Oc04 | Oc05 | Oc06 | Oc10 | Oc12 | Oc13 | Oc14 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Olur10* | $N_A$ | 12 | 22 | 17 | 22 | 11 | 10 | 31 | 25 | 20 | 13 |
| | $R_S$ | 7.048 | 9.382 | 8.418 | 7.890 | 6.415 | 5.555 | 9.486 | 8.296 | 8.238 | 8.800 |
| | $H_E$ | 0.887 | 0.953 | 0.927 | 0.876 | 0.859 | 0.794 | 0.954 | 0.922 | 0.922 | 0.926 |
| | $H_O$ | 0.680 | 0.897 | 0.929 | 0.813 | 0.950 | 0.742 | 0.924 | 0.855 | 0.878 | 0.700 |
| | $F_{IS}$ | 0.237 | 0.061 | -0.001 | 0.074 | -0.109 | 0.067 | 0.031 | 0.073 | 0.049 | 0.254 |
| *Olur11* | $N_A$ | 13 | 10 | 12 | 12 | 3 | 9 | 15 | 12 | 11 | 12 |
| | $R_S$ | 6.318 | 5.328 | 5.568 | 5.799 | 2.992 | 4.720 | 5.470 | 4.828 | 5.577 | 5.804 |
| | $H_E$ | 0.850 | 0.801 | 0.804 | 0.808 | 0.683 | 0.714 | 0.807 | 0.753 | 0.815 | 0.821 |
| | $H_O$ | 0.906 | 0.745 | 0.800 | 0.796 | 0.750 | 0.627 | 0.714 | 0.710 | 0.797 | 0.831 |
| | $F_{IS}$ | -0.066 | 0.071 | 0.005 | 0.015 | -0.100 | 0.123 | 0.115 | 0.057 | 0.021 | -0.012 |
| *Olur12* | $N_A$ | 11 | 12 | 15 | 11 | 11 | 11 | 15 | 13 | 12 | 17 |
| | $R_S$ | 5.802 | 6.008 | 6.858 | 5.216 | 7.266 | 6.339 | 6.400 | 6.061 | 5.767 | 5.856 |
| | $H_E$ | 0.821 | 0.842 | 0.868 | 0.759 | 0.899 | 0.867 | 0.857 | 0.849 | 0.834 | 0.825 |
| | $H_O$ | 0.686 | 0.826 | 0.900 | 0.740 | 0.950 | 0.824 | 0.845 | 0.890 | 0.853 | 0.775 |
| | $F_{IS}$ | 0.166 | 0.019 | -0.037 | 0.025 | -0.059 | 0.051 | 0.013 | -0.048 | -0.023 | 0.060 |
| *Olur13* | $N_A$ | 16 | 18 | 16 | 15 | 12 | 9 | 18 | 18 | 19 | 10 |
| | $R_S$ | 8.732 | 8.268 | 8.252 | 8.089 | 7.321 | 5.174 | 8.264 | 8.198 | 8.562 | 6.926 |
| | $H_E$ | 0.940 | 0.922 | 0.924 | 0.923 | 0.898 | 0.741 | 0.924 | 0.923 | 0.933 | 0.873 |
| | $H_O$ | 0.844 | 1.000 | 0.718 | 0.688 | 0.947 | 0.714 | 0.843 | 0.853 | 0.938 | 0.583 |
| | $F_{IS}$ | 0.104 | -0.086 | 0.225 | **0.258** | **-0.057** | 0.037 | 0.088 | 0.076 | -0.005 | 0.342 |
| *Olur15* | $N_A$ | 17 | 18 | 16 | 17 | 13 | 10 | 18 | 19 | 19 | 17 |
| | $R_S$ | 8.592 | 8.290 | 8.209 | 8.233 | 7.622 | 4.956 | 8.343 | 8.574 | 8.591 | 8.605 |
| | $H_E$ | 0.937 | 0.925 | 0.926 | 0.927 | 0.906 | 0.748 | 0.926 | 0.934 | 0.934 | 0.936 |
| | $H_O$ | 0.843 | 0.957 | 0.820 | 0.860 | 0.950 | 0.725 | 0.879 | 0.939 | 0.946 | 0.864 |
| | $F_{IS}$ | 0.101 | -0.034 | 0.115 | 0.073 | **-0.049** | 0.030 | 0.051 | -0.006 | -0.013 | 0.078 |
| *Olur17* | $N_A$ | 27 | 29 | 25 | 28 | 13 | 16 | 34 | 30 | 26 | 26 |
| | $R_S$ | 8.689 | 8.903 | 8.847 | 9.183 | 7.619 | 7.440 | 8.756 | 8.095 | 7.834 | 8.276 |
| | $H_E$ | 0.933 | 0.935 | 0.933 | 0.943 | 0.904 | 0.890 | 0.935 | 0.913 | 0.902 | 0.922 |
| | $H_O$ | 0.943 | 0.918 | 0.860 | 0.940 | 0.950 | 0.827 | 0.950 | 0.930 | 0.947 | 0.940 |
| | $F_{IS}$ | -0.011 | 0.018 | 0.079 | 0.003 | -0.052 | 0.071 | -0.016 | -0.018 | -0.050 | -0.020 |
| *Olur18* | $N_A$ | 22 | 25 | 24 | 27 | 18 | 22 | 35 | 25 | 25 | 28 |
| | $R_S$ | 8.006 | 8.947 | 8.713 | 8.835 | 8.877 | 8.199 | 8.904 | 7.458 | 8.396 | 8.282 |
| | $H_E$ | 0.914 | 0.943 | 0.935 | 0.938 | 0.938 | 0.922 | 0.940 | 0.899 | 0.929 | 0.921 |
| | $H_O$ | 0.755 | 0.857 | 0.920 | 0.920 | 0.900 | 0.942 | 0.869 | 0.900 | 0.908 | 0.860 |
| | $F_{IS}$ | **0.176** | 0.092 | 0.016 | 0.019 | 0.042 | -0.023 | 0.076 | -0.001 | 0.022 | 0.066 |
| *Olur19* | $N_A$ | 17 | 18 | 16 | 17 | 13 | 11 | 18 | 18 | 19 | 16 |
| | $R_S$ | 8.672 | 8.291 | 8.261 | 8.223 | 7.622 | 5.122 | 8.409 | 8.553 | 8.383 | 8.478 |
| | $H_E$ | 0.939 | 0.925 | 0.927 | 0.927 | 0.906 | 0.757 | 0.928 | 0.934 | 0.928 | 0.932 |
| | $H_O$ | 0.872 | 0.977 | 0.837 | 0.854 | 0.950 | 0.725 | 0.872 | 0.939 | 0.953 | 0.765 |
| | $F_{IS}$ | 0.071 | -0.057 | 0.099 | 0.079 | -0.049 | 0.042 | 0.060 | -0.006 | -0.027 | 0.182 |
| mean | $N_A$ | 16.9 | 19.0 | 17.6 | 18.6 | 11.8 | 12.3 | 23.0 | 20.0 | 18.9 | 17.4 |
| | $R_S$ | 7.732 | 7.927 | 7.891 | 7.684 | 6.967 | 5.938 | 8.004 | 7.508 | 7.669 | 7.628 |
| | $H_E$ | 0.903 | 0.906 | 0.906 | 0.887 | 0.874 | 0.804 | 0.909 | 0.891 | 0.900 | 0.895 |
| | $H_O$ | 0.816 | 0.897 | 0.848 | 0.826 | 0.918 | 0.766 | 0.862 | 0.877 | 0.903 | 0.790 |
| | $F_{IS}$ | **0.097** | 0.010 | 0.064 | **0.070** | **-0.052** | 0.048 | 0.051 | 0.016 | -0.003 | 0.117 |

$R_S$ based on minimum sample size of 6 individuals.

**Table 3.3** Continued.

| Locus | | Sample Group Oc15 | Oc16 | Oc17 | Oc18 | Oc19 | Oc20 | Oc21 | Oc24 | Oc25 | Oc26 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Olur10* | $N_A$ | 13 | 24 | 28 | 30 | 21 | 22 | 24 | 22 | 24 | 26 |
| | $R_S$ | 8.412 | 8.829 | 8.581 | 8.791 | 8.439 | 7.551 | 8.098 | 8.340 | 8.167 | 8.749 |
| | $H_E$ | 0.929 | 0.939 | 0.931 | 0.936 | 0.928 | 0.895 | 0.917 | 0.914 | 0.908 | 0.935 |
| | $H_O$ | 0.923 | 0.932 | 0.917 | 0.933 | 0.936 | 0.857 | 0.930 | 0.824 | 0.843 | 0.920 |
| | $F_{IS}$ | 0.007 | 0.007 | 0.015 | 0.003 | -0.009 | 0.043 | -0.015 | 0.101 | 0.072 | 0.017 |
| *Olur11* | $N_A$ | 9 | 13 | 11 | 9 | 11 | 8 | 10 | 15 | 13 | 9 |
| | $R_S$ | 5.121 | 5.293 | 5.235 | 4.630 | 4.997 | 3.569 | 3.993 | 5.106 | 4.975 | 3.997 |
| | $H_E$ | 0.782 | 0.783 | 0.784 | 0.749 | 0.774 | 0.558 | 0.604 | 0.774 | 0.762 | 0.624 |
| | $H_O$ | 0.813 | 0.771 | 0.730 | 0.760 | 0.750 | 0.600 | 0.620 | 0.758 | 0.791 | 0.685 |
| | $F_{IS}$ | -0.040 | 0.015 | 0.069 | -0.014 | 0.032 | -0.077 | -0.027 | 0.021 | -0.038 | -0.099 |
| *Olur12* | $N_A$ | 11 | 15 | 11 | 13 | 11 | 16 | 16 | 19 | 19 | 14 |
| | $R_S$ | 5.829 | 6.196 | 5.905 | 5.792 | 5.751 | 6.067 | 5.703 | 6.179 | 5.920 | 6.151 |
| | $H_E$ | 0.819 | 0.838 | 0.841 | 0.839 | 0.827 | 0.785 | 0.728 | 0.786 | 0.758 | 0.784 |
| | $H_O$ | 0.854 | 0.857 | 0.869 | 0.890 | 0.848 | 0.776 | 0.760 | 0.783 | 0.770 | 0.796 |
| | $F_{IS}$ | -0.043 | -0.023 | -0.033 | -0.061 | -0.025 | 0.012 | -0.044 | 0.004 | -0.016 | -0.016 |
| *Olur13* | $N_A$ | 15 | 19 | 19 | 20 | 18 | 18 | 21 | 16 | 18 | 15 |
| | $R_S$ | 7.618 | 8.800 | 8.332 | 8.531 | 8.321 | 8.162 | 8.497 | 8.098 | 8.335 | 8.194 |
| | $H_E$ | 0.895 | 0.941 | 0.928 | 0.934 | 0.923 | 0.923 | 0.933 | 0.915 | 0.926 | 0.924 |
| | $H_O$ | 0.650 | 0.782 | 0.925 | 0.908 | 0.826 | 0.740 | 0.960 | 0.658 | 0.830 | 0.843 |
| | $F_{IS}$ | 0.279 | **0.170** | 0.003 | 0.027 | 0.106 | 0.200 | -0.029 | **0.284** | 0.105 | 0.088 |
| *Olur15* | $N_A$ | 19 | 19 | 19 | 20 | 18 | 17 | 21 | 19 | 19 | 16 |
| | $R_S$ | 8.699 | 8.884 | 8.295 | 8.466 | 8.673 | 8.230 | 8.461 | 8.515 | 8.503 | 8.143 |
| | $H_E$ | 0.937 | 0.943 | 0.927 | 0.931 | 0.936 | 0.925 | 0.932 | 0.933 | 0.932 | 0.920 |
| | $H_O$ | 0.927 | 0.956 | 0.959 | 0.937 | 0.895 | 0.880 | 0.960 | 0.859 | 0.892 | 0.870 |
| | $F_{IS}$ | 0.011 | -0.014 | -0.035 | -0.006 | 0.045 | 0.049 | -0.030 | 0.079 | 0.042 | 0.055 |
| *Olur17* | $N_A$ | 26 | 23 | 33 | 32 | 32 | 20 | 29 | 53 | 50 | 24 |
| | $R_S$ | 8.520 | 8.162 | 8.791 | 8.661 | 9.317 | 8.137 | 8.404 | 9.740 | 9.535 | 8.539 |
| | $H_E$ | 0.930 | 0.922 | 0.937 | 0.934 | 0.950 | 0.919 | 0.927 | 0.959 | 0.954 | 0.928 |
| | $H_O$ | 0.918 | 0.930 | 0.920 | 0.950 | 0.939 | 0.880 | 0.950 | 0.969 | 0.966 | 0.981 |
| | $F_{IS}$ | 0.013 | -0.008 | 0.018 | -0.018 | 0.012 | 0.042 | -0.025 | -0.010 | -0.012 | -0.058 |
| *Olur18* | $N_A$ | 21 | 27 | 33 | 35 | 29 | 23 | 32 | 39 | 36 | 24 |
| | $R_S$ | 8.838 | 8.353 | 8.456 | 8.806 | 9.156 | 8.760 | 8.862 | 8.897 | 8.662 | 8.739 |
| | $H_E$ | 0.940 | 0.921 | 0.929 | 0.939 | 0.947 | 0.938 | 0.939 | 0.940 | 0.935 | 0.936 |
| | $H_O$ | 0.880 | 0.958 | 0.870 | 0.970 | 0.940 | 0.796 | 0.910 | 0.922 | 0.853 | 0.926 |
| | $F_{IS}$ | **0.064** | -0.040 | 0.064 | -0.033 | 0.008 | 0.153 | 0.031 | 0.020 | 0.088 | 0.011 |
| *Olur19* | $N_A$ | 18 | 19 | 19 | 20 | 18 | 18 | 21 | 21 | 19 | 16 |
| | $R_S$ | 8.366 | 8.924 | 8.265 | 8.378 | 8.558 | 8.341 | 8.461 | 8.617 | 8.463 | 8.143 |
| | $H_E$ | 0.926 | 0.944 | 0.926 | 0.929 | 0.933 | 0.928 | 0.932 | 0.935 | 0.930 | 0.920 |
| | $H_O$ | 0.892 | 0.925 | 0.949 | 0.929 | 0.955 | 0.820 | 0.960 | 0.906 | 0.886 | 0.870 |
| | $F_{IS}$ | 0.037 | 0.020 | -0.026 | 0.000 | -0.024 | 0.117 | -0.030 | 0.032 | 0.048 | 0.055 |
| mean | $N_A$ | 16.5 | 19.9 | 21.6 | 22.4 | 19.8 | 17.8 | 21.8 | 25.5 | 24.8 | 18.0 |
| | $R_S$ | 7.675 | 7.930 | 7.733 | 7.757 | 7.902 | 7.352 | 7.560 | 7.937 | 7.820 | 7.582 |
| | $H_E$ | 0.895 | 0.904 | 0.900 | 0.899 | 0.902 | 0.859 | 0.864 | 0.895 | 0.888 | 0.872 |
| | $H_O$ | 0.857 | 0.889 | 0.892 | 0.910 | 0.886 | 0.794 | 0.881 | 0.835 | 0.854 | 0.862 |
| | $F_{IS}$ | **0.042** | **0.017** | 0.009 | -0.012 | 0.018 | 0.077 | -0.020 | **0.067** | **0.039** | 0.011 |

$R_S$ based on minimum sample size of 6 individuals.

**Table 3.3** Continued.

| Locus | | Sample Group Oc27 | Oc28 | Oc29 | Oc30 | Oc31 | Oc32 | Oc34 | Oc36 | Oc37 | Oc38 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Olur10* | $N_A$ | 20 | 27 | 23 | 15 | 24 | 20 | 9 | 12 | 31 | 26 |
| | $R_S$ | 8.322 | 8.475 | 8.105 | 7.932 | 9.520 | 8.967 | 9.000 | 8.108 | 9.189 | 8.685 |
| | $H_E$ | 0.926 | 0.926 | 0.916 | 0.913 | 0.954 | 0.939 | 0.955 | 0.926 | 0.947 | 0.934 |
| | $H_O$ | 0.944 | 0.909 | 0.944 | 0.800 | 0.966 | 0.926 | 0.833 | 1.000 | 0.914 | 0.954 |
| | $F_{IS}$ | -0.020 | 0.018 | -0.032 | 0.126 | -0.012 | 0.014 | 0.138 | -0.082 | 0.036 | -0.022 |
| *Olur11* | $N_A$ | 11 | 11 | 10 | 6 | 10 | 11 | 7 | 9 | 14 | 11 |
| | $R_S$ | 4.743 | 4.588 | 3.546 | 3.683 | 5.298 | 6.225 | 5.876 | 5.070 | 4.634 | 4.944 |
| | $H_E$ | 0.695 | 0.660 | 0.578 | 0.576 | 0.741 | 0.838 | 0.847 | 0.777 | 0.677 | 0.770 |
| | $H_O$ | 0.678 | 0.662 | 0.567 | 0.560 | 0.733 | 0.786 | 0.500 | 0.806 | 0.591 | 0.691 |
| | $F_{IS}$ | 0.025 | -0.003 | 0.021 | 0.029 | 0.010 | 0.064 | 0.423 | -0.038 | 0.127 | 0.103 |
| *Olur12* | $N_A$ | 13 | 16 | 15 | 16 | 14 | 11 | 16 | 9 | 18 | 11 |
| | $R_S$ | 6.273 | 6.153 | 6.224 | 7.700 | 6.600 | 6.598 | 8.888 | 5.927 | 7.096 | 5.645 |
| | $H_E$ | 0.816 | 0.778 | 0.790 | 0.903 | 0.830 | 0.838 | 0.941 | 0.819 | 0.864 | 0.828 |
| | $H_O$ | 0.746 | 0.853 | 0.817 | 0.840 | 0.767 | 0.750 | 0.882 | 0.611 | 0.859 | 0.866 |
| | $F_{IS}$ | 0.086 | -0.097 | -0.033 | 0.071 | 0.077 | 0.107 | 0.064 | 0.259 | 0.006 | -0.046 |
| *Olur13* | $N_A$ | 19 | 17 | 18 | 14 | 19 | 19 | 16 | 13 | 19 | 18 |
| | $R_S$ | 8.502 | 8.427 | 7.970 | 8.408 | 9.133 | 8.694 | 9.475 | 7.461 | 8.063 | 8.523 |
| | $H_E$ | 0.931 | 0.932 | 0.914 | 0.933 | 0.949 | 0.936 | 0.958 | 0.903 | 0.920 | 0.933 |
| | $H_O$ | 0.879 | 0.882 | 0.867 | 0.880 | 0.964 | 0.828 | 0.929 | 0.893 | 0.925 | 0.955 |
| | $F_{IS}$ | 0.056 | 0.053 | 0.052 | 0.058 | -0.016 | 0.118 | 0.032 | 0.011 | -0.005 | -0.023 |
| *Olur15* | $N_A$ | 19 | 18 | 18 | 15 | 19 | 19 | 16 | 8 | 19 | 18 |
| | $R_S$ | 8.597 | 8.520 | 8.078 | 8.664 | 9.133 | 8.595 | 9.284 | 6.443 | 8.111 | 8.523 |
| | $H_E$ | 0.934 | 0.934 | 0.919 | 0.940 | 0.949 | 0.934 | 0.954 | 0.877 | 0.922 | 0.933 |
| | $H_O$ | 0.932 | 0.912 | 0.917 | 0.897 | 0.964 | 0.857 | 0.944 | 0.750 | 0.923 | 0.955 |
| | $F_{IS}$ | 0.002 | 0.024 | 0.002 | 0.046 | -0.016 | 0.084 | 0.010 | 0.150 | -0.001 | -0.023 |
| *Olur17* | $N_A$ | 26 | 27 | 30 | 17 | 24 | 29 | 22 | 34 | 37 | 29 |
| | $R_S$ | 8.775 | 8.149 | 8.806 | 7.461 | 9.440 | 10.395 | 9.763 | 8.646 | 9.572 | 8.668 |
| | $H_E$ | 0.939 | 0.920 | 0.936 | 0.896 | 0.953 | 0.974 | 0.960 | 0.924 | 0.955 | 0.933 |
| | $H_O$ | 0.966 | 0.868 | 0.933 | 0.889 | 0.933 | 0.966 | 0.900 | 0.900 | 0.945 | 0.897 |
| | $F_{IS}$ | -0.029 | 0.057 | 0.002 | 0.008 | 0.021 | 0.009 | 0.064 | 0.026 | 0.011 | 0.038 |
| *Olur18* | $N_A$ | 28 | 34 | 25 | 24 | 29 | 22 | 18 | 30 | 50 | 28 |
| | $R_S$ | 8.878 | 8.968 | 8.575 | 9.199 | 10.264 | 9.111 | 8.775 | 8.409 | 9.921 | 8.285 |
| | $H_E$ | 0.940 | 0.940 | 0.932 | 0.945 | 0.971 | 0.945 | 0.937 | 0.928 | 0.963 | 0.924 |
| | $H_O$ | 0.948 | 0.941 | 0.917 | 0.963 | 0.800 | 0.759 | 0.947 | 0.950 | 0.927 | 0.897 |
| | $F_{IS}$ | -0.009 | -0.001 | 0.016 | -0.019 | **0.179** | 0.200 | -0.011 | -0.024 | 0.038 | 0.030 |
| *Olur19* | $N_A$ | 19 | 18 | 18 | 15 | 19 | 18 | 16 | 8 | 19 | 18 |
| | $R_S$ | 8.597 | 8.520 | 8.078 | 8.405 | 9.092 | 8.426 | 9.363 | 6.511 | 8.105 | 8.525 |
| | $H_E$ | 0.934 | 0.934 | 0.919 | 0.931 | 0.948 | 0.930 | 0.956 | 0.880 | 0.921 | 0.933 |
| | $H_O$ | 0.932 | 0.912 | 0.917 | 0.913 | 0.964 | 0.893 | 0.947 | 0.769 | 0.880 | 0.940 |
| | $F_{IS}$ | 0.002 | 0.024 | 0.002 | 0.020 | -0.017 | 0.041 | 0.009 | 0.130 | 0.044 | -0.008 |
| mean | $N_A$ | 19.4 | 21.0 | 19.6 | 15.3 | 19.8 | 18.6 | 15.0 | 15.4 | 25.9 | 19.9 |
| | $R_S$ | 7.836 | 7.725 | 7.423 | 7.682 | 8.560 | 8.376 | 8.803 | 7.072 | 8.086 | 7.725 |
| | $H_E$ | 0.889 | 0.878 | 0.863 | 0.880 | 0.912 | 0.917 | 0.939 | 0.879 | 0.896 | 0.899 |
| | $H_O$ | 0.878 | 0.867 | 0.860 | 0.843 | 0.886 | 0.845 | 0.860 | 0.835 | 0.870 | 0.894 |
| | $F_{IS}$ | 0.013 | 0.012 | 0.004 | 0.043 | **0.028** | 0.079 | 0.085 | 0.051 | 0.029 | 0.005 |

$R_S$ based on minimum sample size of 6 individuals.

**Table 3.3** Continued.

| Locus | | Sample Group Oc39 | Oc40 | Oc41 | Oc42 | Oc43 | Oc44 | Oc45 | Oc46 | Oc47 | Oc49 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Olur10* | $N_A$ | 24 | 23 | 27 | 28 | 32 | 30 | 31 | 34 | 30 | 28 | 22.8 |
| | $R_S$ | 8.722 | 9.067 | 9.129 | 9.359 | 9.280 | 8.442 | 9.291 | 9.942 | 10.221 | 9.672 | 9.394 |
| | $H_E$ | 0.935 | 0.945 | 0.946 | 0.951 | 0.946 | 0.908 | 0.945 | 0.964 | 0.970 | 0.960 | 0.926 |
| | $H_O$ | 0.982 | 0.872 | 0.945 | 0.945 | 0.943 | 0.898 | 0.936 | 0.917 | 0.938 | 0.915 | 0.897 |
| | $F_{IS}$ | -0.051 | 0.079 | 0.000 | 0.006 | 0.003 | 0.011 | 0.010 | 0.049 | 0.034 | 0.047 | 0.032 |
| *Olur11* | $N_A$ | 9 | 10 | 13 | 10 | 14 | 13 | 13 | 14 | 12 | 14 | 11.0 |
| | $R_S$ | 4.346 | 5.062 | 4.887 | 5.011 | 5.590 | 5.710 | 5.505 | 5.280 | 4.795 | 5.886 | 5.309 |
| | $H_E$ | 0.710 | 0.776 | 0.725 | 0.749 | 0.751 | 0.772 | 0.761 | 0.745 | 0.718 | 0.789 | 0.743 |
| | $H_O$ | 0.719 | 0.821 | 0.700 | 0.754 | 0.755 | 0.717 | 0.679 | 0.692 | 0.736 | 0.820 | 0.723 |
| | $F_{IS}$ | -0.014 | -0.059 | 0.034 | -0.007 | -0.005 | 0.072 | 0.109 | 0.071 | -0.026 | -0.040 | 0.026 |
| *Olur12* | $N_A$ | 12 | 10 | 9 | 15 | 16 | 14 | 14 | 17 | 15 | 17 | 13.8 |
| | $R_S$ | 5.824 | 6.012 | 5.467 | 6.904 | 7.123 | 6.968 | 7.019 | 6.895 | 6.883 | 7.423 | 6.603 |
| | $H_E$ | 0.832 | 0.847 | 0.821 | 0.863 | 0.869 | 0.874 | 0.877 | 0.832 | 0.849 | 0.884 | 0.835 |
| | $H_O$ | 0.895 | 0.769 | 0.883 | 0.842 | 0.868 | 0.846 | 0.839 | 0.792 | 0.800 | 0.898 | 0.823 |
| | $F_{IS}$ | -0.076 | 0.093 | -0.077 | 0.024 | 0.001 | 0.032 | 0.043 | 0.048 | 0.058 | -0.016 | 0.014 |
| *Olur13* | $N_A$ | 17 | 18 | 18 | 19 | 21 | 17 | 20 | 18 | 19 | 16 | 17.1 |
| | $R_S$ | 8.753 | 8.466 | 8.516 | 8.538 | 8.829 | 8.538 | 8.863 | 8.487 | 8.471 | 8.487 | 8.760 |
| | $H_E$ | 0.940 | 0.933 | 0.932 | 0.935 | 0.941 | 0.935 | 0.941 | 0.933 | 0.932 | 0.934 | 0.923 |
| | $H_O$ | 0.965 | 0.949 | 0.949 | 0.946 | 0.962 | 0.906 | 1.000 | 0.885 | 0.885 | 0.898 | 0.865 |
| | $F_{IS}$ | -0.026 | -0.017 | -0.019 | -0.013 | -0.023 | 0.031 | -0.063 | 0.052 | 0.051 | 0.039 | **0.063** |
| *Olur15* | $N_A$ | 17 | 18 | 18 | 19 | 19 | 17 | 19 | 18 | 18 | 16 | 17.5 |
| | $R_S$ | 8.759 | 8.466 | 8.536 | 8.565 | 8.650 | 8.585 | 8.790 | 8.529 | 8.563 | 8.492 | 8.744 |
| | $H_E$ | 0.940 | 0.933 | 0.933 | 0.935 | 0.937 | 0.936 | 0.940 | 0.934 | 0.935 | 0.934 | 0.927 |
| | $H_O$ | 0.964 | 0.949 | 0.917 | 0.929 | 0.925 | 0.860 | 0.982 | 0.863 | 0.721 | 0.826 | 0.898 |
| | $F_{IS}$ | -0.026 | -0.017 | 0.018 | 0.007 | 0.013 | 0.082 | -0.045 | 0.077 | **0.231** | **0.117** | **0.032** |
| *Olur17* | $N_A$ | 24 | 28 | 25 | 32 | 33 | 35 | 36 | 32 | 37 | 32 | 29.1 |
| | $R_S$ | 8.409 | 9.269 | 8.508 | 9.973 | 9.914 | 9.557 | 9.720 | 9.415 | 10.020 | 9.752 | 9.422 |
| | $H_E$ | 0.926 | 0.949 | 0.932 | 0.965 | 0.964 | 0.955 | 0.958 | 0.948 | 0.965 | 0.959 | 0.937 |
| | $H_O$ | 0.947 | 1.000 | 0.933 | 0.946 | 0.943 | 0.981 | 0.967 | 0.925 | 1.000 | 0.902 | 0.934 |
| | $F_{IS}$ | -0.023 | -0.054 | -0.002 | 0.020 | 0.022 | **-0.028** | -0.009 | 0.025 | -0.037 | 0.060 | **0.003** |
| *Olur18* | $N_A$ | 28 | 24 | 24 | 39 | 33 | 38 | 40 | 33 | 45 | 27 | 29.2 |
| | $R_S$ | 8.765 | 8.925 | 8.297 | 9.824 | 9.557 | 9.932 | 10.000 | 9.866 | 10.366 | 9.275 | 9.177 |
| | $H_E$ | 0.937 | 0.942 | 0.926 | 0.960 | 0.955 | 0.963 | 0.964 | 0.962 | 0.972 | 0.950 | 0.940 |
| | $H_O$ | 0.947 | 0.769 | 0.800 | 0.839 | 0.792 | 0.778 | 0.850 | 0.788 | 0.943 | 0.612 | 0.876 |
| | $F_{IS}$ | -0.011 | **0.185** | **0.137** | 0.127 | **0.172** | **0.194** | **0.120** | **0.182** | 0.030 | **0.358** | **0.069** |
| *Olur19* | $N_A$ | 17 | 18 | 18 | 19 | 20 | 17 | 20 | 18 | 19 | 17 | 17.6 |
| | $R_S$ | 8.753 | 8.466 | 8.542 | 8.537 | 8.787 | 8.579 | 8.846 | 8.487 | 8.443 | 8.366 | 8.747 |
| | $H_E$ | 0.940 | 0.933 | 0.933 | 0.935 | 0.940 | 0.936 | 0.941 | 0.933 | 0.930 | 0.931 | 0.926 |
| | $H_O$ | 0.965 | 0.949 | 0.950 | 0.947 | 0.943 | 0.887 | 1.000 | 0.885 | 0.824 | 0.940 | 0.905 |
| | $F_{IS}$ | -0.026 | -0.017 | -0.018 | -0.014 | -0.004 | 0.053 | -0.063 | 0.052 | 0.116 | -0.010 | **0.023** |
| mean | $N_A$ | 18.5 | 18.6 | 19.0 | 22.6 | 23.5 | 22.6 | 24.1 | 23.0 | 24.4 | 20.9 | 19.8 |
| | $R_S$ | 7.791 | 7.967 | 7.735 | 8.339 | 8.466 | 8.289 | 8.504 | 8.363 | 8.470 | 8.419 | 8.270 |
| | $H_E$ | 0.895 | 0.907 | 0.893 | 0.912 | 0.913 | 0.910 | 0.916 | 0.906 | 0.909 | 0.918 | 0.895 |
| | $H_O$ | 0.923 | 0.885 | 0.885 | 0.894 | 0.892 | 0.859 | 0.907 | 0.843 | 0.856 | 0.851 | 0.865 |
| | $F_{IS}$ | -0.032 | **0.025** | **0.010** | 0.020 | **0.024** | **0.056** | **0.010** | **0.070** | **0.059** | **0.073** | **0.033** |

$R_S$ based on minimum sample size of 6 individuals.

**Table 3.4** Global genetic diversity parameter summary for Olympia oyster microsatellite loci. ($N_A$) number of alleles; ($H_E$) expected heterozygosity; ($H_O$) observed heterozygosity; ($F_{IS}$) fixation index or the deviation between expected and observed heterozygote proportions. Significant Bonferroni corrected heterozygote deficits from HWE using Rousset & Raymond's (1995) unconditional exact test are highlighted bold.

| Locus | $N_A$ | $H_E$ | $H_O$ | $F_{IS}$ |
|---|---|---|---|---|
| C105 | 51 | 0.9511 | 0.9074 | **0.0459** |
| C122 | 20 | 0.7866 | 0.7283 | **0.0741** |
| C123 | 31 | 0.8505 | 0.8235 | 0.0318 |
| D3 | 27 | 0.9398 | 0.8808 | **0.0628** |
| D8 | 26 | 0.9394 | 0.9028 | **0.0390** |
| D101 | 93 | 0.9514 | 0.9374 | 0.0148 |
| D104 | 107 | 0.9462 | 0.8834 | **0.0664** |
| D107 | 25 | 0.9395 | 0.9086 | **0.0329** |
| mean | 47.5 | 0.9131 | 0.8715 | **0.0455** |

**Table 3.5** Pairwise $F_{ST}$ values (below the diagonal) and degree of significance (above the diagonal). Table 3.1 Sample group code used to identify individual sampling events. (NS) $F_{ST}$ non-significant $P > 0.05$; (*) $F_{ST}$ significant $P \leq 0.05$; (**) $F_{ST}$ significant $P \leq 0.01$; (***) $F_{ST}$ significant $P \leq 0.001$; (****) $F_{ST}$ significant after Bonferroni correction $P \leq 0.000064$.

| Sample Group | code | Oc01 | Oc02 | Oc03 | Oc04 | Oc05 | Oc06 | Oc10 | Oc12 | Oc13 | Oc14 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Klaskino | Oc01 | | NS | * | NS | **** | **** | *** | **** | **** | NS |
| Amai | Oc02 | 0.0029 | | NS | NS | **** | **** | * | **** | **** | NS |
| Port Eliza | Oc03 | 0.0011 | 0.0007 | | NS | **** | **** | **** | **** | **** | NS |
| Torquart Bay | Oc04 | 0.0086 | 0.0101 | 0.0144 | | **** | **** | *** | **** | **** | * |
| Shoal Bay1 | Oc05 | 0.0183 | 0.0246 | 0.0192 | 0.0230 | | *** | **** | **** | **** | **** |
| Shoal Bay 2 | Oc06 | 0.0583 | 0.0681 | 0.0625 | 0.0549 | 0.0197 | | **** | **** | **** | **** |
| Discovery Bay | Oc10 | 0.0107 | 0.0017 | 0.0093 | 0.0150 | 0.0210 | 0.0555 | | **** | **** | * |
| Clam Bay 1 | Oc12 | 0.0111 | 0.0117 | 0.0121 | 0.0169 | 0.0219 | 0.0611 | 0.0066 | | NS | * |
| Clam Bay 2 | Oc13 | 0.0133 | 0.0096 | 0.0090 | 0.0169 | 0.0237 | 0.0634 | 0.0061 | 0.0024 | | * |
| Triton Cove 1 | Oc14 | 0.0097 | 0.0179 | 0.0127 | 0.0186 | 0.0274 | 0.0671 | 0.0149 | 0.0109 | 0.0158 | |
| Triton Cove 2 | Oc15 | 0.0132 | 0.0119 | 0.0152 | 0.0121 | 0.0280 | 0.0641 | 0.0086 | 0.0133 | 0.0169 | 0.0062 |
| Seal Rock | Oc16 | 0.0123 | 0.0122 | 0.0109 | 0.0169 | 0.0249 | 0.0579 | 0.0057 | 0.0089 | 0.0109 | 0.0047 |
| North Bay | Oc17 | 0.0116 | 0.0091 | 0.0116 | 0.0146 | 0.0192 | 0.0580 | 0.0050 | 0.0046 | 0.0022 | 0.0120 |
| Oakland Bay | Oc18 | 0.0139 | 0.0118 | 0.0144 | 0.0169 | 0.0202 | 0.0591 | 0.0062 | 0.0060 | 0.0049 | 0.0156 |
| Oyster Bay | Oc19 | 0.0132 | 0.0075 | 0.0125 | 0.0175 | 0.0271 | 0.0657 | 0.0045 | 0.0068 | 0.0031 | 0.0117 |
| Willapa Bay 1 | Oc20 | 0.0199 | 0.0261 | 0.0146 | 0.0455 | 0.0348 | 0.0932 | 0.0369 | 0.0427 | 0.0376 | 0.0380 |
| Willapa Bay 2 | Oc21 | 0.0197 | 0.0208 | 0.0152 | 0.0418 | 0.0425 | 0.0936 | 0.0339 | 0.0399 | 0.0369 | 0.0383 |
| Yaquina Bay 1 | Oc24 | 0.0118 | 0.0141 | 0.0113 | 0.0332 | 0.0254 | 0.0751 | 0.0205 | 0.0235 | 0.0246 | 0.0176 |
| Yaquina Bay 2 | Oc25 | 0.0124 | 0.0147 | 0.0114 | 0.0319 | 0.0258 | 0.0763 | 0.0229 | 0.0262 | 0.0262 | 0.0246 |
| CB North Bend | Oc26 | 0.0211 | 0.0222 | 0.0155 | 0.0443 | 0.0364 | 0.0939 | 0.0324 | 0.0376 | 0.0347 | 0.0340 |
| CB Citrus Dock | Oc27 | 0.0135 | 0.0160 | 0.0118 | 0.0337 | 0.0251 | 0.0730 | 0.0249 | 0.0308 | 0.0275 | 0.0292 |
| CB Blossom Creek | Oc28 | 0.0167 | 0.0180 | 0.0141 | 0.0386 | 0.0340 | 0.0846 | 0.0283 | 0.0352 | 0.0310 | 0.0316 |
| CB Shinglehouse | Oc29 | 0.0226 | 0.0212 | 0.0176 | 0.0399 | 0.0341 | 0.0922 | 0.0328 | 0.0401 | 0.0357 | 0.0397 |
| Coos Bay | Oc30 | 0.0153 | 0.0195 | 0.0118 | 0.0339 | 0.0260 | 0.0811 | 0.0268 | 0.0328 | 0.0255 | 0.0276 |
| Tomales Bay East | Oc31 | 0.0164 | 0.0117 | 0.0082 | 0.0338 | 0.0365 | 0.0908 | 0.0196 | 0.0281 | 0.0203 | 0.0295 |
| Tomales Bay West | Oc32 | 0.0104 | 0.0156 | 0.0063 | 0.0316 | 0.0348 | 0.0829 | 0.0214 | 0.0264 | 0.0193 | 0.0277 |
| Redwood Port | Oc34 | 0.0109 | 0.0093 | 0.0081 | 0.0243 | 0.0288 | 0.0728 | 0.0160 | 0.0262 | 0.0228 | 0.0252 |
| Toquaht River | Oc36 | 0.0110 | 0.0110 | 0.0123 | 0.0013 | 0.0263 | 0.0699 | 0.0180 | 0.0236 | 0.0237 | 0.0227 |
| Sailing Lake | Oc37 | 0.0254 | 0.0197 | 0.0209 | 0.0423 | 0.0372 | 0.0893 | 0.0285 | 0.0348 | 0.0285 | 0.0455 |
| Henderson Inlet | Oc38 | 0.0091 | 0.0075 | 0.0105 | 0.0164 | 0.0180 | 0.0618 | 0.0068 | 0.0069 | 0.0062 | 0.0141 |
| Eld Inlet | Oc39 | 0.0146 | 0.0124 | 0.0151 | 0.0206 | 0.0224 | 0.0578 | 0.0053 | 0.0060 | 0.0055 | 0.0163 |
| Liberty Bay 1 | Oc40 | 0.0089 | 0.0072 | 0.0077 | 0.0167 | 0.0249 | 0.0707 | 0.0034 | -0.0010 | 0.0015 | 0.0067 |
| Liberty Bay 2 | Oc41 | 0.0135 | 0.0150 | 0.0134 | 0.0138 | 0.0187 | 0.0527 | 0.0066 | 0.0034 | 0.0028 | 0.0147 |
| Humbolt Bay | Oc42 | 0.0137 | 0.0104 | 0.0087 | 0.0289 | 0.0214 | 0.0669 | 0.0152 | 0.0222 | 0.0149 | 0.0273 |
| Tomales Bay South | Oc43 | 0.0172 | 0.0187 | 0.0107 | 0.0323 | 0.0285 | 0.0776 | 0.0246 | 0.0316 | 0.0228 | 0.0285 |
| Tomales Bay North | Oc44 | 0.0190 | 0.0201 | 0.0122 | 0.0363 | 0.0335 | 0.0838 | 0.0263 | 0.0338 | 0.0237 | 0.0290 |
| Drakes Bay | Oc45 | 0.0109 | 0.0091 | 0.0088 | 0.0293 | 0.0228 | 0.0683 | 0.0133 | 0.0215 | 0.0149 | 0.0244 |
| Elkhorn Slough | Oc46 | 0.0163 | 0.0139 | 0.0100 | 0.0341 | 0.0260 | 0.0729 | 0.0221 | 0.0326 | 0.0250 | 0.0325 |
| Point Orient | Oc47 | 0.0192 | 0.0141 | 0.0127 | 0.0367 | 0.0308 | 0.0771 | 0.0240 | 0.0335 | 0.0272 | 0.0357 |
| Candlestick Park | Oc49 | 0.0169 | 0.0141 | 0.0109 | 0.0336 | 0.0297 | 0.0793 | 0.0224 | 0.0282 | 0.0246 | 0.0306 |

**Table 3.5** Continued.

| Sample Group | code | Oc15 | Oc16 | Oc17 | Oc18 | Oc19 | Oc20 | Oc21 | Oc24 | Oc25 | Oc26 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Klaskino | Oc01 | *** | **** | **** | **** | *** | **** | **** | **** | **** | **** |
| Amai | Oc02 | *** | *** | *** | *** | * | **** | **** | ** | **** | **** |
| Port Eliza | Oc03 | ** | *** | **** | **** | *** | *** | **** | *** | **** | **** |
| Torquart Bay | Oc04 | ** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Shoal Bay1 | Oc05 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Shoal Bay 2 | Oc06 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Discovery Bay | Oc10 | ** | *** | **** | **** | ** | **** | **** | **** | **** | **** |
| Clam Bay 1 | Oc12 | *** | **** | *** | **** | ** | **** | **** | **** | **** | **** |
| Clam Bay 2 | Oc13 | **** | **** | * | **** | NS | **** | **** | **** | **** | **** |
| Triton Cove 1 | Oc14 | NS | NS | ** | ** | NS | ** | **** | * | * | *** |
| Triton Cove 2 | Oc15 | | NS | *** | *** | * | **** | **** | *** | **** | **** |
| Seal Rock | Oc16 | 0.0030 | | **** | **** | **** | **** | **** | **** | **** | **** |
| North Bay | Oc17 | 0.0124 | 0.0075 | | NS | NS | **** | **** | **** | **** | **** |
| Oakland Bay | Oc18 | 0.0152 | 0.0092 | 0.0005 | | NS | **** | **** | **** | **** | **** |
| Oyster Bay | Oc19 | 0.0120 | 0.0091 | 0.0016 | 0.0022 | | **** | **** | *** | **** | **** |
| Willapa Bay 1 | Oc20 | 0.0437 | 0.0386 | 0.0386 | 0.0380 | 0.0387 | | NS | *** | *** | NS |
| Willapa Bay 2 | Oc21 | 0.0433 | 0.0391 | 0.0365 | 0.0375 | 0.0349 | 0.0003 | | **** | **** | * |
| Yaquina Bay 1 | Oc24 | 0.0261 | 0.0266 | 0.0223 | 0.0253 | 0.0216 | 0.0117 | 0.0109 | | NS | ** |
| Yaquina Bay 2 | Oc25 | 0.0262 | 0.0289 | 0.0242 | 0.0262 | 0.0217 | 0.0099 | 0.0086 | -0.0004 | | *** |
| CB North Bend | Oc26 | 0.0405 | 0.0368 | 0.0351 | 0.0335 | 0.0348 | 0.0004 | 0.0019 | 0.0062 | 0.0063 | |
| CB Citrus Dock | Oc27 | 0.0339 | 0.0280 | 0.0268 | 0.0258 | 0.0268 | 0.0008 | 0.0012 | 0.0055 | 0.0057 | 0.0000 |
| CB Blossom Creek | Oc28 | 0.0389 | 0.0337 | 0.0313 | 0.0309 | 0.0294 | -0.0008 | -0.0010 | 0.0086 | 0.0066 | 0.0018 |
| CB Shinglehouse | Oc29 | 0.0425 | 0.0377 | 0.0338 | 0.0336 | 0.0362 | 0.0015 | 0.0018 | 0.0122 | 0.0099 | 0.0019 |
| Coos Bay | Oc30 | 0.0338 | 0.0259 | 0.0287 | 0.0273 | 0.0269 | -0.0007 | 0.0069 | 0.0152 | 0.0135 | 0.0086 |
| Tomales Bay East | Oc31 | 0.0292 | 0.0263 | 0.0248 | 0.0258 | 0.0196 | 0.0156 | 0.0121 | 0.0121 | 0.0117 | 0.0112 |
| Tomales Bay West | Oc32 | 0.0295 | 0.0268 | 0.0236 | 0.0251 | 0.0195 | 0.0195 | 0.0190 | 0.0140 | 0.0137 | 0.0171 |
| Redwood Port | Oc34 | 0.0253 | 0.0235 | 0.0225 | 0.0231 | 0.0215 | 0.0257 | 0.0262 | 0.0180 | 0.0201 | 0.0246 |
| Toquaht River | Oc36 | 0.0151 | 0.0192 | 0.0238 | 0.0262 | 0.0217 | 0.0386 | 0.0379 | 0.0300 | 0.0274 | 0.0353 |
| Sailing Lake | Oc37 | 0.0438 | 0.0374 | 0.0315 | 0.0303 | 0.0317 | 0.0299 | 0.0261 | 0.0261 | 0.0255 | 0.0262 |
| Henderson Inlet | Oc38 | 0.0128 | 0.0112 | 0.0002 | 0.0035 | 0.0040 | 0.0326 | 0.0320 | 0.0167 | 0.0186 | 0.0264 |
| Eld Inlet | Oc39 | 0.0155 | 0.0086 | 0.0010 | 0.0009 | 0.0016 | 0.0412 | 0.0390 | 0.0228 | 0.0244 | 0.0363 |
| Liberty Bay 1 | Oc40 | 0.0127 | 0.0069 | 0.0000 | 0.0028 | 0.0020 | 0.0379 | 0.0362 | 0.0183 | 0.0224 | 0.0320 |
| Liberty Bay 2 | Oc41 | 0.0110 | 0.0076 | 0.0016 | 0.0029 | 0.0036 | 0.0441 | 0.0421 | 0.0260 | 0.0277 | 0.0380 |
| Humbolt Bay | Oc42 | 0.0254 | 0.0228 | 0.0146 | 0.0139 | 0.0136 | 0.0198 | 0.0198 | 0.0142 | 0.0132 | 0.0179 |
| Tomales Bay South | Oc43 | 0.0347 | 0.0289 | 0.0273 | 0.0262 | 0.0230 | 0.0156 | 0.0163 | 0.0137 | 0.0130 | 0.0144 |
| Tomales Bay North | Oc44 | 0.0349 | 0.0306 | 0.0285 | 0.0285 | 0.0234 | 0.0198 | 0.0204 | 0.0157 | 0.0156 | 0.0183 |
| Drakes Bay | Oc45 | 0.0285 | 0.0220 | 0.0180 | 0.0191 | 0.0155 | 0.0189 | 0.0181 | 0.0122 | 0.0141 | 0.0144 |
| Elkhorn Slough | Oc46 | 0.0338 | 0.0324 | 0.0281 | 0.0278 | 0.0262 | 0.0139 | 0.0131 | 0.0139 | 0.0133 | 0.0123 |
| Point Orient | Oc47 | 0.0393 | 0.0318 | 0.0291 | 0.0294 | 0.0281 | 0.0193 | 0.0172 | 0.0181 | 0.0193 | 0.0173 |
| Candlestick Park | Oc49 | 0.0330 | 0.0288 | 0.0265 | 0.0275 | 0.0239 | 0.0240 | 0.0217 | 0.0180 | 0.0183 | 0.0212 |

**Table 3.5** Continued.

| Sample Group | code | Oc27 | Oc28 | Oc29 | Oc30 | Oc31 | Oc32 | Oc34 | Oc36 | Oc37 | Oc38 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Klaskino | Oc01 | **** | **** | **** | ** | **** | *** | ** | NS | **** | *** |
| Amai | Oc02 | **** | *** | **** | ** | **** | **** | * | NS | **** | ** |
| Port Eliza | Oc03 | **** | **** | **** | ** | *** | *** | ** | * | **** | **** |
| Torquart Bay | Oc04 | **** | **** | **** | *** | **** | **** | ** | NS | **** | **** |
| Shoal Bay1 | Oc05 | **** | **** | **** | **** | **** | **** | ** | **** | **** | **** |
| Shoal Bay 2 | Oc06 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Discovery Bay | Oc10 | **** | **** | **** | **** | **** | **** | *** | *** | **** | *** |
| Clam Bay 1 | Oc12 | **** | **** | **** | **** | **** | **** | **** | **** | **** | *** |
| Clam Bay 2 | Oc13 | **** | **** | **** | **** | **** | **** | **** | **** | **** | ** |
| Triton Cove 1 | Oc14 | *** | ** | *** | ** | ** | *** | ** | *** | **** | * |
| Triton Cove 2 | Oc15 | **** | **** | **** | **** | *** | **** | * | *** | **** | ** |
| Seal Rock | Oc16 | **** | **** | **** | *** | **** | **** | **** | **** | **** | **** |
| North Bay | Oc17 | **** | **** | **** | **** | **** | **** | *** | **** | **** | NS |
| Oakland Bay | Oc18 | **** | **** | **** | **** | **** | **** | **** | **** | **** | NS |
| Oyster Bay | Oc19 | **** | **** | **** | **** | **** | **** | ** | **** | **** | NS |
| Willapa Bay 1 | Oc20 | NS | NS | NS | NS | **** | **** | *** | **** | **** | **** |
| Willapa Bay 2 | Oc21 | NS | NS | NS | NS | **** | **** | **** | **** | **** | **** |
| Yaquina Bay 1 | Oc24 | ** | **** | *** | *** | *** | **** | ** | **** | **** | **** |
| Yaquina Bay 2 | Oc25 | *** | **** | **** | *** | **** | **** | *** | **** | **** | **** |
| CB North Bend | Oc26 | NS | * | * | * | **** | **** | *** | **** | **** | **** |
| CB Citrus Dock | Oc27 | | NS | NS | NS | **** | **** | **** | **** | **** | **** |
| CB Blossom Creek | Oc28 | 0.0004 | | NS | NS | **** | **** | *** | **** | **** | **** |
| CB Shinglehouse | Oc29 | 0.0002 | 0.0013 | | NS | **** | **** | *** | **** | **** | **** |
| Coos Bay | Oc30 | 0.0026 | 0.0036 | 0.0048 | | **** | **** | ** | **** | **** | **** |
| Tomales Bay East | Oc31 | 0.0116 | 0.0110 | 0.0154 | 0.0159 | | NS | NS | **** | **** | **** |
| Tomales Bay West | Oc32 | 0.0159 | 0.0188 | 0.0289 | 0.0170 | -0.0007 | | * | **** | **** | **** |
| Redwood Port | Oc34 | 0.0180 | 0.0222 | 0.0281 | 0.0144 | 0.0046 | 0.0056 | | **** | NS | *** |
| Toquaht River | Oc36 | 0.0316 | 0.0335 | 0.0327 | 0.0299 | 0.0333 | 0.0340 | 0.0263 | | **** | **** |
| Sailing Lake | Oc37 | 0.0210 | 0.0257 | 0.0244 | 0.0215 | 0.0060 | 0.0146 | 0.0077 | 0.0462 | | **** |
| Henderson Inlet | Oc38 | 0.0215 | 0.0286 | 0.0311 | 0.0252 | 0.0220 | 0.0190 | 0.0192 | 0.0239 | 0.0313 | |
| Eld Inlet | Oc39 | 0.0287 | 0.0330 | 0.0375 | 0.0313 | 0.0289 | 0.0258 | 0.0260 | 0.0273 | 0.0343 | 0.0029 |
| Liberty Bay 1 | Oc40 | 0.0282 | 0.0311 | 0.0371 | 0.0272 | 0.0225 | 0.0166 | 0.0198 | 0.0222 | 0.0311 | 0.0008 |
| Liberty Bay 2 | Oc41 | 0.0305 | 0.0376 | 0.0391 | 0.0336 | 0.0302 | 0.0270 | 0.0271 | 0.0197 | 0.0394 | 0.0026 |
| Humbolt Bay | Oc42 | 0.0133 | 0.0167 | 0.0173 | 0.0110 | 0.0046 | 0.0068 | 0.0032 | 0.0291 | 0.0056 | 0.0134 |
| Tomales Bay South | Oc43 | 0.0099 | 0.0128 | 0.0156 | 0.0093 | 0.0007 | 0.0052 | 0.0057 | 0.0329 | 0.0093 | 0.0246 |
| Tomales Bay North | Oc44 | 0.0143 | 0.0162 | 0.0209 | 0.0129 | 0.0028 | 0.0044 | 0.0104 | 0.0396 | 0.0124 | 0.0273 |
| Drakes Bay | Oc45 | 0.0110 | 0.0141 | 0.0192 | 0.0122 | 0.0021 | 0.0035 | 0.0057 | 0.0296 | 0.0096 | 0.0155 |
| Elkhorn Slough | Oc46 | 0.0106 | 0.0118 | 0.0139 | 0.0139 | 0.0004 | 0.0080 | 0.0040 | 0.0299 | 0.0102 | 0.0235 |
| Point Orient | Oc47 | 0.0130 | 0.0154 | 0.0176 | 0.0128 | 0.0018 | 0.0091 | -0.0003 | 0.0347 | 0.0027 | 0.0271 |
| Candlestick Park | Oc49 | 0.0165 | 0.0196 | 0.0228 | 0.0143 | 0.0019 | 0.0057 | -0.0015 | 0.0325 | 0.0039 | 0.0254 |

**Table 3.5** Continued.

| Sample Group | code | Oc39 | Oc40 | Oc41 | Oc42 | Oc43 | Oc44 | Oc45 | Oc46 | Oc47 | Oc49 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Klaskino | Oc01 | *** | *** | *** | **** | **** | **** | **** | **** | **** | **** |
| Amai | Oc02 | *** | ** | *** | **** | **** | **** | *** | **** | **** | **** |
| Port Eliza | Oc03 | **** | *** | *** | **** | **** | **** | **** | **** | **** | **** |
| Torquart Bay | Oc04 | **** | **** | *** | **** | **** | **** | **** | **** | **** | **** |
| Shoal Bay1 | Oc05 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Shoal Bay 2 | Oc06 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Discovery Bay | Oc10 | ** | ** | ** | **** | **** | **** | **** | **** | **** | **** |
| Clam Bay 1 | Oc12 | *** | NS | * | **** | **** | **** | **** | **** | **** | **** |
| Clam Bay 2 | Oc13 | ** | NS | NS | **** | **** | **** | **** | **** | **** | **** |
| Triton Cove 1 | Oc14 | * | NS | NS | ** | *** | *** | *** | *** | **** | *** |
| Triton Cove 2 | Oc15 | ** | ** | ** | **** | **** | **** | **** | **** | **** | **** |
| Seal Rock | Oc16 | **** | **** | *** | **** | **** | **** | **** | **** | **** | **** |
| North Bay | Oc17 | NS | * | NS | **** | **** | **** | **** | **** | **** | **** |
| Oakland Bay | Oc18 | NS | ** | ** | **** | **** | **** | **** | **** | **** | **** |
| Oyster Bay | Oc19 | NS | NS | NS | **** | **** | **** | **** | **** | **** | **** |
| Willapa Bay 1 | Oc20 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Willapa Bay 2 | Oc21 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Yaquina Bay 1 | Oc24 | **** | *** | **** | **** | **** | **** | **** | **** | **** | **** |
| Yaquina Bay 2 | Oc25 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| CB North Bend | Oc26 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| CB Citrus Dock | Oc27 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| CB Blossom Creek | Oc28 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| CB Shinglehouse | Oc29 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Coos Bay | Oc30 | **** | **** | **** | **** | *** | *** | **** | **** | *** | **** |
| Tomales Bay East | Oc31 | **** | **** | **** | * | NS | NS | NS | NS | NS | NS |
| Tomales Bay West | Oc32 | **** | **** | **** | ** | * | * | NS | ** | ** | *** |
| Redwood Port | Oc34 | *** | *** | ** | NS | NS | * | NS | NS | NS | NS |
| Toquaht River | Oc36 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| Sailing Lake | Oc37 | **** | **** | **** | **** | **** | **** | **** | **** | ** | **** |
| Henderson Inlet | Oc38 | NS | * | NS | **** | **** | **** | **** | **** | **** | **** |
| Eld Inlet | Oc39 | | NS | NS | **** | **** | **** | **** | **** | **** | **** |
| Liberty Bay 1 | Oc40 | 0.0006 | | * | **** | **** | **** | **** | **** | **** | **** |
| Liberty Bay 2 | Oc41 | 0.0009 | 0.0029 | | **** | **** | **** | **** | **** | **** | **** |
| Humbolt Bay | Oc42 | 0.0173 | 0.0155 | 0.0212 | | *** | **** | * | *** | NS | ** |
| Tomales Bay South | Oc43 | 0.0288 | 0.0266 | 0.0298 | 0.0049 | | NS | NS | ** | * | *** |
| Tomales Bay North | Oc44 | 0.0321 | 0.0277 | 0.0328 | 0.0084 | -0.0013 | | *** | *** | *** | **** |
| Drakes Bay | Oc45 | 0.0243 | 0.0172 | 0.0230 | 0.0034 | 0.0031 | 0.0064 | | *** | ** | *** |
| Elkhorn Slough | Oc46 | 0.0309 | 0.0265 | 0.0332 | 0.0038 | 0.0052 | 0.0110 | 0.0053 | | NS | **** |
| Point Orient | Oc47 | 0.0318 | 0.0263 | 0.0369 | 0.0026 | 0.0054 | 0.0091 | 0.0053 | 0.0006 | | NS |
| Candlestick Park | Oc49 | 0.0295 | 0.0231 | 0.0329 | 0.0035 | 0.0038 | 0.0090 | 0.0056 | 0.0057 | 0.0009 | |

*Regional-scale genetic differentiation*

Hierarchical AMOVA based on broad geographical regions found evidence for genetic differentiation at this scale ($F_{ST\ Total}$ = 0.024183, $P \leq 0.01$) and among regions ($F_{ST\ Among}$ = 0.017408, $P \leq 0.01$), corresponding to approximately 72% of the total genetic variation being distributed among the eight geographical regions (Vancouver Island BC, Puget Sound WA, Willapa Bay WA, Yaquina Bay OR, Coos Bay OR, Tomales Bay CA, San Francisco Bay CA and coastal California estuaries surrounding San Francisco Bay, which included Humboldt Bay, Drakes Bay and Elkhorn Slough) and 28% within populations. A neighbor-joining tree arbitrarily rooted at Yaquina Bay, OR (Figure 3.7), reveals five distinct groups, largely in accordance with regions (Vancouver Island, Puget Sound, Willapa/Coos Bays, Yaquina Bay, and all California Bays).

The non-significant ($P > 0.05$) pairwise $F_{ST}$ values between all combinations of Willapa and Coos Bays populations (Table 3.5) and a non-significant factorial correspondence analysis ($F_{ST}$ = -0.0008; $P$ = 0.8200) based on pooled sample groups within individual bays (Figure 3.8) supports our belief that these geographically distant populations are genetically similar and their clustering is correctly depicted as a single group based on genetic distances (Figure 3.7).

A significant ($P \leq 0.000001$) positive Spearman's Rank correlation (Mantel test) between geographic and genetic distances ($F_{ST}/(1- F_{ST})$ across all population pairs (Figure 3.9) indicates a strong pattern of isolation by distance among Olympia oyster populations despite the poor fit of the linear model ($R^2 = 0.1577$). This correlation remained significant ($P \leq 0.00160$) across all pooled population pairs (Figure 3.10) after conservatively accounting for non-independence among sampling groups by pooling all genetically non-differentiated samples within any geographic location or sub-region (Table 3.6).

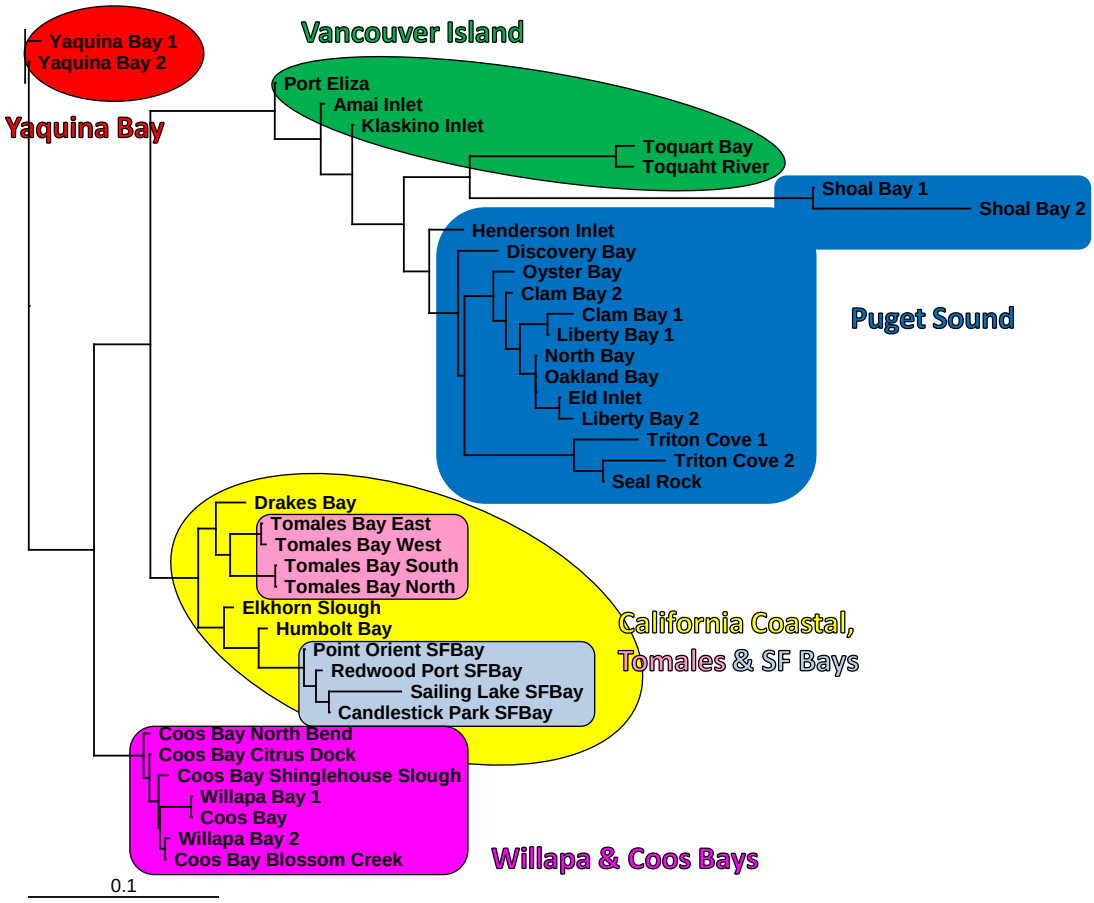


**Figure 3.7** Neighbor-joining tree based on Nei's (1978) genetic distance and arbitrarily rooted at Yaquina Bay OR for all Olympia oyster populations. Sites ranged from the northern tip of Vancouver Island BC southward to Elkhorn Slough CA. Populations clustered into five groups, largely in accordance with major geographic regions (Vancouver Island, Puget Sound, Willapa/Coos Bays, Yaquina Bay, and all California Bays). Approximately 72% of the total genetic variation was distributed among the eight defined geographical regions.

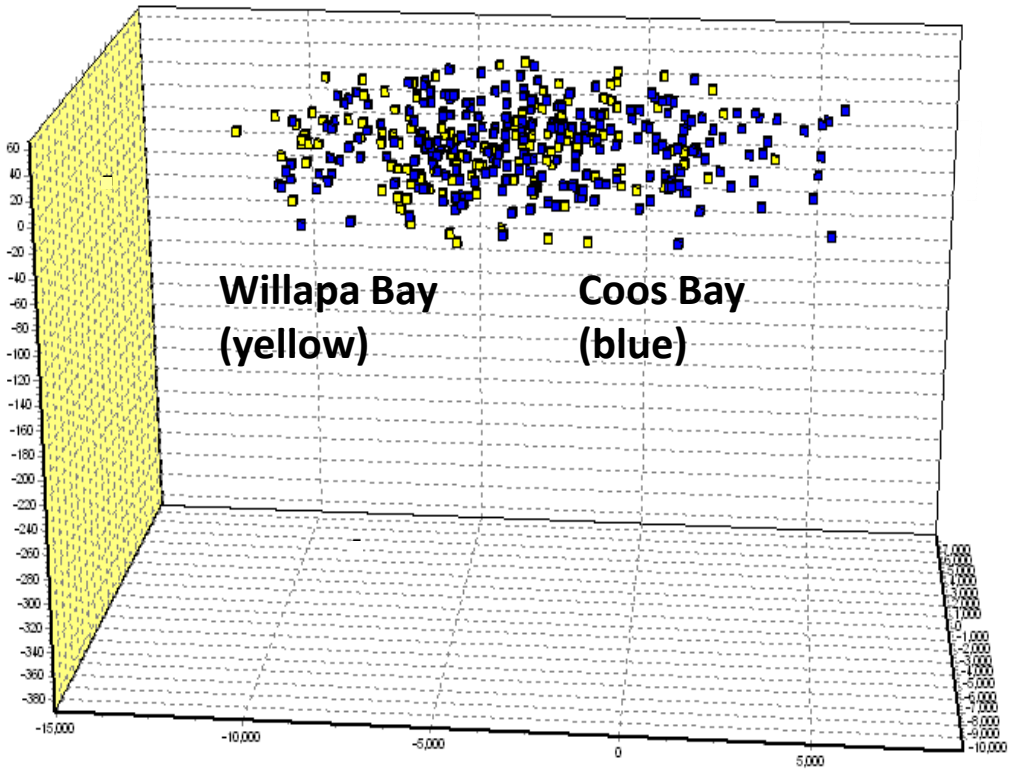


**Figure 3.8** Factorial correspondence analysis between Willapa Bay WA and Coos Bay OR. Our results support the premise that the Coos Bay OR population, which was non-existent 30 years ago, is the result of an introduction event from Willapa Bay WA ($F_{ST}$ = -0.0008; $P$ = 0.8200).

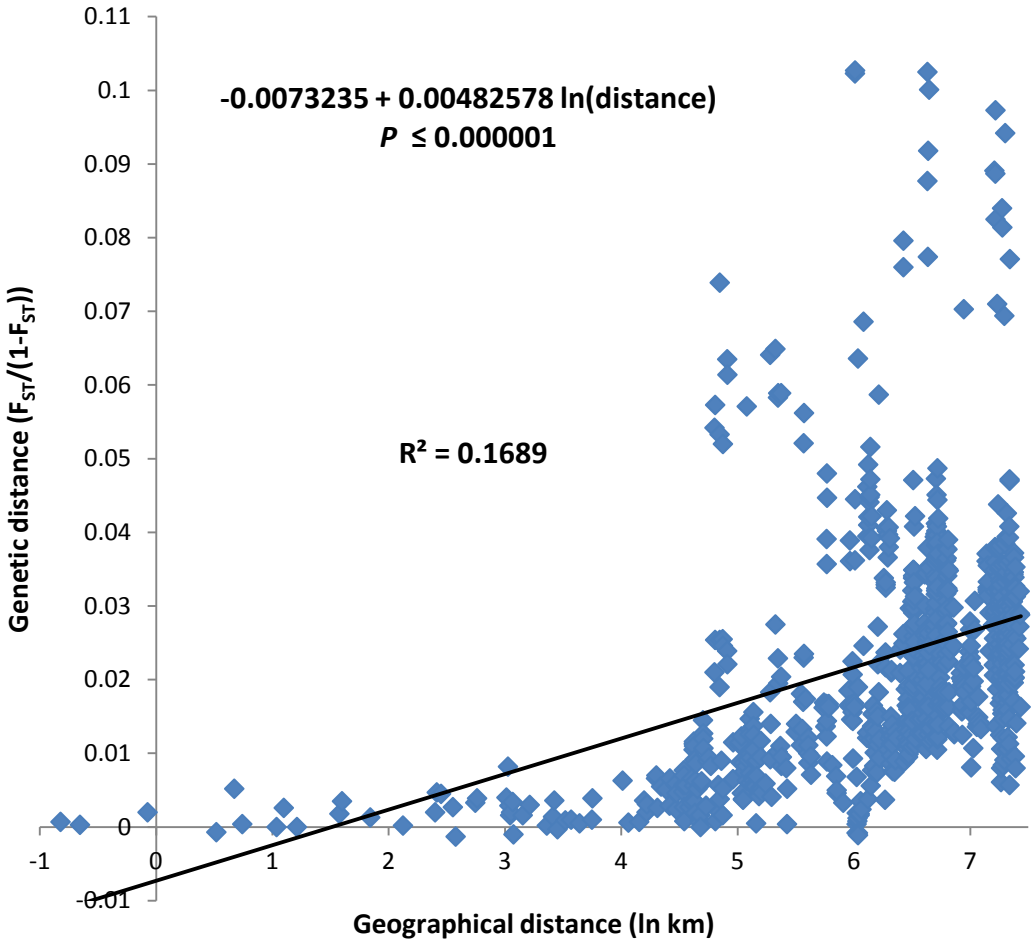


**Figure 3.9** Significant relationship between genetic distance ($F_{ST}/(1-F_{ST})$) and geographical distance (ln km) for non-pooled Olympia oyster populations.

**Table 3.6** Mantel test sample group pools. We pooled all samples within any geographic location or sub-region not found to be genetically differentiated. We also considered samples originating from Coos Bay OR to be the result of an introduction event from Willapa Bay WA**.**

| Mantel Pool | Sample Group | General Population Location |
|---|---|---|
| 1 | Oc01 | Klaskino Inlet, Vancouver Island, BC (near Port Alice) |
| 2 | Oc02 | Amai Inlet, Vancouver Island, BC |
| 3 | Oc03 | Port Eliza, Vancouver Island, BC |
| 4 | Oc04 | Toquart Bay, Vancouver Island, BC (Lucky Creek, Barkley Sound) |
| 4 | Oc36 | Toquaht River, Vancouver Island, BC |
| 5 | Oc05 | Shoal Bay, Lopez Island, WA (North Puget Sound) |
| 5 | Oc06 | Shoal Bay, Lopez Island, WA (North Puget Sound) |
| 6 | Oc10 | Discovery Bay, WA (Striaght of Juan de Fuca) |
| 7 | Oc12 | Clam Bay, Rich Passage, WA (Puget Sound Central Basin) |
| 7 | Oc13 | Clam Bay, Rich Passage, WA (Puget Sound Central Basin) |
| 7 | Oc40 | Liberty Bay, WA (Puget Sound Central Basin) |
| 7 | Oc41 | Liberty Bay, WA (Puget Sound Central Basin) |
| 8 | Oc14 | Triton Cove, WA (Hood Canal) |
| 8 | Oc15 | Triton Cove, WA (Hood Canal) |
| 8 | Oc16 | Seal Rock, WA (Hood Canal) |
| 9 | Oc17 | North Bay, Case Inlet, WA (South Puget Sound) |
| 9 | Oc18 | Oakland Bay, Hammersley Inlet, WA (South Puget Sound) |
| 9 | Oc19 | Oyster Bay, Totten Inlet, WA (South Puget Sound) |
| 9 | Oc38 | Henderson Inlet, WA (South Puget Sound) |
| 9 | Oc39 | Eld Inlet, WA (South Puget Sound) |
| 10 | Oc20 | Willapa Bay, Nahcotta, WA |
| 10 | Oc21 | Willapa Bay, Nahcotta, WA |
| 10 | Oc26 | Coos Bay, North Bend, OR |
| 10 | Oc27 | Coos Bay, Citrus Dock, OR |
| 10 | Oc28 | Coos Bay, Blossom Creek Boardwalk, OR |
| 10 | Oc29 | Coos Bay, Shinglehouse Slough, OR |
| 10 | Oc30 | Coos Bay, North Bend, OR |
| 11 | Oc24 | Yaquina Bay, Oregon Oyster, OR |
| 11 | Oc25 | Yaquina Bay, Oregon Oyster, OR |
| 12 | Oc31 | Tomales Bay, CA (East Bay) |
| 12 | Oc32 | Tomales Bay, CA (West Bay) |
| 12 | Oc43 | Tomales Bay, CA (South Bay) |
| 12 | Oc44 | Tomales Bay, CA (North Bay) |
| 13 | Oc34 | San Francisco Bay, Redwood Port, CA |
| 13 | Oc37 | San Francisco Bay, Sailing Lake, CA |
| 13 | Oc47 | San Francisco Bay, Point Orient, CA |
| 13 | Oc49 | San Francisico Bay, Candlestick Park, CA |
| 14 | Oc42 | Humbolt Bay, Mad River Estuary, CA |
| 15 | Oc45 | Drakes Bay, CA |
| 16 | Oc46 | Elkhorn Slough, CA |

BC, British Columbia; WA, Washington; OR, Oregon; CA, California.

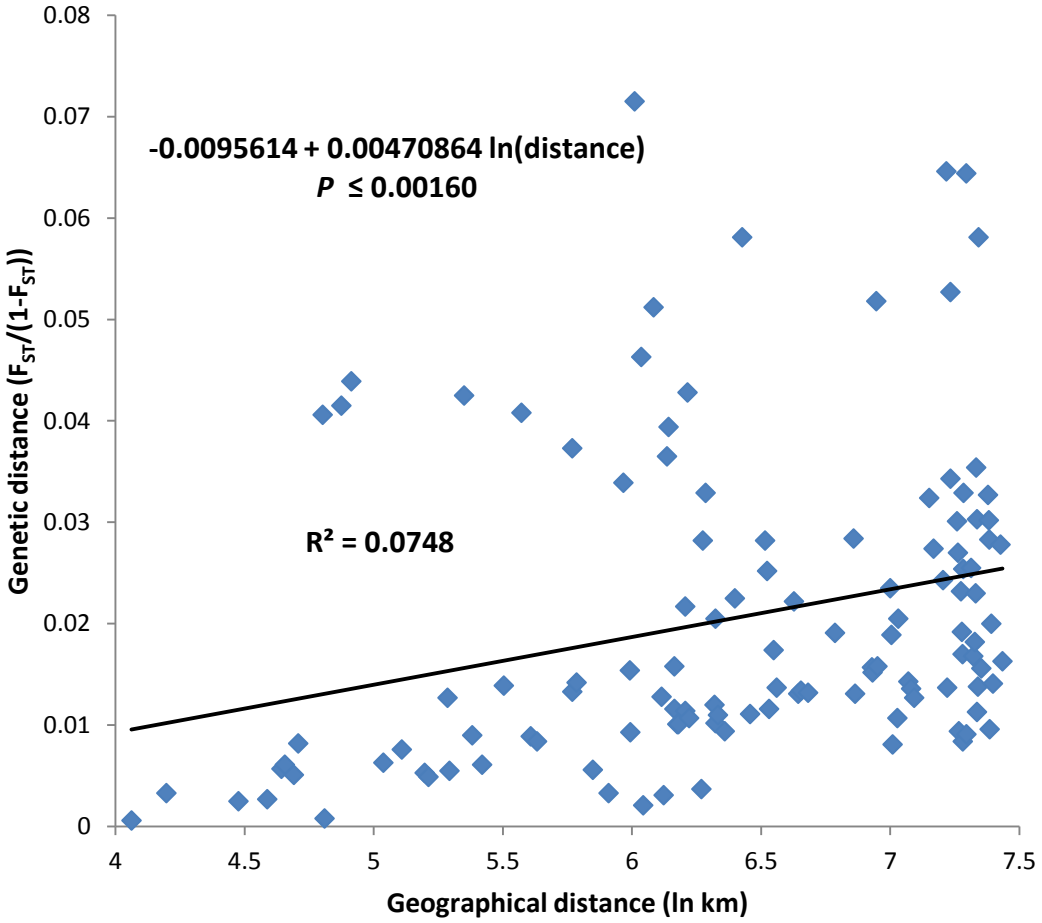


**Figure 3.10** Significant relationship between genetic distance ($F_{ST}$/(1-$F_{ST}$)) and geographical distance (ln km) for pooled Olympia oyster populations. The Mantel test remained significant ($P \leq 0.00160$) after pooling all samples within any geographic location or sub-region not found to be genetically differentiated (Table 3.6) to account for non-independent sampling.

*Sub-regional scale genetic differentiation*

We detected significant genetic differentiation between populations within both the Puget Sound and California regions. We found significant overall genetic differentiation for all Puget Sound populations $F_{ST\ PSTotal}$ = 0.014218 ($P \leq 0.01$) and among geographical Puget Sound sub-regions $F_{ST\ PS\ Among}$ = 0.010696 ($P \leq 0.01$), which corresponds to approximately 75% of the total genetic variation attributed to the 4 sub-regions (North Puget Sound, Hood Canal, Central Basin and South Puget Sound) and 25% within populations. A neighbor-joining tree arbitrarily rooted at Discovery Bay, WA (Figure 3.11), revealed 4 distinct groups, again largely in accordance with Puget Sound sub-regions.

Although populations from the South Puget Sound and Central Basin formed distinct groups based on genetic distances (Figure 3.11), closer examination of pairwise $F_{ST}$ estimates (Table 3.5) and bootstrapping of the neighbor-joining trees provided no consensus supporting genetic differentiation between these two sub-regions. Liberty Bay 2 samples (Central Basin) were adult Olympia oysters that colonized discarded shell from a defunct Pacific oyster processing plant, while Liberty Bay 1 samples were second-year set from an ongoing Olympia oyster habitat enhancement project (Davis *et al.* 2010). These two sites are separated by less than 150 meters and were assumed to be a single

population. However, these two sample groups differ significantly ($F_{ST}$ = 0.029; $P$ = 0.01776).

Because the North Puget Sound populations are so genetically dissimilar from any other Puget Sound site, we questioned whether the Shoal Bay samples were driving our overall significant results and examined the possibility of genetic differentiation among the three remaining geographical Puget Sound regions (Hood Canal, Central Basin and South Puget Sound). However, removal of the Shoal Bay samples from the dataset did not affect our conclusions; we still found significant overall differentiation ($F_{ST\ PS\text{-}\ Total}$ = 0.006587; $P \leq 0.01$) and differentiation among the remaining geographical regions ($F_{ST\ PS\text{-}\ Among}$ = 0.003678, $P \leq 0.01$), corresponding to approximately 56% of the total genetic variation attributed to the three Puget Sound regions of Hood Canal, Central Basin and South Puget Sound while 44% was within populations.

There is some indication that gene flow may be limited between north and south Vancouver Island, BC and that our North Puget Sound samples are more genetically similar to samples from south Vancouver Island than to the rest of the Puget Sound (Figure 3.7; Table 3.5). We did not, however, have adequate sampling from these regions to resolve this consideration.

All California populations grouped together in the regional analysis (Figure 3.7). However, because the Tomales Bay sample groups were genetically more similar to other samples from that bay, we tested for potential genetic substructure among remaining California populations by sub-partitioning the total genetic variation within the California region using AMOVA. Again, we found significant overall genetic differentiation ($F_{ST\ CA\ Total}$ = 0.006668; $P \leq 0.01$) and among geographical regions ($F_{ST\ CA\ Among}$ = 0.003733; $P \leq 0.01$), corresponding to approximately 56% of the total genetic variation attributed to the 3 geographic regions (Tomales Bay, San Francisco Bay and coastal California estuaries, which included Humboldt Bay, Drakes Bay and Elkhorn Slough) and 44% within populations. Figure 3.12 is a neighbor-joining tree arbitrarily rooted at Humboldt Bay, CA showing these three distinct genetic groups.

AMOVA analysis of sites within San Francisco Bay found genetic differentiation among populations ($F_{ST\ SB\ Total}$ = 0.002816; $P \leq 0.01$) but not among the arbitrarily created north and south bay regions ($F_{ST\ SB\ Among}$ = 0.000425, $P > 0.05$), corresponding to approximately 15% of total genetic variation attributed to the North and South San Francisco Bay regions and 85% within subpopulations. Closer examination of pairwise $F_{ST}$ values for San Francisco Bay populations (Table 3.5) reveals that Sailing Lake samples were significantly different, even after Bonferroni correction, from all other San Francisco Bay subpopulations indicating that our failure to identify structure may be due to uninformative

partitioning of the genetic variance in our AMOVA caused by inadequate sampling from this region (Figure 3.13).

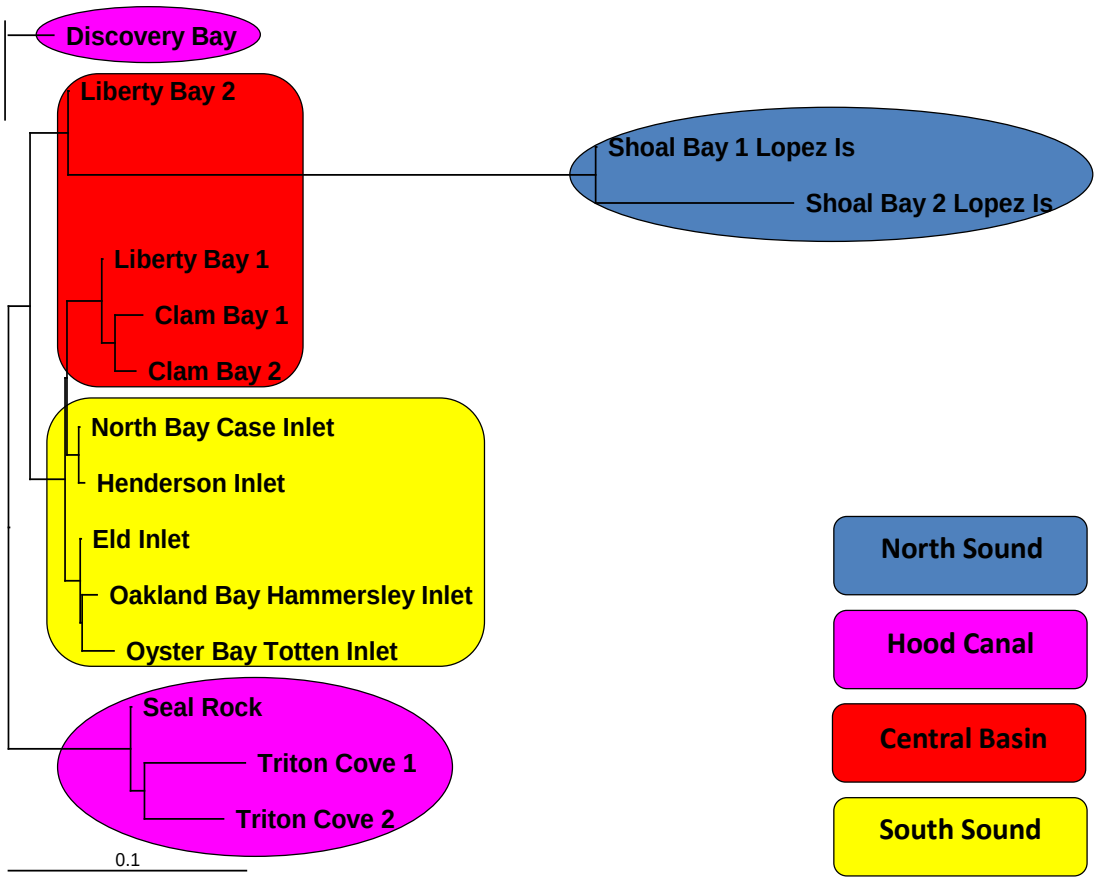


**Figure 3.11** Neighbor-joining tree based on Nei's (1978) genetic distance and arbitrarily rooted at Discovery Bay WA for 15 Puget Sound Olympia oyster populations. Populations clustered into four groups, largely in accordance with major geographic sub-regions of the sound (North Puget Sound, Hood Canal, Central Basin and South Puget Sound). Approximately 75% of the total genetic variation was distributed among the four defined geographical sub-regions. However, there was no evidence of significant genetic differentiation between any South Puget Sound population and Central Basin population with the exception of the Liberty Bay 2 site.

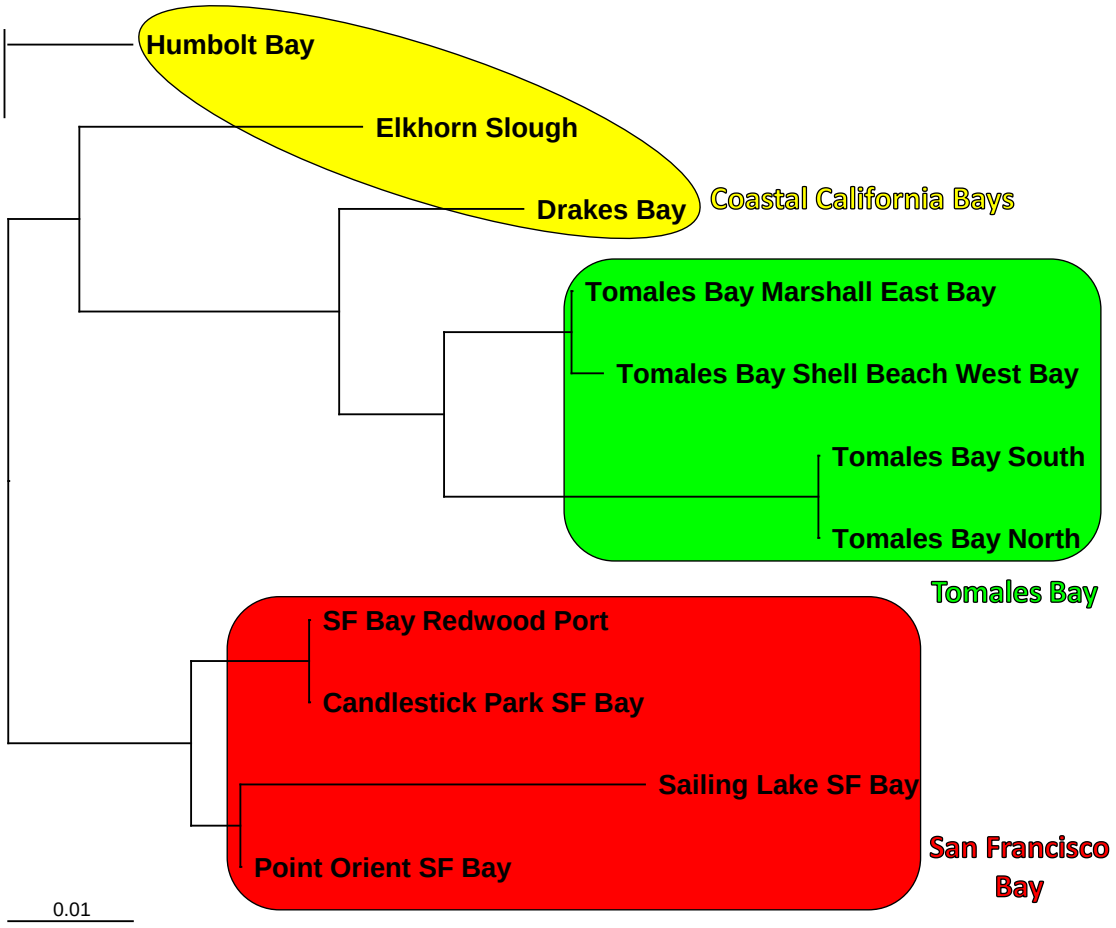


**Figure 3.12** Neighbor-joining tree based on Nei's (1978) genetic distance and arbitrarily rooted at Humboldt Bay CA for 11 California Olympia oyster populations. Populations clustered into three groups, largely in accordance with major geographic regions of the area (Tomales Bay, San Francisco Bay and the coastal bays of Humboldt Bay, Drakes Bay and Elkhorn Slough). Approximately 56% of the total genetic variation was attributed to the three defined geographical regions.

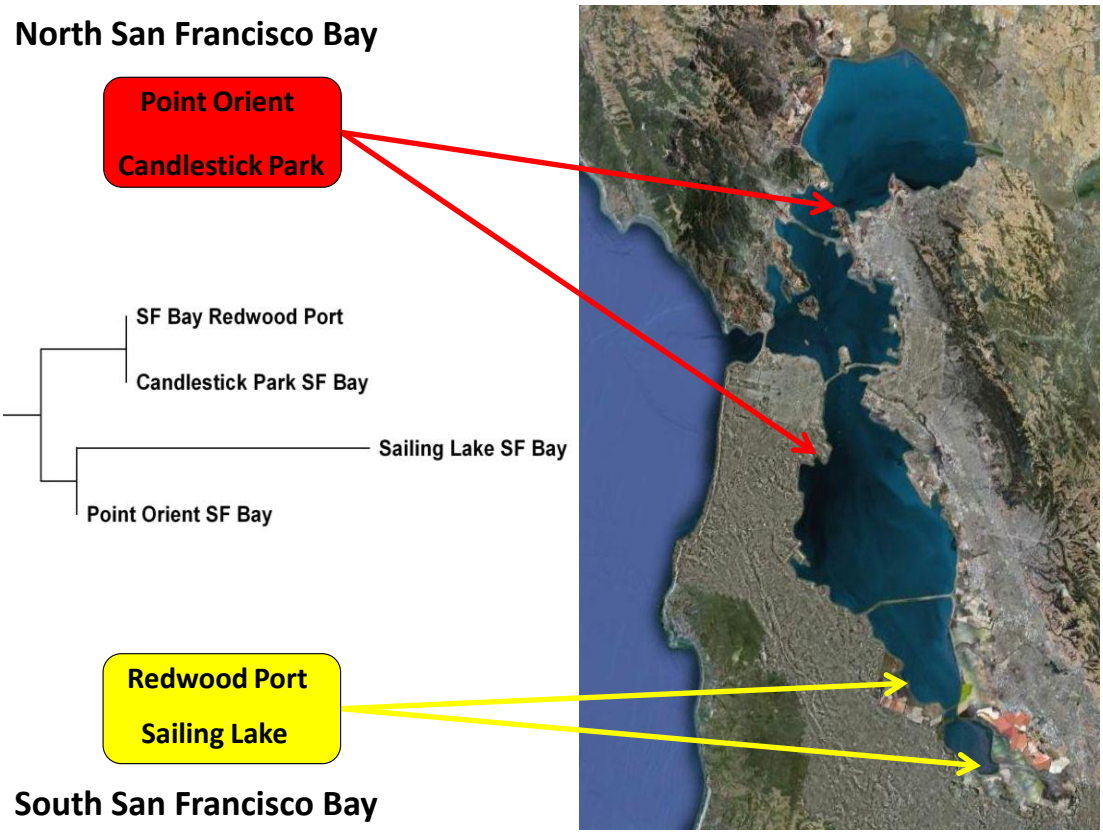


**Figure 3.13** General location of surveyed San Francisco Bay CA Olympia oyster (*O. lurida*) populations. The San Francisco Bay hierarchical AMOVA partitioned the total genetic variation into components corresponding to two broad geographical regions created without any genetic preconceptions (North and South San Francisco Bay). However, only 15% of the total genetic differentiation variation was among these arbitrary regions. Sailing Lake was, however, genetically different from all other surveyed populations based on $F_{ST}$ values.

## Discussion

The potential ecological benefits provided by oyster beds and their cultural significance to Pacific Northwest Native American tribes has motivated restoration and supplementation efforts for the Olympia oyster. These range from simple habitat restoration projects that provide suitable substrate for local larval recruitment to projects utilizing hatchery-propagated juvenile seed from broodstock sources that may or may not be closely associated with the restoration or supplementation site. However, these efforts have proceeded without a clear understanding of existing genetic structure among extant populations. The existence of genetic structure may influence decision-makers overseeing ongoing Olympia oyster restoration efforts.

The loci we used were generally well behaved and lacked any negative microsatellite characteristic that would interfere with our ability to resolve population structure. Overall, our data appeared reliable and non-neutrality, linkage disequilibrium (LD) and deviations from Hardy-Weinberg Equilibrium (HWE) caused by null alleles or high genetic load were not observed. Microsatellite variation is assumed to be selectively neutral and has generally been shown to be uncorrelated with potentially adaptive, quantitative genetic variation (Reed& Frankham 2001). Although linkage of loci to adaptive genes is

possible, comparison of observed allelic distribution across populations (data not provided) discount this possibility.

Despite global heterozygote deficiencies for all loci ($P \leq 0.05$), we found no systematic issues with either LD or HWE within populations that could bias our results. Oyster genotypic frequencies commonly deviate from HWE expectations due to the presence of null alleles (McGoldrick *et al.* 2000; Reece *et al.* 2004) or as a consequence of high genetic load (Launey& Hedgecock 2001) but there is little evidence that this dramatically compromises their utility for resolving population differentiation (Carlsson 2008; Chapuis& Estoup 2007). Wahlund effects, inbreeding and selection can also lead to heterozygote deficiencies. Although they cannot be discounted, we observed no consistent trends in our results indicating any of these effects influenced our analyses or conclusions.

Without historical samples, we are not able to determine if the current differentiation we found reflects allelic and genotypic changes largely driven by anthropogenic or natural causes. Historical accounts indicate that all of the populations surveyed experienced periods of greatly reduced census numbers but we found no evidence of recent genetic bottlenecks using the program BOTTLENECK (Cornuet& Luikart 1996). In a recently bottlenecked population, expected gene diversity should be less than observed gene diversity because any recent reduction in effective population size would reduce allelic diversity more

than the observed genotypic diversity. However, when population numbers are small, as may be the case for many Olympia oyster populations, the migration of even a few individuals can quickly reduce the genetic bottleneck signature (Busch *et al.* 2007). It is also plausible that *O. lurida's* inherent genetic diversity quickly mitigates the bottleneck signature; reducing the already short, transient time period of bottleneck-induced heterozygous excess just as historical bottlenecks are not detectable after a few generations (Luikart& Cornuet 1998). Although contemporary population densities do not represent historical totals, consistent natural recruitment in most extant populations (Gillespie 2009; Groth& Rumrill 2009; Polson& Zacherl 2009) indicates densities of sufficient size to mitigate or completely negate any genetic differentiation caused by the effects of genetic drift.

High variation in reproductive success among individuals within a population (Hedgecock 1994) can also reduce the genetic diversity within any recruitment class relative to the population as a whole and therefore, drive differentiation between different recruitment cohorts collected from a common location. We sampled two or more temporally separated recruitment cohorts in eight bays (Shoal Bay WA, Clam Bay WA, Triton Cove WA, Willapa Bay WA, Yaquina Bay OR, Coos Bay OR, Tomales Bay and San Francisco Bay CA). Pairwise $F_{ST}$ values between these cohorts were not significant (Table 3.5) and provide no support of sweepstake recruitment events. Although the pairwise $F_{ST}$ value for samples

collected from Shoal Bay WA at two different times was significant ($P$ = 0.00058), this population has recently become extirpated and any perceived differentiation may have been indicative of this ongoing extirpative process.

We found surprisingly high levels of genetic structure among and within major geographical regions covering most of *O. lurida's* contemporary range. Our regional-scale AMOVA found that approximately 72% of the total genetic variation is among eight arbitrarily-defined geographic regions. The fact that *O. lurida's* preferred habitat is fragmented and patchily distributed may help, in part, to explain this finding. Most moderately stable polyhaline coastal bays and estuaries that experience periods of low salinity and also have substrate suitable for larval settlement are geographically separated by several hundred kilometers of ocean, preventing consistent gene flow from one population to another. Wind-driven currents off the Pacific Northwest coastline move surface water a maximum of approximately 30 km in a given day (Jay Peterson, pers. comm.; http://bragg.oce.orst.edu), requiring the pelagic larvae to survive extended periods of time at sub-optimal water temperatures (Hopkins 1937).

With the notable exception of Coos Bay OR, the genetic signature of every coastal estuary/bay from the Strait of Juan de Fuca southward differs from adjacent populations. Our results support the hypothesis that the present-day Coos Bay population is the result of a recent introduction event from Willapa Bay WA

(Baker *et al.* 1999). Thorough sampling of both bays (271 individuals from five sites within South Horn and Isthmus Sough region of Coos Bay and 150 individuals from two sites located within the Willapa Bay Oyster Reserves) found no evidence of genetic differentiation among sites within either bay or between the two regions as a whole. Furthermore, widespread biotic surveys thirty years ago found no evidence of any live Olympia oysters in Coos Bay (Baker *et al.* 1999; Groth& Rumrill 2009).

Puget Sound (Figure 3.2) gene flow population dynamics were largely considered panmictic (Blake& Peabody 2006) until this hypothesis was questioned by Stick *et al.* (2007). Our AMOVA sub-analysis supports the hypothesis that geographical regions within Puget Sound are genetically distinct. Sub-regions accounted for approximately 75% of that area's total genetic variation. Hydrology may, in part, explain the differentiation between the North Puget Sound/Hood Canal from the Central Basin and South Sound regions, but other factors may also play a role. Independent evidence for limited *O. lurida* larval dispersal and isolated recruitment (Dinnel 2010) is consistent with our observation of genetic differentiation at smaller than expected spatial scales and significant isolation by distance.

All populations within the Puget Sound's Central Basin and South Sound regions are genetically homogeneous, with the exception of the Central Basin Liberty Bay

2 site. Although populations from each sub-region were genetically more similar to one another, pairwise $F_{ST}$ values were not significantly different and bootstrapping of the neighbor-joining trees did not provide evidence of genetic differentiation between these two sub-regions. Historically, Puget Sound oyster operations were predominantly associated with the South Sound region and commercial producers routinely moved stocks throughout the area from sites favorable for larval setting to locations conducive for growth (Steele 1964) resulting in over one-hundred years of intensive anthropogenic admixture. Furthermore, warmer water temperatures and high tidal exchange within the relatively shallow South Puget Sound may promote larval survival and facilitate dispersal, both of which would tend to maintain genetic similarity within this region compared to the rest of the Sound.

The unique history of the Liberty Bay 2 oysters may provide some explanation as to why this population sample differs significantly from the other Central Basin populations. Dogfish Bay is a small harbor at the inlet of Liberty Bay that has been a site of an Olympia oyster restoration effort using hatchery-produced seed from Bainbridge Island broodstock (Betsy Peabody, Puget Sound Restoration Fund pers. comm.). Both hatchery records and genetic analysis of multiple groups of hatchery-produced seed (Stick, unpublished data) support the hypothesis that the current Bainbridge population consists of oysters introduced from the North Sound region. It is possible that Liberty Bay 2's samples are the result of a natural

spawn originating from oysters planted as part of this restoration effort but we could not confirm this due to inadequate sampling.

Depleted San Francisco oyster beds were widely supplemented using oysters harvested from many bays located throughout the Pacific Northwest starting in 1851 in response to excessive commercial exploitation. As a result, we expected to find substantial genetic similarity between these source populations and the recipient San Francisco Bay population. However, this was not the case. All potential source populations (Puget Sound, Willapa Bay and Yaquina Bay populations) are genetically distinct from the San Francisco Bay populations (allelic data not shown). Although our data strongly support a genetic isolation by geographic distance model of gene flow, it is possible part of our poor coefficient of determination ($R^2 = 0.17$) can be attributed to these historic San Francisco Bay admixture events.

Furthermore, although we found evidence of limited gene flow between all major geographic regions and between geographically separated populations within the larger, more complex bays, this does not necessarily imply that gene flow between non-differentiated populations is the result of a natural phenomenon. Palsboll *et al*. (2006) reported that even infrequent migration events consisting of a limited number of individuals can sufficiently prevent the accumulation of large genetic differences between populations. Although documented

contemporary relocation events involving Olympia oysters are rare, unintentional relocations associated with commercial Pacific oyster practices may generate substantial anthropogenic gene flow (Baker *et al.* 1999).

Finally, it is important to note that our results are based on differences in allelic frequency of selectively neutral microsatellite markers. Although these neutral markers can be used to quantify genetic differentiation and gene flow, they do not tell us whether any of the observed differentiation is adaptive or if the current differentiation is a result of anthropogenic or natural forces. As a result, restoration efforts should consider the full implication of using either broodstock collected from geographically distant populations or transplanting natural set/adults among genetically differentiated sites with minimal gene flow until further research can determine whether these populations are locally adapted (Camara& Vadopalas 2009). In cases where broodstock is either nonexistent or numbers are limited, then efforts to maximize genetic diversity of appropriate introduced stocks must be considered.

## Conclusions

Gene flow among geographically separated extant Olympia oyster populations is surprisingly limited for a marine invertebrate species whose free-swimming larvae are capable of wide-spread dispersal as long as favorable water conditions exist. We found a significant correlation between geographic and genetic distances supporting the premise that coastal populations are isolated. Genetic structure among remnant populations was not limited to broad geographic regions but was also present at sub-regional scales in both the Puget Sound WA and San Francisco Bay CA. Until it can be determined whether the genetically differentiated *O. lurida* populations are locally adapted, restoration projects and resource managers should be cautious of random, injudicious mixing or transplantation among stocks where there is no evidence for gene flow by natural processes.

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# Chapter 4
# Development of an integrated genetic linkage map based on microsatellite and AFLP markers for the Pacific oyster, *Crassostrea gigas*

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**Abstract**

Traditional quantitative trait locus (QTL) mapping strategies use crosses among inbred lines to create segregating populations. Unfortunately, even low levels of inbreeding in the Pacific oyster (*Crassostrea gigas*), the most widely cultivated aquaculture species in the world, can substantially depress economically important quantitative traits such as yield, survival and fertility, potentially complicating subsequent QTL analyses. To circumvent this problem, we constructed an integrated linkage map for Pacific oysters, consisting of 65 microsatellite (18 of which were previously unmapped) and 212 AFLP markers using a full-sib cross between phenotypically differentiated outbred families. We identified 10 linkage groups spanning 710.48 cM, with an average genomic coverage of 91.39% and an average distance between markers of 2.62 cM. Average marker saturation was 27.7 per linkage group, ranging between 19 (LG9) and 36 markers (LG3). Three of the ten linkage groups (LG1, LG2 and LG6) contained markers with significant distortions from Mendelian segregation patterns, suggesting an association with survival-related genes. Our decision to use the integrated full-sib outcross mapping strategy based on phenotypically differentiated outbred families has proven to be an efficient and reliable mapping methodology, which can be used in the search for QTLs.

## Introduction

The Pacific oyster, *Crassostrea gigas,* is the most widely cultivated aquaculture species by weight with worldwide production exceeding 4.5 million metric tons valued at over $3.6 billion (US) per year (www.FAO.org FAO 2007 Fisheries and Aquaculture production statistics). The farm-gate value of the United States West Coast shellfish industry exceeds $117 million (US) annually, of which 62% ($73 million) consists of Pacific oysters (www.pcsga.org Pacific Coast Shellfish Growers Association 2009 production statistics). The total economic impact of West Coast oyster production, assuming a modest economic multiplier of 2.5 for support industries and larvae and seed sales, exceeds $200 million.

Genetic improvement programs in several countries are working to domesticate the Pacific oyster and increase production efficiency. Although substantial genetic gains have been achieved through traditional genetic improvement strategies such as selection (Dégremont *et al.* 2010; Langdon *et al.* 2003) and cross-breeding (Hedgecock& Davis 2007), additional improvement may be possible if quantitative trait loci (QTL) for economic traits can be identified and mapped to enable marker-assisted selection (Sonesson 2007; Yu& Guo 2006).

Traditional QTL mapping strategies in most livestock and crop species use crosses among highly inbred lines to create segregating populations with high

levels of linkage disequilibrium between marker loci and QTL. Unfortunately, due to the extraordinarily high genetic load carried by Pacific oysters (Launey& Hedgecock 2001) even low levels of inbreeding cause substantial depression of economically important quantitative traits such as yield, survival and fertility (Camara *et al.* 2008; Evans *et al.* 2004; McGoldrick& Hedgecock 1997), potentially complicating subsequent QTL analyses (Freyer *et al.* 2009). Furthermore, crossing even minimally inbred oysters can result in substantial heterosis (Hedgecock& Davis 2007; Hedgecock *et al.* 1996), whose non-additive genetic effects are not useful to selection programs designed to maximize additive genetic gains (Hedgecock *et al.* 1995). To minimize these inbreeding and heterosis-related issues, we utilized an integrated mapping strategy for crosses between outbred parents (Wu *et al.* 2002a) based on both double pseudo-test cross (Grattapaglia& Sederoff 1994) and intercross mapping markers (Barreneche *et al.* 1998; Garcia *et al.* 2006), as implemented in the program ONEMAP (Margarido *et al.* 2007).

Because the Pacific oyster genome is one of the most variable among animal species, linkage maps in this species will likely be specific to the mapping family used, limiting the utility of a consensus linkage map constructed from many different mapping families (Hedgecock *et al.* 2005). Multiple linkage maps for the Pacific oyster have been reported but their creation has always been based on a single marker type, either microsatellites (Hubert *et al.* 2009; Hubert&

Hedgecock 2004) or AFLPs (Li& Guo 2004). Microsatellites are attractive because they are co-dominant and easily transferred between different mapping families, but they are costly to develop and assay. AFLP markers, in contrast, require little development and are inexpensive because many loci can be scored from a single PCR reaction, but they cannot be transferred between families. Combining readily transferrable microsatellite markers with cross-specific AFLP markers, therefore, can cost-effectively increase marker saturation for species where the availability of co-dominant markers may be limiting, while still anchoring observed linkage groups to any previously reported map and thus, facilitate the mapping of QTL (Maliepaard *et al.* 1998).

Here we report the first linkage map for Pacific oysters developed from an outcrossed family and integrating microsatellite and AFLP markers. The map consists of 65 microsatellite markers (18 of which were previously unmapped) and 212 AFLP markers, which can be used in the search for QTLs.

## Materials and Methods

*Selection of broodstock for creation of mapping families*

We identified phenotypically divergent full-sib families within Cohort 14 of the Molluscan Broodstock Program (MBP) based on harvest yield and survival data collected from a standard MBP field trial conducted at 3 growout sites (Yaquina Bay OR; Totten Bay WA; Westcott Bay WA). To do this, we ranked all 52 families based on mean percent survival and mean individual weight (calculated by dividing the family-specific aggregate live weight by the number of surviving oysters in each growout unit) across growout sites and classified the 23 most phenotypically extreme families as high or low for survival or growth, resulting in four phenotypic classes: High-growth (HG), low-growth (LG), high-survival (HS) and low-survival (LS) (Figure 4.1). Only Family 37 was categorized with multiple descriptive classifications as both a high-survival and low-growth family.

Surviving oysters from low-survival families may not fully retain the genetic signature that made them low-surviving after a substantial mortality event because the alleles that reduce survival could be eliminated from the population. We attempted to mitigate this effect by utilizing broodstock maintained at the MBP Broodstock Repository located in Yaquina Bay OR, a “safe site” that historically has not experienced seasonal mortality events.

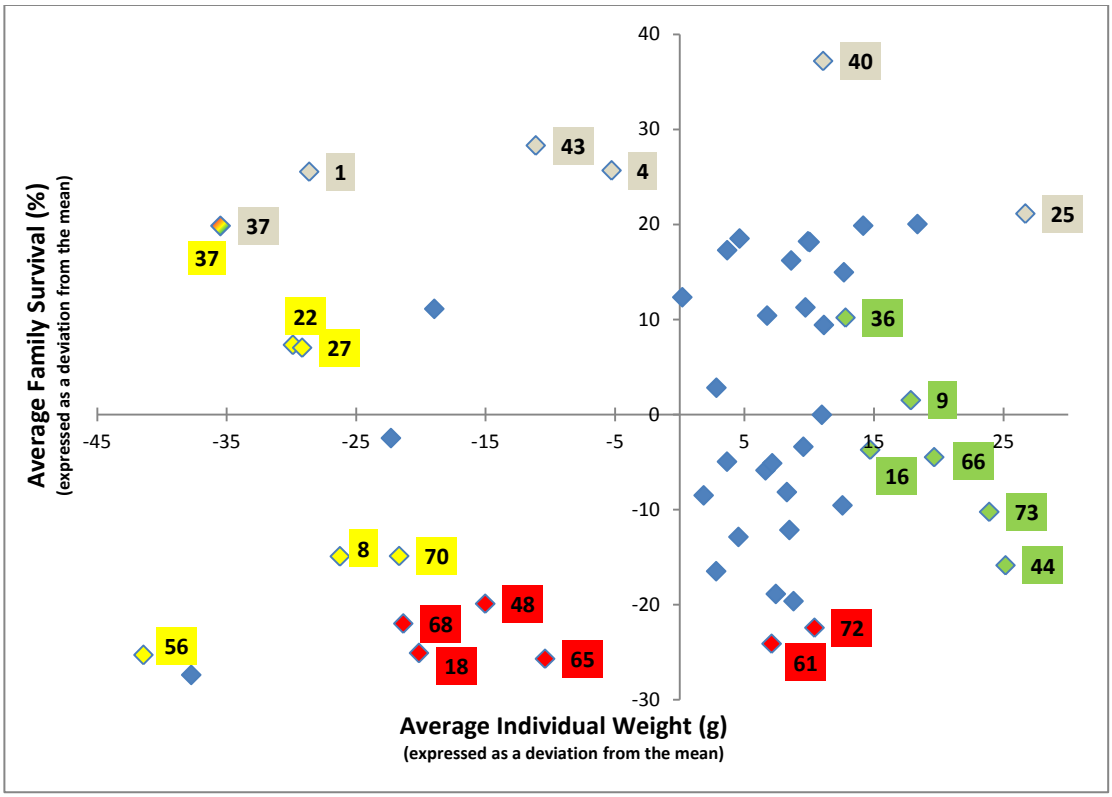


**Figure 4.1** Mean performance of Cohort 14 families expressed as deviations from the average family survival (%) and individual weight (g) across 3 growout sites. High-surviving families selected as broodstock are depicted in gray; Low-surviving families in red; High-growth families in green; Low-growth families in yellow. Family 37 was categorized as both a high-surviving and low-growth family.

*Mating design*

We created 12 outbred full-sib families in May 2005 using a mating strategy designed to maximize the within-family genetic variance by crossing the most phenotypically extreme families (Figure 4.2). We replicated those crosses where the likelihood of QTL segregation for either growth or survival was increased due to our mating design (green cells in Figure 4.2). We included secondary crosses capable of evaluating whether a completely different suite of genes are important when considering genotype- or genotype-by-environmental interactions for either growth or survival (yellow cells in Figure 4.2).

We also imposed a strict within-family selective criterion to further maximize the phenotypic difference in size between divergent parents and thus further increase the within-family genetic variance in their progeny beyond that created by the mating design. For example, we selected the largest appropriately sexed oyster for all HG crosses and the smallest for all LG crosses, regardless of the phenotypic classification of its mate. For the HS x LS crosses, we simply maximized the size difference among available, appropriately sexed parental oysters. Table 4.1 summarizes the pedigree, line classification, within-family phenotypic diversification criteria and the phenotypic measurements highlighting differences between parental broodstock of the 12 outbred full-sib mapping families.

| | | | female parental family | | | |
|---|---|---|---|---|---|---|
| | | | growth | | survival | |
| | | | high | low | high | low |
| male parental family | growth | high | | 2 X | | 1 X |
| | | low | 2 X | | | 1 X |
| | survival | high | 1 X | 1 X | | 2 X |
| | | low | | | 2 X | |

**Figure 4.2** Outbred mating strategy designed to maximize the within-family genetic variance by crossing phenotypically differentiated families. Crosses expected to have an increased likelihood of QTL segregation due to the mating design (green cells) were replicated. Some potentially interesting correlated crosses capable of identifying genotypic interactions among growth and survival were included in the design (yellow cells).

**Table 4.1** Pedigree information, phenotypic line classification and descriptive measurements for the families and broodstock of the outbred full-sib mapping families. High-surviving (HS) families are depicted in gray; Low-surviving (LS) families in red; High-growth (HG) families in green; Low-growth (LG) families in yellow. We maximized phenotypic differences between parents in an attempt to maximize genetic differences. Length was measured from anterior hinge to posterior shell margin; width was measured perpendicular to length at the widest dorsal-ventral margin; depth was measured as the greatest vertical distance between the two valves.

| Cross | Sex | Phenotypic Class | Family | Family Mean Survival (%) | Family Mean Weight (g) | Within Family Criterion | Live Wet Weight (g in shell) | Meat Weight (g) | Length (mm) | Width (mm) | Depth (mm) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | HG | 44 | 14.20 | 125.90 | large | 279.2 | 74.7 | 156.42 | 85.54 | 30.11 |
| 1 | F | LG | 56 | 4.76 | 59.31 | small | 159.6 | 40.2 | 123.61 | 62.73 | 35.44 |
| 2 | M | HG | 73 | 19.82 | 124.65 | large | 175.8 | 41.3 | 118.09 | 82.50 | 30.36 |
| 2 | F | LG | 37 | 49.91 | 65.28 | small | 87.2 | 25.4 | 97.71 | 68.08 | 26.32 |
| 3 | M | HG | 36 | 40.27 | 113.53 | large | 189.7 | 52.5 | 165.65 | 69.55 | 27.78 |
| 3 | F | LS | 48 | 10.17 | 85.72 | small | 125.0 | 33.7 | 105.17 | 65.80 | 33.76 |
| 4 | M | LG | 22 | 37.39 | 70.85 | small | 51.1 | 10.5 | 84.39 | 53.82 | 16.68 |
| 4 | F | HG | 66 | 25.58 | 120.40 | large | 207.7 | 54.1 | 138.46 | 82.44 | 30.61 |
| 5 | M | LG | 8 | 15.13 | 74.49 | small | 103.7 | 18.2 | 75.97 | 74.22 | 35.70 |
| 5 | F | HG | 9 | 31.59 | 118.59 | large | 235.6 | 65.8 | 149.90 | 70.40 | 37.71 |
| 6 | M | LG | 27 | 37.11 | 71.57 | small | 86.0 | 22.8 | 88.43 | 62.54 | 26.80 |
| 6 | F | LS | 68 | 8.06 | 79.38 | large | 231.2 | 59.5 | 118.44 | 100.05 | 45.65 |
| 7 | M | HS | 37 | 49.91 | 65.28 | small | 104.0 | 28.1 | 118.14 | 53.79 | 31.69 |
| 7 | F | HG | 16 | 26.37 | 115.45 | large | 275.9 | 70.5 | 145.32 | 71.42 | 38.03 |
| 8 | M | HS | 25 | 51.19 | 127.43 | large | 137.0 | 36.2 | 146.41 | 54.57 | 28.52 |
| 8 | F | LG | 70 | 15.19 | 79.06 | small | 87.3 | 24.7 | 110.98 | 62.30 | 27.92 |
| 9 | M | HS | 40 | 67.27 | 111.81 | large | 153.2 | 29.7 | 119.80 | 69.55 | 30.46 |
| 9 | F | LS | 65 | 4.38 | 90.34 | small | 77.2 | 19.0 | 79.90 | 58.55 | 20.15 |
| 10 | M | HS | 43 | 58.37 | 89.61 | large | 232.8 | 41.7 | 167.91 | 60.33 | 34.61 |
| 10 | F | LS | 18 | 4.96 | 80.59 | small | 119.5 | 27.0 | 94.14 | 63.09 | 30.99 |
| 11 | M | LS | 61 | 5.96 | 107.84 | small | 68.5 | 16.5 | 82.19 | 60.98 | 18.79 |
| 11 | F | HS | 4 | 55.74 | 95.46 | large | 261.1 | 50.1 | 175.60 | 78.51 | 39.13 |
| 12 | M | LS | 72 | 7.63 | 111.15 | large | 184.6 | 35.6 | 108.52 | 70.50 | 32.71 |
| 12 | F | HS | 1 | 55.63 | 72.11 | small | 88.3 | 21.1 | 104.38 | 51.24 | 27.59 |

*Husbandry practices*

We anesthetized all potential broodstock in a 7% $MgCl_2$ bath at 18°C and determined the sex of partially conditioned individual oysters by evaluating small gamete samples collected via gonadal puncture microscopically prior to the May 2005 spawn.

Broodstock conditioning, spawning practices and larval culture methods largely followed previously reported husbandry procedures (Camara *et al.* 2008; Langdon *et al.* 2003) at the MBP hatchery (Hatfield Marine Science Center, Newport OR). Modifications included: An extended conditioning period of 10 weeks at 19°C (instead of 6 weeks at 18°C), 30-L static larval culture tanks (instead of 100-L), Day 1 stocking density of 3 normal D-larvae $mL^{-1}$ (instead of 10 normal D-larvae $mL^{-1}$), Day 6 stocking density of 1 normal 80 µm veliger larvae $mL^{-1}$ (instead of 3 normal 80 µm veliger larvae $mL^{-1}$), 1ppm Chloramphenicol and 5ppm Calcium-montmorillonite treatments at every water change, supplementation of standard algal diet mixture consisting of equal concentrations of *Isochrysis galbana* (Tahitian strain; T-*ISO*) and *Chaetoceros calcitrans* (Japanese source; *Cc*j) with an equal component concentration of *Chaetoceros gracile* (*Cg*) after day 8, an enhanced general cleaning routine including water changes every two days (instead of twice a week), and we randomized culture position within the hatchery after every water change.

Nursery culture of the juvenile oysters also largely followed standard MBP operating practices (Langdon *et al.* 2003) at the USDA Shellfish Genetics Facility (Hatfield Marine Science Center, Newport OR). Modifications included: Supplementation of standard algal diet mixture consisting of equal concentrations of T-*ISO* and *Cc*j with an equal component concentration of *Cg,* spat were down-welled for 10 days in a partially recirculating nursery system before thinning cultures to 5000 individual oysters /cross and reversing water flow to the standard upwelling conditions (at which time water temperature was decreased by 1°C/day to ambient), culture units were periodically split to reduce density, culture units were also randomly rotated within the system to eliminate environmental effects and greatly enhanced general cleaning protocols were utilized including daily cleaning.

We transferred crosses at 80 days of age to AquaPurse growout units (http://www.ttpplastics.com.au), which were held under flow-through raceway conditions designed to maximize early growth at the MBP facility for an additional 50-75 days. When oysters reached roughly 3 cm in length (average weight 2.37 g; length 31.27 mm; width 20.94 mm; depth 7.52 mm), we randomly assigned 240 oysters from each cross (2880 total) to pearl oyster panel net pockets (http://www.orca-marine.com), which were planted subtidally in Yaquina Bay OR.

*DNA extraction*

We preserved adductor muscle (1cm x 1cm) from all parents in 95% ethanol. After 1 year in the field, we anesthetized all offspring in a 7% $MgCl_2$ bath at 18°C and from the left valve excised a small (2mm x 2mm) piece of the posterior mantle, which was preserved in 95% ethanol. We extracted genomic DNA from preserved samples (both parental and offspring) using DNeasy® 96 Blood and Tissue Digestion Kits (Qiagen Inc., Valencia CA) following a slightly modified protocol. Modifications included: Overnight (16-18 hr) sample lysis at 56°C, rinsing samples twice with AW2 buffer to reduce salt carryover and incubating columns uncovered at 70°C for 10 min to ensure evaporation of residual ethanol prior to elution. We quantified the DNA concentration using a NanoDrop ® ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and standardized all samples to working concentrations of 10 ng/µL using a Beckman Coulter Biomek FX liquid handling system (Beckman Coulter Inc., Fullerton CA).

*Microsatellite marker analysis*

We optimized 19 unreported (Masashi Sekino pers. comm.) and re-optimized 95 previously published microsatellites (Huvet *et al.* 2000; Li *et al.* 2003; Liu& Li 2008; Magoulas *et al.* 1998; McGoldrick 1997; McGoldrick *et al.* 2000; Wang *et al.* 2008; Yu& Li 2007) and screened the parental genotypes for informative allelic

combinations (see *Marker segregation* for description of informative allelic combinations) (Table 4.2). Primary polymerase chain reaction (PCR) reagents ($MgCl_2$, buffer, dNTPs, Taq) were purchased from Promega Inc. (Madison WI). We amplified loci individually in 10 µL PCR reactions containing the following components: locus-specific $MgCl_2$ concentration (Table 4.2), 1x GoTaq® FlexiPCR buffer pH 8.5, 0.15 mM dNTPs, locus-specific concentrations of 5'-fluorescently labeled forward primer (ABI, Foster City CA) and unlabeled reverse primer (Integrated DNA Technologies Inc., Coralville IA) (Table 4.2) and 0.025 U/µL GoTaq® FlexiDNA Polymerase on a GeneAmp® PCR 9700 thermocycler (ABI, Foster City CA) running the following program: 1) initial denaturing for 5 min at 94°C, 2) amplification using locus-specific number of cycles of 30 sec at 94°C followed by 30 sec at the locus-specific annealing temperature and 45 sec at 72°C (Table 4.2), 3) final extension for 10 min at 72°C and 4) a final hold at 10°C. We resolved products using an ABI 3730xl automated DNA sequencer with GeneScan® 500 LIZ size standard (ABI, Foster City, CA) and scored them using GeneMapper® version 3.7 (ABI, Foster City, CA). We confirmed genotypic calls by independently amplifying and genotyping all parental and a random selection of offspring samples multiple times.

**Table 4.2** Optimized PCR parameters and characterization for 114 Pacific oyster (*C. gigas*) microsatellites.

| Locus | Literature Source | GenBank Assession # | 5' Label | Annealing Temp (°C ) | [$MgCl_2$] (mM) | [Primer] (µM) | # PCR Cycles |
|---|---|---|---|---|---|---|---|
| **Cg001c** | McGoldrick (1997) | AF201460 | FAM | 60 | 1.5 | 0.2 | 40 |
| **Cg001u** | McGoldrick *et al.* (2000) | N/A | VIC | 58 | 1.5 | 0.4 F 0.2 R | 40 |
| **Cg002** | McGoldrick *et al.* (2000) | N/A | FAM | 56 | 1.5 | 0.4 | 40 |
| **Cg003c** | McGoldrick *et al.* (2000) | AF201462 | NED | 56 | 1.5 | 0.2 | 40 |
| **Cg003u** | McGoldrick *et al.* (2000) | N/A | NED | 50 | 1.5 | 0.2 | 35 |
| **Cg006** | McGoldrick *et al.* (2000) | AF051172 | FAM | 60 | 2.0 | 0.4 | 40 |
| **Cg008** | McGoldrick *et al.* (2000) | AF051175 | NED | 54 | 2.0 | 0.2 | 40 |
| **Cg014** | McGoldrick *et al.* (2000) | AF051174 | VIC | 54 | 2.5 | 0.4 | 40 |
| **Cg018** | McGoldrick *et al.* (2000) | N/A | NED | 52 | 1.5 | 0.4 | 35 |
| **Cg021** | McGoldrick *et al.* (2000) | N/A | VIC | 50 | 1.5 | 0.4 | 40 |
| **Cg022** | McGoldrick *et al.* (2000) | N/A | FAM | 48 | 1.5 | 0.4 | 40 |
| **Cg024** | McGoldrick (1997) | N/A | NED | 56 | 1.5 | 0.4 F 0.2 R | 40 |
| **Cg044** | Magoulas *et al.* (1998) | Y12085 | VIC | 50 | 2.0 | 0.4 | 40 |
| **Cg049** | Magoulas *et al.* (1998) | Y12086 | FAM | 52 | 1.5 | 0.4 | 40 |
| **Cg061** | McGoldrick *et al.* (2000) | AF204062 | VIC | 50 | 1.5 | 0.4 | 40 |
| **Cg108** | Magoulas *et al.* (1998) | Y12087 | NED | 54 | 1.5 | 0.4 | 40 |
| **Cg109** | Li *et al.* (2003) | AF468525 | VIC | 54 | 1.5 | 0.4 | 35 |
| **Cg117** | Li *et al.* (2003) | AF468528 | NED | 56 | 2.0 | 0.4 | 35 |
| **Cg119** | Li *et al.* (2003) | AF468529 | VIC | 50 | 1.5 | 0.4 | 35 |
| **Cg120** | Li *et al.* (2003) | AF468530 | FAM | 50 | 2.0 | 0.4 | 40 |
| **Cg126** | Li *et al.* (2003) | AF468532 | PET | 56 | 2.0 | 0.4 | 40 |
| **Cg129** | Li *et al.* (2003) | AF468534 | VIC | 56 | 1.5 | 0.4 | 40 |
| **Cg130** | Li *et al.* (2003) | AF468535 | NED | 60 | 1.5 | 0.4 | 40 |
| **Cg131** | Li *et al.* (2003) | AF468536 | FAM | 54 | 2.5 | 0.4 | 40 |
| **Cg133** | Li *et al.* (2003) | AF468537 | VIC | 54 | 2.0 | 0.2 | 40 |
| **Cg134** | Li *et al.* (2003) | AF468538 | PET | 58 | 2.5 | 0.4 | 40 |
| **Cg136** | Li *et al.* (2003) | AF468540 | FAM | 58 | 1.5 | 0.4 | 40 |
| **Cg138** | Li *et al.* (2003) | AF468542 | NED | 60 | 1.5 | 0.2 | 40 |
| **Cg139** | Li *et al.* (2003) | AF468543 | PET | 56 | 1.5 | 0.4 | 40 |
| **Cg140** | Li *et al.* (2003) | AF468544 | FAM | 60 | 2.0 | 0.4 | 40 |
| **Cg141c** | McGoldrick *et al.* (2000) | AF204060 | FAM | 54 | 1.5 | 0.2 | 40 |
| **Cg141u** | Li *et al.* (2003) | AF468545 | PET | 56 | 1.5 | 0.2 | 40 |
| **Cg142** | Li *et al.* (2003) | AF468546 | PET | 54 | 2.0 | 0.4 | 40 |
| **Cg145** | Li *et al.* (2003) | AF468547 | NED | 56 | 1.5 | 0.2 F 0.4 R | 40 |
| **Cg146** | Li *et al.* (2003) | AF468548 | PET | 58 | 1.5 | 0.2 | 40 |
| **Cg147** | Li *et al.* (2003) | AF468549 | PET | 56 | 1.5 | 0.2 | 40 |
| **Cg148** | Li *et al.* (2003) | AF468550 | NED | 56 | 1.5 | 0.4 F 0.2 R | 40 |
| **Cg149** | Li *et al.* (2003) | AF468551 | NED | 54 | 2.0 | 0.4 | 40 |

**Table 4.2** Continued.

| Locus | Literature Source | GenBank Assession # | 5' Label | Annealing Temp (°C ) | [$MgCl_2$] (mM) | [Primer] (µM) | # PCR Cycles |
|---|---|---|---|---|---|---|---|
| **Cg150** | Li *et al.* (2003) | AF468552 | PET | 56 | 2.0 | 0.4 | 40 |
| **Cg151c** | McGoldrick *et al.* (2000) | N/A | NED | 56 | 1.5 | 0.2 | 40 |
| **Cg151u** | Li *et al.* (2003) | AF468553 | VIC | 60 | 1.5 | 0.2 | 40 |
| **Cg152** | Li *et al.* (2003) | AF468554 | PET | 58 | 1.5 | 0.2 | 40 |
| **Cg153** | Li *et al.* (2003) | AF468555 | VIC | 58 | 2.0 | 0.4 | 35 |
| **Cg155** | Li *et al.* (2003) | AF468556 | FAM | 56 | 1.5 | 0.4 | 40 |
| **Cg156** | Li *et al.* (2003) | AF468557 | NED | 60 | 2.0 | 0.4 | 40 |
| **Cg157** | Li *et al.* (2003) | AF468558 | FAM | 60 | 2.0 | 0.4 | 40 |
| **Cg158** | Li *et al.* (2003) | AF468559 | VIC | 58 | 1.5 | 0.4 | 40 |
| **Cg160** | Li *et al.* (2003) | AF468560 | FAM | 56 | 2.0 | 0.2 | 40 |
| **Cg161** | Li *et al.* (2003) | AF468561 | PET | 56 | 1.5 | 0.4 | 40 |
| **Cg162** | Li *et al.* (2003) | AF468562 | FAM | 56 | 1.5 | 0.4 | 40 |
| **Cg165** | Li *et al.* (2003) | AF468565 | VIC | 56 | 2.0 | 0.4 | 40 |
| **Cg166** | Li *et al.* (2003) | AF468566 | PET | 56 | 1.5 | 0.4 | 40 |
| **Cg171** | Li *et al.* (2003) | AF468569 | FAM | 54 | 1.5 | 0.2 | 40 |
| **Cg172** | Li *et al.* (2003) | AF468570 | FAM | 58 | 2.0 | 0.4 | 40 |
| **Cg173** | Li *et al.* (2003) | AF468571 | VIC | 56 | 2.0 | 0.2 | 40 |
| **Cg175** | Li *et al.* (2003) | AF468573 | NED | 60 | 2.5 | 0.2 | 40 |
| **Cg176** | Li *et al.* (2003) | AF468574 | PET | 58 | 2.0 | 0.4 F 0.2 R | 40 |
| **Cg177** | Li *et al.* (2003) | AF468575 | PET | 54 | 2.0 | 0.4 | 40 |
| **Cg178** | Li *et al.* (2003) | AF468576 | VIC | 60 | 2.0 | 0.2 | 40 |
| **Cg181** | Li *et al.* (2003) | AF468579 | NED | 60 | 1.5 | 0.4 | 40 |
| **Cg184** | Li *et al.* (2003) | AF468582 | FAM | 58 | 1.5 | 0.4 F 0.2 R | 40 |
| **Cg185** | Li *et al.* (2003) | AF468583 | PET | 54 | 2.0 | 0.4 | 40 |
| **Cg186** | Li *et al.* (2003) | AF468584 | NED | 54 | 2.0 | 0.4 | 40 |
| **Cg189** | Li *et al.* (2003) | AF468587 | NED | 50 | 1.5 | 0.2 | 40 |
| **Cg191** | Li *et al.* (2003) | AF468589 | NED | 60 | 2.0 | 0.4 | 35 |
| **Cg192** | Li *et al.* (2003) | AF468590 | VIC | 58 | 2.0 | 0.4 | 40 |
| **Cg193** | Li *et al.* (2003) | AF468591 | VIC | 60 | 2.0 | 0.2 | 40 |
| **Cg194** | Li *et al.* (2003) | AF468592 | NED | 54 | 2.5 | 0.2 | 40 |
| **Cg195** | Li *et al.* (2003) | AF468593 | NED | 60 | 2.0 | 0.4 | 40 |
| **Cg196** | Li *et al.* (2003) | AF468594 | PET | 58 | 2.0 | 0.4 | 40 |
| **Cg197** | Li *et al.* (2003) | AF468595 | NED | 52 | 1.5 | 0.2 | 35 |
| **Cg198** | Li *et al.* (2003) | AF468596 | FAM | 60 | 1.5 | 0.4 | 40 |
| **Cg199** | Li *et al.* (2003) | AF468597 | VIC | 54 | 1.5 | 0.2 | 40 |
| **Cg200** | Li *et al.* (2003) | AF468598 | NED | 58 | 1.5 | 0.4 | 40 |
| **Cg201** | Li *et al.* (2003) | AF468599 | PET | 60 | 2.5 | 0.40 | 40 |
| **Cg202** | Li *et al.* (2003) | AF468600 | FAM | 56 | 2.0 | 0.4 F 0.2 R | 40 |
| **Cg203** | Li *et al.* (2003) | AF468601 | FAM | 60 | 2.0 | 0.4 | 40 |

**Table 4.2** Continued.

| Locus | Literature Source | GenBank Assession # | 5' Label | Annealing Temp (°C) | [$MgCl_2$] (mM) | [Primer] (µM) | # PCR Cycles |
|---|---|---|---|---|---|---|---|
| **CgE001** | Yu & Li (2007) | AJ566607 | FAM | 52 | 1.5 | 0.2 | 40 |
| **CgE004** | Yu & Li (2007) | BQ426454 | FAM | 54 | 2.5 | 0.4 | 40 |
| **CgE005** | Yu & Li (2007) | BQ426816 | FAM | 52 | 1.5 | 0.4 | 35 |
| **CgE009** | Yu & Li (2007) | CX068958 | FAM | 56 | 1.5 | 0.4 | 40 |
| **CgE027** | Liu & Li (2008) | ES789161 | VIC | 56 | 2.0 | 0.3 | 40 |
| **CgE032** | Liu & Li (2008) | CX068987 | PET | 54 | 1.5 | 0.4 | 40 |
| **CgE104** | Wang *et al.* (2008) | BQ426867 | VIC | 62 | 1.5 | 0.4 | 40 |
| **CgE204** | Wang *et al.* (2008) | ES789535 | PET | 54 | 1.5 | 0.4 | 40 |
| **CgE206** | Wang *et al.* (2008) | BQ426867 | PET | 60 | 1.5 | 0.4 | 40 |
| **CgE207** | Wang *et al.* (2008) | BQ426523 | FAM | 46 | 1.5 | 0.4 | 40 |
| **CgE208** | Wang *et al.* (2008) | DW713975 | NED | 50 | 2.0 | 0.4 | 40 |
| **CgE214** | Wang *et al.* (2008) | ES789767 | VIC | 54 | 2.0 | 0.4 | 40 |
| **CgE218** | Wang *et al.* (2008) | ES789575 | PET | 54 | 2.0 | 0.2 | 40 |
| **CgG002** | Liu & Li (2008) | AY765366 | NED | 52 | 1.5 | 0.4 | 40 |
| **CgG003** | Liu & Li (2008) | AJ242657 | VIC | 60 | 1.5 | 0.2 | 40 |
| **Cgi1** | Blouin (pers. comm.) | N/A | VIC | 48 | 2.0 | 0.4 | 40 |
| **Crgi168** | Sekino (pers. comm.) | N/A | FAM | 52 | 2.0 | 0.4 | 40 |
| **Crgi183** | Sekino (pers. comm.) | N/A | FAM | 56 | 2.5 | 0.4 | 40 |
| **Crgi184** | Sekino (pers. comm.) | N/A | FAM | 58 | 1.5 | 0.4 | 40 |
| **Crgi190** | Sekino (pers. comm.) | N/A | FAM | 54 | 2.0 | 0.2 | 40 |
| **Crgi196** | Sekino (pers. comm.) | N/A | FAM | 52 | 2.0 | 0.4 | 40 |
| **Crgi203** | Sekino (pers. comm.) | N/A | FAM | 56 | 2.0 | 0.4 | 40 |
| **Crgi204** | Sekino (pers. comm.) | N/A | FAM | 58 | 2.0 | 0.4 | 35 |
| **Crgi218** | Sekino (pers. comm.) | N/A | FAM | 62 | 2.0 | 0.2 | 40 |
| **Crgi224** | Sekino (pers. comm.) | N/A | FAM | 58 | 2.0 | 0.4 | 40 |
| **Crgi236** | Sekino (pers. comm.) | N/A | FAM | 54 | 1.5 | 0.2 | 40 |
| **Crgi238** | Sekino (pers. comm.) | N/A | FAM | 54 | 1.5 | 0.4 | 40 |
| **Crgi244** | Sekino (pers. comm.) | N/A | FAM | 54 | 2.0 | 0.4 | 40 |
| **Crgi246** | Sekino (pers. comm.) | N/A | FAM | 60 | 1.5 | 0.2 | 40 |
| **Crgi247** | Sekino (pers. comm.) | N/A | FAM | 56 | 2.0 | 0.2 | 40 |
| **Crgi257** | Sekino (pers. comm.) | N/A | FAM | 56 | 1.5 | 0.4 | 35 |
| **Crgi261** | Sekino (pers. comm.) | N/A | FAM | 54 | 2.5 | 0.4 | 40 |
| **Crgi263** | Sekino (pers. comm.) | N/A | FAM | 58 | 1.5 | 0.4 | 40 |
| **Crgi282** | Sekino (pers. comm.) | N/A | FAM | 58 | 1.5 | 0.4 | 35 |
| **Crgi301** | Sekino (pers. comm.) | N/A | FAM | 52 | 2.0 | 0.2 | 40 |
| **GL10** | Huvet *et al.* (2000) | AF170850 | NED | 54 | 1.5 | 0.4 | 40 |
| **GL48** | Huvet *et al.* (2000) | AF170852 | PET | 60 | 1.5 | 0.4 F 0.2 R | 40 |

*AFLP marker analysis*

We utilized a modification of Vos *et al.*'s (1995) AFLP protocol as proposed by Wilding *et al.* (2001) and Yu and Guo (2003). Restriction enzymes, T4 ligase, buffers and bovine serum albumin (BSA) were purchased from New England Biolabs (NEB, Ipswich MA) and primary PCR reagents ($MgCl_2$, buffer, dNTPs, Taq) were purchased from Promega Inc. (Madison WI). Adaptors, pre-selective and selective primers were purchased from Integrated DNA Technologies Inc. (IDT, Coralville IA) and are provided in Table 4.3. Briefly, genomic DNA (500 ng) was digested with 5 U *Eco*RI and 5 U *Mse*I restriction enzymes, 0.5x NEB buffer #2, 1x BSA and simultaneously ligated with 5 µM EcoRI and 50 µM MseI adaptors, 100 U T4 DNA ligase, 1x T4 ligase buffer in 50 µL reactions for 16 hr at 16°C.

We then performed 50 µL pre-selective PCR reactions on 5 µL diluted AFLP digestion/ligation product (1:9 with 1x reduced EDTA -$TE_{0.1}$) containing the following components: 1.5 mM $MgCl_2$, 1x GoTaq® FlexiPCR buffer, pH 8.5, 0.10 mM dNTPs, 10 µM EcoRI+A and MseI+C pre-selective primers and 0.020 U/µL GoTaq® FlexiDNA Polymerase on a GeneAmp® PCR 9700 thermocycler (ABI, Foster City CA) running the following program: 1) an initial hold for 2 min at 72°C followed by denaturing for 30 sec at 94°C, 2) amplification using 20 cycles of 30 sec at 94°C followed by 1 min at 56°C and 1 min at 72°C, 3) final extension for 5 min at 72°C and 4) a final hold at 10°C.

We performed fifteen 10 µL selective PCR reactions on 2.5µL diluted AFLP pre-selective product (1:9 with 1x reduced EDTA -$TE_{0.1}$) containing the following components: 1.5 mM $MgCl_2$, 1x GoTaq® FlexiPCR buffer, pH 8.5, 0.20 mM dNTPs, 5 µM EcoRI+3 (5'-FAM fluorescently labeled) and MseI+3 selective primers and 0.050 U/µL GoTaq® FlexiDNA Polymerase on a GeneAmp® PCR 9700 thermocycler (ABI, Foster City CA) running the following program: 1) initial denaturing for 5 min at 94°C, 2) amplification using 12 cycles of 30 sec at 94°C followed by 30 sec of touchdown-specific annealing temperatures (starting at 65°C with a 0.7°C reduction in annealing temperature each touchdown cycle) and 1 min at 72°C, 3) continued amplification using 25 cycles of 30 sec at 94°C followed by 30 sec of 56°C and 1 min at 72°C, 4) final extension for 3 min at 72°C and 4) a final hold at 10°C. We resolved products using an ABI 3730xl automated DNA sequencer with GeneScan® 500 LIZ size standard (ABI, Foster City, CA) and scored them using GeneMapper® version 3.7 (ABI, Foster City, CA).

**Table 4.3** AFLP adapter, pre-selective and selective primer sequences. All *Eco* RI selective primers 5'-FAM fluorescently labeled. *AFLP primer combination nomenclature follows International AFLP naming rules (http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.html).

| AFLP primers, adapters and combinations | | |
|---|---|---|
| Adapters | | |
| | *Eco* RI | F: 5'-CTC GTA GAC TGC GTA CC-3' |
| | | R: 5'-AAT TGG TAC GCA GTC TAC-3' |
| | *Mse* I | F: 5'-GAC GAT GAG TCC TGA G-3' |
| | | R: 5'-TAC TCA GGA CTC AT-3' |
| Preselective primers | | |
| | *Eco* RI+A | 5'-GAC TGC GTA CCA ATT CA-3' |
| | *Mse* I+C | 5'-GAT GAG TCC TGA GTA AC-3' |
| Selective primers | | |
| | *Eco* RI+AAC | 5'-GAC TGC GTA CCA ATT CAA C-3' |
| | *Eco* RI+AAG | 5'-GAC TGC GTA CCA ATT CAA G-3' |
| | *Eco* RI+ACA | 5'-GAC TGC GTA CCA ATT CAC A-3' |
| | *Eco* RI+ACC | 5'-GAC TGC GTA CCA ATT CAC C-3' |
| | *Eco* RI+ACT | 5'-GAC TGC GTA CCA ATT CAC T-3' |
| | *Mse* I+CACT | 5'-GAT GAG TCC TGA GTA ACA CT-3' |
| | *Mse* I+CTAG | 5'-GAT GAG TCC TGA GTA ACT AG-3' |
| | *Mse* I+CTAT | 5'-GAT GAG TCC TGA GTA ACT AT-3' |
| | *Mse* I+CACG | 5'-GAT GAG TCC TGA GTA ACA CG-3' |
| | *Mse* I+CAG | 5'-GAT GAG TCC TGA GTA ACA G-3' |
| | *Mse* I+CTA | 5'-GAT GAG TCC TGA GTA ACT A-3' |
| AFLP primer combinations* | | |
| E32M238 | *Eco* RI+AAC | *Mse* I+CACT |
| E32M281 | *Eco* RI+AAC | *Mse* I+CTAG |
| E32M282 | *Eco* RI+AAC | *Mse* I+CTAT |
| E33M49 | *Eco* RI+AAG | *Mse* I+CAG |
| E33M59 | *Eco* RI+AAG | *Mse* I+CTA |
| E35M49 | *Eco* RI+ACA | *Mse* I+CAG |
| E35M59 | *Eco* RI+ACA | *Mse* I+CTA |
| E36M49 | *Eco* RI+ACC | *Mse* I+CAG |
| E36M59 | *Eco* RI+ACC | *Mse* I+CTA |
| E36M237 | *Eco* RI+ACC | *Mse* I+CACG |
| E36M238 | *Eco* RI+ACC | *Mse* I+CACT |
| E36M281 | *Eco* RI+ACC | *Mse* I+CTAG |
| E36M282 | *Eco* RI+ACC | *Mse* I+CTAT |
| E38M49 | *Eco* RI+ACT | *Mse* I+CAG |
| E38M59 | *Eco* RI+ACT | *Mse* I+CTA |

*Marker segregation*

We only utilized markers whose parental alleles segregated in either a fully informative or partially but still very informative manner for each respective marker type, microsatellite or AFLP (Maliepaard *et al.* 1998; Wu *et al.* 2002a). These parental marker configurations are summarized in Table 4.4, which was adapted from Margarido *et al*. (2007). Briefly, an A1 classification is a fully informative microsatellite genotypic configuration in which both parents are heterozygous and all alleles involved are unique (AB x CD). An A3 classification is also a fully informative microsatellite genotypic configuration in which again both parents are heterozygous and all alleles are still unique but one allele is actually a null allele (-) making it appear that one of the parental genotypes is homozygous (AB x C-). For both A1 and A3 marker classifications, 4 genotypic combinations segregate in equal proportions in the offspring. Microsatellite markers in either of the less informative D1.9 (AB x CC) or D2. 14 (CC x AB) classification have only 2 genotypic configurations that segregate in the offspring. In order to identify the less informative D1.12 (B- x AA) and D2.17 (AA x B-) genotypic classifications, we genotyped 12 offspring for all microsatellite markers whose parental genotypes appeared homozygous for different alleles in order to identify parental allelic combinations that included a single segregating null allele. We only used the partially but most informative AFLP genotypic states of either D1.13 (A*o* x *oo*) or D2.18 (*oo* x A*o*).

Any marker with more than 10 and any individual with more than 15 missing genotypic data points was not utilized. We tested for segregation distortion in all markers using contingency table analysis comparing observed offspring genotypic frequencies against Mendelian expectation based on parental genotypes. However, because any deviation could be a result of linkage between the marker and a neighboring viability QTL, we deleted only those markers that exceeded significance after Bonferroni correction by an order of magnitude and any marker that did deviate from expected genotypic ratios was recorded as such in the final map.

**Table 4.4** Informative marker segregation types used in linkage map creation. Cross-type classification code adapted from Margarido *et al*. (2007). The parental microsatellite null condition is depicted by "-", whereas the AFLP null condition is depicted by "*o*".

| Cross-type Classification Code | Actual Parental Genotypic Cross | Observed Parental Genotypes | Observed Offspring Genotypes | Expected Offspring Segregation | Marker Type | Degree of Informativeness |
|---|---|---|---|---|---|---|
| **A1** | AB x CD | AB x CD | AC, AD, BC, BD | 1:1:1:1 | microsat | Full |
| **A3** | AB x C- | AB x C | AC, A, BC, B | 1:1:1:1 | microsat | Full |
| **D1.9** | AB x CC | AB x C | AC, BC | 1:1 | microsat | Partial |
| **D1.12** | B- x AA | B x A | AB, A | 1:1 | microsat | Partial |
| **D1.13** | A*o* x *oo* | A x *o* | A, *o* | 1:1 | AFLP | Partial |
| **D1.14** | CC x AB | C x AB | AC, BC | 1:1 | microsat | Partial |
| **D1.17** | AA x B- | A x B | AB, A | 1:1 | microsat | Partial |
| **D1.18** | *oo* x A*o* | *o* x A | A, *o* | 1:1 | AFLP | Partial |

*Mapping family selection*

Financial constraints limited analysis to a single mapping family although data was collected and tissue retained for the remaining 11 crosses. We selected Cross 11 as our mapping family based on multiple criteria that promote accurate QTL identification and mapping, including high within full-sib family variance for many of the growth traits evaluated (Chatziplis& Haley 2000; Chatziplis *et al.* 2001), a moderate level of mortality in field trials across 3 sites that could be caused by the segregation of divergent genotypes and the identification of a large proportion of co-dominant markers segregating in a highly-informative manner (Garcia *et al.* 2006; Wu *et al.* 2002a). All future discussion will be in reference to Cross 11.

*Linkage analysis*

We utilized an integrated mapping strategy based on both double pseudo-test cross (Grattapaglia& Sederoff 1994) and intercross mapping markers (Barreneche *et al.* 1998; Garcia *et al.* 2006) developed for crosses between outbred parents by Wu *et al.* (2002a). This strategy simultaneously estimates linkage and linkage phases between all markers and is implemented in the R based program ONEMAP version 1.01 (Margarido *et al.* 2007) available at www.r-project.org. We used a conservative minimum LOD (base 10 logarithm of

odds) score threshold of 5 and maximum recombination fraction of 0.25 for the analysis due to the large number of markers and different marker types utilized.

Linkage maps were drawn with the program MAPCHART version 2.1(Voorrips 2002). Map distances are expressed in centiMorgans (cM) and were estimated based on the recombination fraction using the Kosambi function. Because we anchored our map with previously mapped microsatellites, we were able to link and name our linkage groups corresponding to previous Pacific oyster microsatellite mapping studies (Hubert *et al.* 2009; Hubert& Hedgecock 2004).

*Genomic coverage*

We estimated the total genomic coverage by dividing the observed genome length by the average of the expected genome length ($G_e$) calculated using two different methods: (1) Fishman *et al*. (2001) estimated $G_e$ by adding 2 times the average framework marker density for each linkage group to its observed length and (2) Chakravarti *et al*. (1991) increased the size of each linkage group by $(m+1)/(m-1)$, where $m$ is the number of unique loci mapped.

**Results**

We evaluated a total of 349 markers; 114 microsatellites (Table 4.2) and 235 AFLPs identified as potentially informative (Table 4.3). Of the 114 microsatellites, 76 were informative (67%). However, three of these failed to amplify in 10 or more individuals and we deleted them from dataset as unreliable. We also excluded 3 microsatellites and 23 AFLPs that deviated significantly from Mendelian expectations. Five microsatellites could not be assigned to linkage groups in our analysis (Cg001c, Cg002, Cg129, Cg157 and Cg189) and were not included in the final map.

Our final integrated linkage map (Figure 4.3) consists of 277 markers (65 microsatellites and 212 AFLPs) on 10 linkage groups (LG1 –LG10) spanning 710.48 cM, which resulted in an average genomic coverage of 91.39% and an average distance between markers (marker density) of 2.62 cM (Table 4.5). Not all mapped markers, however, were unique (*m*) and the realized average framework marker density increased to 3.28 cM due to our failure to observe any recombination event between these tightly-linked markers in our sampled offspring. In spite of these reasonable marker densities, markers were not evenly distributed within any individual linkage group, with an average largest unmapped interval of 14.55 cM and a range between 10.02 and 22.24 cM.

Table 4.5 summarizes the descriptive characteristics of the linkage groups. Average marker saturation was 27.7 per linkage group ranging between a low of 19 (LG9) and high of 36 markers (LG3). Although the average number of AFLPs per linkage group (21.2 markers), was roughly three times that observed for microsatellites (6.5 markers), the different marker types were not evenly distributed among linkage groups. For example, LG6 consisted of 42% microsatellites and 58% AFLPs whereas LG10 was 13% microsatellite and 87% AFLPs.

Three of the ten linkage groups (LG1, LG2 and LG6) contained markers that deviated significantly from Mendelian genotypic proportions. This is especially true of LG6, where 81% of all markers exhibited genotypic distortion.

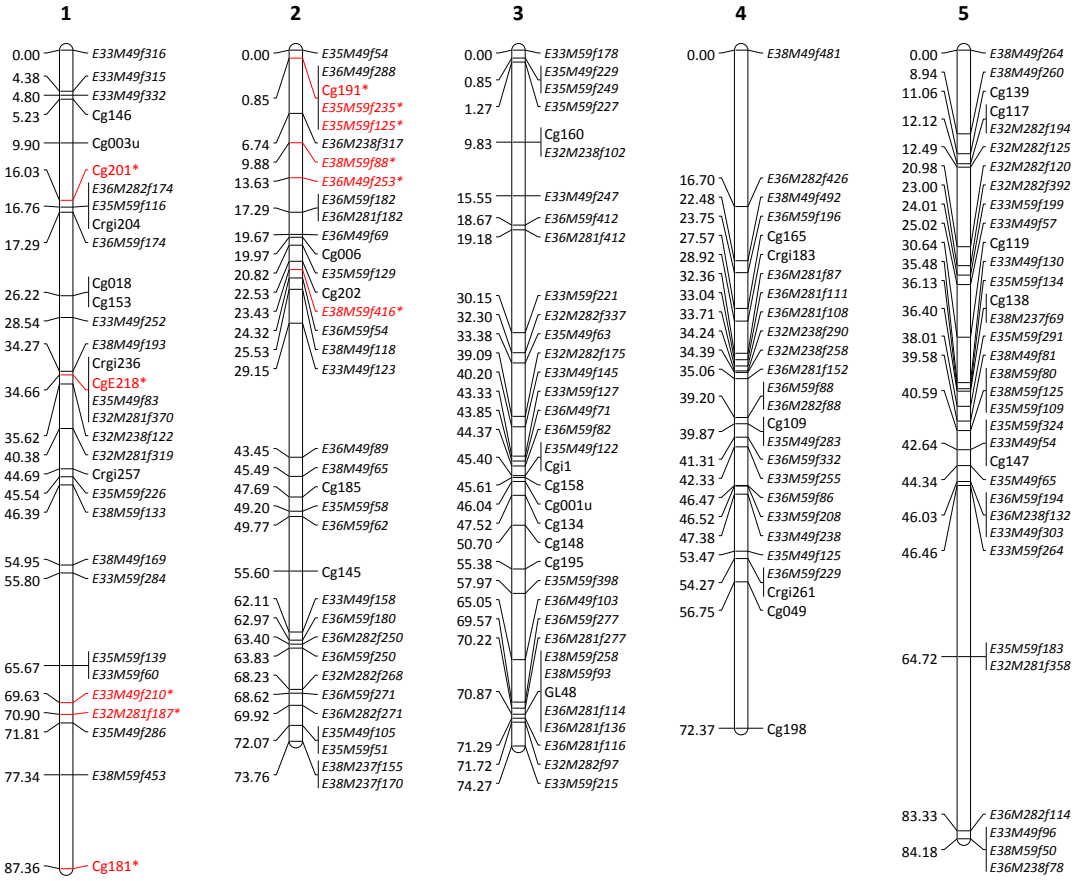


**Figure 4.3** Integrated microsatellite and AFLP based linkage maps of the Pacific oyster. Map is based on genotypes for 277 markers (65 microsatellites and 212 AFLPs) in 237 outcrossed progeny from parental cross 11 and covers 710.48 cM across 10 linkage groups. Linkage group nomenclature corresponds with previous Pacific oyster microsatellite mapping studies (Hubert *et al.* 2009; Hubert& Hedgecock 2004). Markers are indicated on the right and distances in cM estimated using the Kosambi mapping function is on the left of each linkage group. AFLP markers follow International AFLP naming rules and are italicized. *Markers in red deviate from Mendelian segregation expectations but did not exceed Bonferroni correction by more than an order of magnitude to warrant deletion from the analysis.

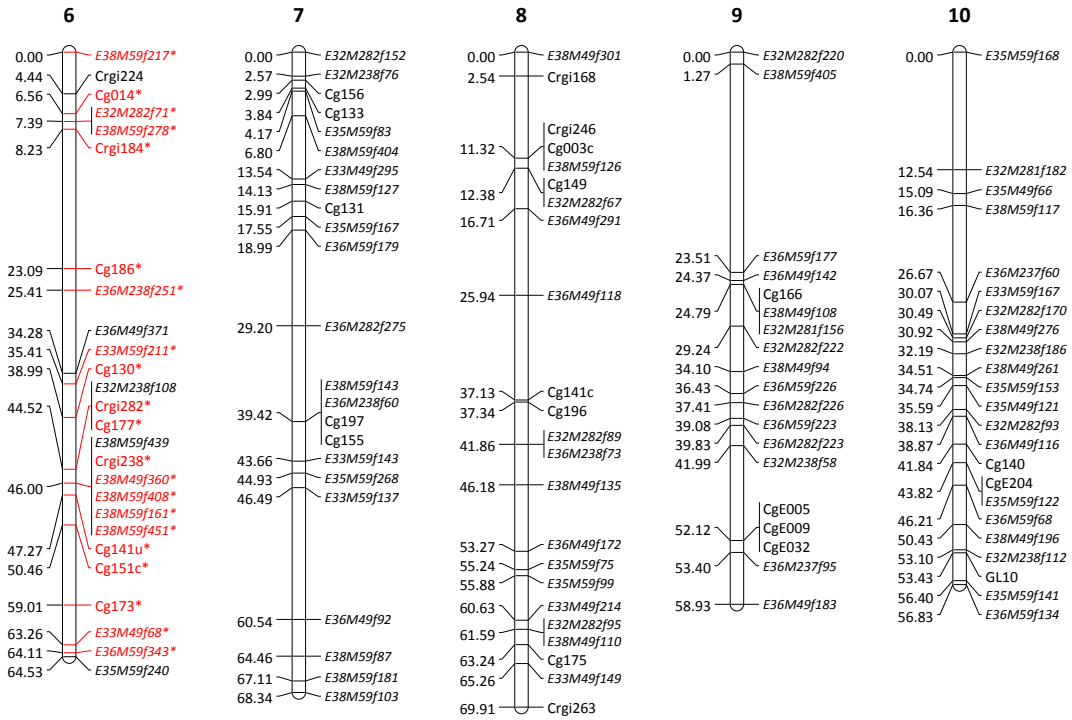


**Figure 4.3** Continued.

**Table 4.5** Descriptive characteristics of Pacific oyster linkage groups. All map distances and largest unmapped interval are in centiMorgans (cM) estimated using the Kosambi mapping function. Average density is calculated as total linkage group (LG) distance divided by total markers, whereas average framework density is the total linkage group distance divided by total number of unique markers (*m*). Number distorted refers to the number of markers that failed to segregate in expected Mendelian genotypic proportions.

| | | | Per Linkage Group | | | | | Map Length | | Average | Largest |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Linkage Group (LG) | Total Markers | Total Unlinked (*m*) | Number Microsats | Proportion Microsat (%) | Number AFLPs | Proportion AFLP (%) | Number Distorted | Total Distance (cM) | Average Density (cM) | Framework Density (cM) | Unmapped Interval (cM) |
| **LG1** | 32 | 25 | 10 | 31% | 22 | 69% | 5 | 87.36 | 2.73 | 3.49 | 10.02 |
| **LG2** | 35 | 29 | 5 | 14% | 30 | 86% | 6 | 73.76 | 2.11 | 2.54 | 14.30 |
| **LG3** | 36 | 29 | 8 | 22% | 28 | 78% | 0 | 74.27 | 2.06 | 2.56 | 10.97 |
| **LG4** | 26 | 23 | 6 | 23% | 20 | 77% | 0 | 72.37 | 2.78 | 3.15 | 16.70 |
| **LG5** | 34 | 23 | 5 | 15% | 29 | 85% | 0 | 84.18 | 2.48 | 3.66 | 18.61 |
| **LG6** | 26 | 18 | 11 | 42% | 15 | 58% | 21 | 64.53 | 2.48 | 3.59 | 14.86 |
| **LG7** | 23 | 20 | 5 | 22% | 18 | 78% | 0 | 68.34 | 2.97 | 3.42 | 14.05 |
| **LG8** | 23 | 18 | 8 | 35% | 15 | 65% | 0 | 69.91 | 3.04 | 3.88 | 11.19 |
| **LG9** | 19 | 15 | 4 | 21% | 15 | 79% | 0 | 58.93 | 3.10 | 3.93 | 22.24 |
| **LG10** | 23 | 22 | 3 | 13% | 20 | 87% | 0 | 56.83 | 2.47 | 2.58 | 12.54 |
| **total** | 277 | 222 | 65 | | 212 | | 32 | 710.48 | | | |
| **average** | 27.7 | 22.2 | 6.5 | 24% | 21.2 | 76% | 3.2 | 71.05 | 2.62 | 3.28 | 14.55 |

**Discussion**

Moderately dense linkage maps (>100 markers) for the Pacific oyster have been previously reported but their creation has always been based on a single marker type, either microsatellites (Hubert *et al.* 2009; Hubert& Hedgecock 2004) or AFLPs (Li& Guo 2004). Using both marker types to create a single map can cost-effectively increase marker saturation while still providing a means to anchor linkage groups to other studies and thus facilitate in the mapping of QTLs (Maliepaard *et al.* 1998).

Pacific oysters have 10 pairs of chromosomes (Ahmed& Sparks 1967; Hubert *et al.* 2009; Leitao *et al.* 1999) in agreement with our results of 10 linkage groups. Assuming an average of 1.1 to 1.2 chiasmata per chromosome, the estimated theoretical genetic map length of the Pacific oyster would be between 550-600 cM (Hedgecock *et al.* 2005; Hubert& Hedgecock 2004). All previously reported maps are 32-99% larger than the estimated cytological genome length and this size discrepancy has been attributed to the low marker density of these maps (Hubert& Hedgecock 2004; Li& Guo 2004) and a recombination frequency greater than what direct physical observation of chiasmata would suggest (Hubert *et al.* 2009). In spite of integrating two marker types in our analysis, effectively tripling the marker density over any previously reported map based on a single marker type, our total map length of 710 cM still exceeds the

theoretical expectation by roughly 18-29%, supporting Hubert *et al.*'s (2009) belief that recombination occurs far more frequently in the Pacific oyster than what would be expected based only on chiasmata numbers. Average genomic coverage for our integrated linkage map was estimated at 91.39%, greatly exceeding the 70% coverage reported by Hubert and Hedgecock (2004) for their female microsatellite linkage map and the 81% coverage reported by Li and Guo (Li& Guo 2004) for their male AFLP map.

Segregation distortion is common in Pacific oysters and is usually attributed to high genetic load and selection against deleterious recessive alleles carried by the oyster (Launey& Hedgecock 2001). Meticulous evaluation during the genotypic collection phase of the project, the removal of any questionable marker from the data set and replication of random offspring and all parental genotypes greatly reduced the probability that any observed segregation distortion was due to technical genotyping artifacts. As a result, only 11.5% of the markers we used did not segregate in expected Mendelian genotypic ratios and all of these distorted markers, both microsatellite and AFLP which occurred in roughly equal frequencies, were deficient in homozygote genotypes. This proportion of distorted loci is roughly half of what was reported in previous studies (Hubert& Hedgecock 2004; Li& Guo 2004).

All of our distorted markers mapped to 3 linkage groups (LG1, LG2 and LG6), with roughly 81% of the markers on LG6 not following expected Mendelian genotypic proportions. The inclusion of distorted markers in a linkage analysis may be useful for mapping of genes that affect fitness and survival (Li& Guo 2004) or for loci dominated by additive effects (Xu 2008). However, significant segregation distortion can bias the estimation of the recombination frequency between markers making them appear closely linked. This in turn, reduces the power to detect QTL with dominance effects (Xu 2008) and creates bias when estimating any punitive QTL position and effect by shifting the observed QTL location away from the distorted marker toward the non-distorted chromosome region and even though it is easier to find QTLs with smaller effects, these effects may be overestimated as much as 10 times (Doerge *et al.* 1997). As a result, we were very conservative in our analysis, mapping only those distorted markers that did not cause conflicts in map position with other markers.

Previous Pacific oyster mapping studies reported differences in recombination frequency between the sexes, with females displaying higher recombination rates than males (Hubert& Hedgecock 2004; Li& Guo 2004). As a result, female linkage maps were as much as 25% longer over corresponding male maps. It is not uncommon to find differences in recombination rates between sexes of higher-ordered domesticated animals (Barendse *et al.* 1994; Ellegren *et al.* 1994). However, Hedrick (2007) hypothesized that the evolutionary factors influencing

the difference in recombination rates between sexes in hermaphroditic species without sex-chromosomes or sex-determining genes are very different from those factors associated with either pleiotropy (Haldane 1922) or simple meiotic processes (Kong *et al.* 2010), such as sexual selection (Trivers 1988) or reproductive success (Burt *et al.* 1991). Therefore, excluding these potential evolutionary influences, the difference in recombination rates between sexes of the protandric Pacific oyster (Guo *et al.* 1998) should be minimal (Hubert& Hedgecock 2004) supporting our decision to create a fully integrated linkage map combining not only different marker types but also different sexes into a single common map.

The power of a QTL sib-analysis is dependent upon the magnitude of the parental phenotypic differences (Chatziplis& Haley 2000; Chatziplis *et al.* 2001; Muranty 1996). Furthermore, full-sib analyses provide almost twice as much power as half-sib analyses (Knott *et al.* 1996; Knott& Haley 1992; Knott *et al.* 1992; Martinez *et al.* 2002). This supports our decision to not only utilize a full-sib analysis but also to utilize phenotypically differentiated outbred families imposed with a strict within-family selective criterion to further maximize the phenotypic difference in size between divergent parents.

Our use of the integrated mapping strategy based on crosses between phenotypically differentiated outbred families (Wu *et al.* 2002a) containing both

double pseudo-test cross (Grattapaglia& Sederoff 1994) and intercross mapping markers (Barreneche *et al.* 1998; Garcia *et al.* 2006) to circumvent issues related to inbreeding or heterosis (Freyer *et al.* 2009) has proven to be a viable and efficient method for the creation of linkage maps in Pacific oysters. The design permits simultaneous estimation of linkage and linkage phases between multiple marker types (Garcia *et al.* 2006; Wu *et al.* 2002a) and combines information between sexes (Wu *et al.* 2002b), increasing our overall effective sample size. Although questions concerning the utility of a single consensus linkage map constructed from many different mapping families exist (Hedgecock *et al.* 2005), this strategy also permits the inclusion of genotypic data from multiple full-sib families (Wu *et al.* 2002a).

**Conclusion**

We have created the first fully-integrated linkage map consisting of 65 microsatellite (18 of which were previously unmapped) and 212 AFLP markers based on an outcross design using phenotypically differentiated outbred families for the Pacific oyster capable of being utilized for QTL analyses.

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# Chapter 5
# Identification and mapping of growth-related QTL using microsatellite and AFLP markers for the Pacific oyster, *Crassostrea gigas*

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**Abstract**

We identified 12 quantitative trait loci (QTLs) and 5 potential QTLs in an F1 population of 236 Pacific oysters using an integrated mapping strategy designed to maximize the within-family genetic variance by crossing phenotypically divergent parents from differentiated outbred families. We evaluated four growth-related morphometric measures, including individual wet live weight, shell length measured from anterior hinge to posterior shell margin, shell width measured perpendicular to shell length between the widest dorsal-ventral margins, and shell depth measured as the greatest vertical distance between the two valves at four post-fertilization time points: plant-out (average age of 140 days), first year interim (average age of 358 days), second year interim (average age of 644 days) and harvest (average age of 950 days). Mapped QTLs and potential QTLs accounted for an average of 11.2% of the total phenotypic variation and ranged between 2.1 and 33.1%. Although QTL or potential QTL were mapped to all Pacific oyster linkage groups with the exception of LG2, LG8 and LG9, three groups (LG4, LG10 and LG5) were associated with three of more QTL or potential QTL. We conclude that alleles accounting for a significant proportion of the total phenotypic variation for morphometric measures that influence harvest yield remain segregating within the broodstock of West Coast Pacific oyster selective breeding programs.

## Introduction

The Pacific oyster, *Crassostrea gigas,* is the most widely cultivated aquaculture species by weight with worldwide production exceeding 4.5 million metric tons valued at over $3.6 billion (US) per year (www.FAO.org FAO 2007 Fisheries and Aquaculture production statistics). The farm-gate value of the United States West Coast shellfish industry exceeds $117 million (US) annually, of which 62% ($73 million) consists of Pacific oysters (www.pcsga.org Pacific Coast Shellfish Growers Association 2009 production statistics). The total economic impact of West Coast oyster production, assuming a modest economic multiplier of 2.5 for support industries as well as larvae and seed sales, exceeds $200 million.

Genetic improvement programs in several countries are working to domesticate the Pacific oyster and increase production efficiency. Although substantial genetic gains have been achieved through traditional genetic improvement strategies such as selection (Dégremont *et al.* 2010; Langdon *et al.* 2003) and cross-breeding (Hedgecock& Davis 2007), additional improvement may be possible if quantitative trait loci (QTLs) for economic traits can be identified and mapped to enable marker-assisted selection (Sonesson 2007; Yu& Guo 2006).

Pacific Northwest oyster breeding and genetic improvement programs have focused primarily on improving the economic trait harvest live weight or yield.

Yield is a composite trait that combines the effects of growth and survival (Langdon *et al.* 2003) and is complicated by interactions with environmental factors (Evans& Langdon 2006b; Shi *et al.* 2009). Because oyster survival is largely dependent on transient environmental interactions (Dégremont *et al.* 2010; Degremont *et al.* 2007; Evans& Langdon 2006b) while growth rate, assuming food availability is not limiting, is not (Evans& Langdon 2006a), the identification of QTL affecting individual growth characteristics may be of value for shellfish breeding programs.

QTL have been successfully mapped in bivalves, including disease resistance in Eastern oysters, *Crassostrea virginica* (Yu& Guo 2006) and European flat oysters, *Ostrea edulis* (Lallias *et al.* 2009), shell color and size in bay scallops, *Argopectin irradians* (Qin *et al.* 2007a, b) and Zhikong scallops, *Chlamys farreri* (Zhan *et al.* 2009) and for heterosis affecting yield in Pacific oysters, *C. gigas* (Hedgecock *et al.* 2005). However, these projects have been based on traditional QTL mapping strategies that utilize crosses among inbred lines to create segregating populations or have relied on less inefficient mapping family methodologies (Knott *et al.* 1996; Knott& Haley 1992; Knott *et al.* 1992; Martinez *et al.* 2002).

Our use of an integrated mapping strategy based on crosses between phenotypically divergent parents from differentiated outbred families (Wu *et al.* 2002) containing both double pseudo-test cross (Grattapaglia& Sederoff 1994)

and intercross mapping markers (Barreneche *et al.* 1998; Garcia *et al.* 2006) to circumvent issues related to inbreeding or heterosis (Freyer *et al.* 2009) has proven to be a viable and efficient method for the creation of linkage maps in Pacific oysters (see Chapter 4). Here, we use this map to identify QTL associated with growth-related traits that influence yield.

## Materials and Methods

*Mapping family mating strategy and design*

The mating strategy and design used to create the mapping family is fully described in Chapter 4. Briefly, we created a full-sib family (Cross 11) in May 2005 using a mating strategy designed to maximize the within-family genetic variance by crossing two families within Cohort 14 of the Molluscan Broodstock Program (MBP) identified as being phenotypically divergent for survival across three growout sites (Yaquina Bay OR; Totten Bay WA; Westcott Bay WA). We classified parental Family 14.061 as a low-surviving (LS) family (average survival 5.96%) and parental Family 14.004 as a high-surviving (HS) family (average survival of 55.74%) and the resultant full-sib mapping family was Cross 11 (Chapter 4 Table 4.1).

Surviving oysters from low-surviving families may not fully retain the genetic signature that made them low-surviving after a substantial mortality event because the alleles that reduce survival could have been eliminated. We attempted to mitigate this effect by utilizing broodstock maintained at the MBP broodstock repository in Yaquina Bay OR, a “safe site” that historically has not experienced seasonal mortality events.

We also imposed a strict within-family selective criterion to further maximize the phenotypic difference in size between the parents of divergent families and thus further increase the within-family genetic variance in their progeny beyond that created by the mating design. In order to mitigate potential density effects on the family mean individual weight caused by differential survival (LS Family 14.061 mean individual weight was 107.84 g compared to HS Family 14.004 was 95.46 g), we selected the smallest oyster (68.5 g), which happened to be male, from Family 14.061 and therefore, the largest female oyster (261.1 g) from Family 14.004.

*Husbandry practices*

Broodstock conditioning, spawning, larval and nursery culture methods largely followed previously reported husbandry procedures (Camara *et al.* 2008; Langdon *et al.* 2003) at the MBP hatchery and USDA Shellfish Genetics facility located in the Hatfield Marine Science Center (HMSC), Newport OR. These procedures are fully described including all modifications in Chapter 4.

We transferred oysters at 80 days of age to AquaPurse growout units (http://www.ttpplastics.com.au), which were held under flow-through raceway conditions designed to maximize early growth at the MBP facility. When oysters reached roughly 3 cm in length (average age of 140 days; average weight 2.37 g

(*SD* = 1.03 g); length 31.27 mm (*SD* = 6.31 mm); width 20.94 mm (*SD* = 3.83 mm); depth 7.52 mm (*SD* = 1.50 mm)), we randomly assigned 2 oysters to each of 120 pearl oyster panel net pockets (http://www.orca-marine.com), which were planted subtidally at 2 locations in the Yaquina Bay OR (60 nets at both Oregon Oyster Farms and USDA-ARS repository). The polyhaline Oregon Oyster site is located approximately 8 kM upriver of the predominantly euhaline USDA-ARS site located near the mouth of the river at the Hatfield Marine Science Center in Newport OR, and can experience water temperatures 8-10°C warmer. We removed all fouling organisms from nets and oysters twice a year (spring and fall) and interim measurements were collected once a year (spring only) until project termination after 3 growing seasons (average age of 950 days).

*Quantitative traits evaluated*

We measured four morphological traits that directly reflect growth at four post-fertilization time points: plant-out (average age of 140 days), first year interim (average age of 358 days), second year interim (average age of 644 days) and harvest (average age of 950 days). Total time required to process each site varied between 5 and 7 days. We measured individual wet live weight, shell length measured from anterior hinge to posterior shell margin, shell width measured perpendicular to shell length between the widest dorsal-ventral margins, and shell depth measured as the greatest vertical distance between the two valves. All

weight measurements were recorded to the nearest 0.01 g at plant-out and 0.1 g at harvest. All shell dimensions were recorded to the nearest 0.01 mm at plant-out with digital Vernier calipers and to the nearest 1.0 mm at harvest with a SciElex shellfish measuring board (www.scielex.com). We calculated morphometric shape indices for both shell shape (shell length/shell width) (Galtsoff 1964) and cup shape (shell width/shell depth and shell length/shell depth).

*Markers and linkage analysis*

We utilized the genetic linkage map created in Chapter 4 for the QTL analysis.

*QTL analysis*

We estimated summary statistics describing the data's distribution using SAS software, Version 9.2 and JMP Version 8.0.2 (SAS Institute Inc., Cary NC). QTL mapping analyses were performed using the program MapQTL 6 (Van Ooijen 2009). We determined LOD thresholds for identifying statistically significant QTL (genome-wide $\alpha = 0.05$;chromosome-wide $\alpha = 0.01$) by permuting the quantitative trait values among individuals 10,000 times while keeping the marker data fixed to estimate the LOD test statistic distribution under the null hypothesis of no segregating QTL (Churchill& Doerge 1994; Doerge& Churchill

1996). QTL analyses were performed using both regression (Haley& Knott 1992) and maximum likelihood mixture models based on EM algorithms using 1.0 cM mapping steps, a maximum of 5 neighboring markers, a maximum of 200 iterations, a functional tolerance of 1.0e-08 and a *P*-value of 0.02 for automatic marker co-factor selection. We used both interval mapping (IM) (Lander& Botstein 1989; Van Ooijen 1992) and the multiple-QTL model (MQM) mapping method (Jansen 1993; Jansen& Stam 1994), which is similar to composite interval mapping (CIM) (Zeng 1993, 1994) and simultaneously accounts for genotypic differences among alleles from both parents as well as intra-locus interactions (dominance) among alleles. Both MQM and CIM analysis methodologies increase the power of the QTL search by fitting markers that account for a large proportion of the phenotypic variance as model cofactors, allowing any linked marker to be used in subsequent analyses to search for other segregating QTLs.

We removed two biologically important sources of variation from our analyses to improve our statistical power and precision. In all models we fitted the expected QTL genotypic mean for individual *n* in all models by blocking for the fixed effect "Site" in the experimental design using

(1)

where  is the overall QTL genotypic mean and $\kappa_i$ is the mean for Site *i.* The use of Site in analysis of Day 140 data accounts for the time difference required to measure and plant out the oysters between sites and is not equivalent to all other time point analyses where it also accounts for differences among grow out sites. Additionally, we accounted for potential hatchery, nursery and initial growout effects prior to plant out by using Day 140 live wet weight as a design covariate in all models evaluating the non-weight-based Day 140 morphometric measures (length, width and depth) and any subsequent weight analysis. We did this by adjusting the expected QTL genotypic mean for individual *n* using

(2)

where  is the overall QTL genotypic mean, $\tau_n$ is the covariate Day 140 live wet weight for individual *n* and $\beta$ is the estimated regression coefficient.

**Results**

Statistics describing the data's distribution at each time point are summarized in Table 5.1. The variance associated with the morphometric measures of live wet weight, shell length, shell width and shell depth increased with age while the variance associated with shape indices were consistent among sampling points. Most distributions were slightly positively skewed with the tail favoring larger observations. Kurtosis associated with the distributions of the shape indices largely reflected the kurtosis associated with the component measures of the indices (shell length, width and depth). Shell shape (shell length/shell width), however, tended to be leptokurtic indicating infrequent deviations of the data from the mean.

LOD significance thresholds for the quantitative traits are summarized in Table 5.2. Genome-wide LOD significance threshold values ($\alpha = 0.05$) were used to detect QTL anywhere in the entire genome while chromosome-wide LOD thresholds ($\alpha = 0.01$) were used to detect QTL only within a specific linkage group. Although many analyzed genomic regions had LOD scores greater than 3.0 indicating potential QTLs (Lander& Botstein 1989; Liu 1998), we followed a strict criterion that a region must exceed the genome-wide LOD significance threshold before calling the region a QTL. If the region exceeded the chromosome-wide but

not the genome-wide LOD significance threshold, we refer to the QTL as "potential".

**Table 5.1** Summary statistics describing the data's distribution for the evaluated quantitative traits at each measured time point (plant-out average age 140 days, first year interim average age 358 days, second year interim average age 644 days and harvest average age 950 days). N is the number of observations. Shell shape (L/W) is the index of shell length/shell width; Cup shape indices (W/D and L/D) are shell width/shell depth and shell length/shell depth, respectively. Min and Max are the minimum and maximum observations.

| Trait | Time Point | N | Mean | Variance | Min | Max | Skewness | Kurtosis |
|---|---|---|---|---|---|---|---|---|
| **weight (g)** | Day 140 | 236 | 2.90 | 1.12 | 0.61 | 6.79 | 0.77 | 0.42 |
| **length (mm)** | Day 140 | 236 | 34.95 | 33.71 | 19.65 | 51.05 | 0.15 | -0.66 |
| **width (mm)** | Day 140 | 236 | 24.57 | 14.35 | 13.87 | 37.08 | 0.29 | 0.14 |
| **depth (mm)** | Day 140 | 236 | 7.87 | 1.70 | 4.83 | 12.73 | 0.52 | 0.83 |
| **shellshape (L/W)** | Day 140 | 236 | 1.43 | 0.04 | 1.02 | 2.74 | 1.65 | 7.62 |
| **cupshapeA (W/D)** | Day 140 | 236 | 3.17 | 0.27 | 1.88 | 5.31 | 0.47 | 0.65 |
| **cupshapeB (L/D)** | Day 140 | 236 | 4.82 | 0.37 | 3.03 | 5.90 | -0.04 | -0.47 |
| | | | | | | | | |
| **weight (g)** | Day 358 | 236 | 14.60 | 34.83 | 2.98 | 35.87 | 0.97 | 1.05 |
| **length (mm)** | Day 358 | 236 | 62.13 | 99.57 | 33.53 | 91.07 | 0.14 | -0.01 |
| **width (mm)** | Day 358 | 236 | 47.97 | 62.87 | 29.07 | 72.26 | 0.36 | 0.14 |
| **depth (mm)** | Day 358 | 236 | 13.63 | 3.96 | 7.92 | 18.53 | 0.16 | -0.30 |
| **shellshape (L/W)** | Day 358 | 236 | 1.31 | 0.04 | 0.75 | 1.82 | 0.12 | -0.35 |
| **cupshapeA (W/D)** | Day 358 | 236 | 3.55 | 0.27 | 2.14 | 5.14 | 0.21 | -0.12 |
| **cupshapeB (L/D)** | Day 358 | 236 | 4.58 | 0.28 | 3.29 | 6.52 | 0.16 | 0.12 |
| | | | | | | | | |
| **weight (g)** | Day 644 | 202 | 82.69 | 494.08 | 31.30 | 142.20 | 0.22 | -0.28 |
| **length (mm)** | Day 644 | 202 | 105.09 | 327.26 | 55 | 144 | -0.19 | -0.29 |
| **width (mm)** | Day 644 | 202 | 71.86 | 126.91 | 40 | 108 | 0.29 | 0.21 |
| **depth (mm)** | Day 644 | 202 | 24.28 | 8.44 | 16 | 33 | 0.04 | 0.33 |
| **shellshape (L/W)** | Day 644 | 202 | 1.47 | 0.05 | 0.67 | 2.13 | 0.30 | 0.81 |
| **cupshapeA (W/D)** | Day 644 | 202 | 2.97 | 0.18 | 2.15 | 4.48 | 0.76 | 0.98 |
| **cupshapeB (L/D)** | Day 644 | 202 | 4.34 | 0.36 | 2.75 | 5.87 | -0.23 | -0.27 |
| | | | | | | | | |
| **weight (g)** | Day 950 | 174 | 174.61 | 1702.11 | 63.20 | 301.70 | 0.27 | -0.13 |
| **length (mm)** | Day 950 | 174 | 133.83 | 297.44 | 63 | 173 | -0.36 | 0.78 |
| **width (mm)** | Day 950 | 174 | 89.22 | 115.72 | 66 | 128 | 0.56 | 0.95 |
| **depth (mm)** | Day 950 | 174 | 29.99 | 8.57 | 22 | 40 | 0.29 | 0.33 |
| **shellshape (L/W)** | Day 950 | 174 | 1.51 | 0.05 | 0.91 | 2.44 | 0.76 | 2.22 |
| **cupshapeA (W/D)** | Day 950 | 174 | 2.99 | 0.16 | 1.89 | 4.56 | 0.54 | 1.06 |
| **cupshapeB (L/D)** | Day 950 | 174 | 4.49 | 0.39 | 2.10 | 6.00 | -0.17 | 0.61 |

**Table 5.2** LOD significance thresholds (genome-wide α = 0.05; chromosome-wide α = 0.01). Thresholds estimated by permutation of the phenotypic data among individuals 10,000 times for evaluated quantitative traits (individual wet live weight, shell length measured from anterior hinge to posterior shell margin, shell width measured perpendicular to length between the widest dorsal-ventral margins, shell depth measured as the greatest vertical distance between the two valves and morphometric shape indices for both shell shape (shell length/shell width) and cup shape (shell width/shell depth and shell length/shell depth)).

| Trait | Time Point | Linkage Group | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LG1 | LG2 | LG3 | LG4 | LG5 | LG6 | LG7 | LG8 | LG9 | LG10 | GW |
| weight (g) | Day 140 | 4.1 | 4.0 | 3.9 | 3.8 | 3.9 | 3.9 | 3.9 | 3.9 | 3.7 | 3.5 | 4.2 |
| length (mm) | Day 140 | 4.0 | 4.0 | 3.8 | 3.7 | 3.9 | 3.9 | 3.9 | 3.9 | 3.7 | 3.6 | 4.2 |
| width (mm) | Day 140 | 4.1 | 4.0 | 3.9 | 3.8 | 4.0 | 4.0 | 4.0 | 4.0 | 3.7 | 3.5 | 4.3 |
| depth (mm) | Day 140 | 4.0 | 4.0 | 3.8 | 3.9 | 4.0 | 3.9 | 3.9 | 4.1 | 3.7 | 3.5 | 4.2 |
| shellshape (L/W) | Day 140 | 5.0 | 4.8 | 5.5 | 4.3 | 4.5 | 4.6 | 4.5 | 4.3 | 3.9 | 3.7 | 5.1 |
| cupshapeA (W/D) | Day 140 | 4.1 | 4.1 | 4.1 | 3.9 | 4.0 | 3.9 | 3.9 | 3.9 | 3.7 | 3.6 | 4.3 |
| cupshapeB (L/D) | Day 140 | 3.9 | 4.0 | 3.9 | 3.9 | 3.9 | 3.8 | 3.8 | 4.0 | 3.6 | 3.5 | 4.2 |
| weight (g) | Day 358 | 4.3 | 4.2 | 4.2 | 4.1 | 4.1 | 4.1 | 4.1 | 4.1 | 3.9 | 3.6 | 4.4 |
| length (mm) | Day 358 | 4.0 | 4.0 | 3.8 | 3.9 | 3.9 | 3.8 | 3.9 | 4.0 | 3.7 | 3.5 | 4.2 |
| width (mm) | Day 358 | 4.1 | 4.0 | 3.9 | 3.9 | 3.9 | 3.8 | 3.8 | 4.0 | 3.7 | 3.4 | 4.2 |
| depth (mm) | Day 358 | 4.0 | 4.0 | 3.8 | 3.9 | 3.9 | 3.8 | 3.8 | 3.9 | 3.7 | 3.5 | 4.2 |
| shellshape (L/W) | Day 358 | 4.0 | 4.0 | 3.9 | 3.9 | 3.9 | 3.8 | 3.9 | 3.9 | 3.7 | 3.6 | 4.2 |
| cupshapeA (W/D) | Day 358 | 4.0 | 4.0 | 3.8 | 3.8 | 3.9 | 3.9 | 3.9 | 3.9 | 3.6 | 3.4 | 4.2 |
| cupshapeB (L/D) | Day 358 | 4.1 | 4.0 | 4.0 | 3.8 | 3.9 | 3.9 | 3.9 | 4.0 | 3.7 | 3.5 | 4.3 |
| weight (g) | Day 644 | 4.0 | 3.9 | 3.9 | 3.9 | 3.9 | 3.8 | 3.9 | 3.9 | 3.7 | 3.5 | 4.2 |
| length (mm) | Day 644 | 4.1 | 4.1 | 3.9 | 4.0 | 4.0 | 4.1 | 4.0 | 4.0 | 3.7 | 3.6 | 4.3 |
| width (mm) | Day 644 | 4.0 | 4.0 | 3.9 | 3.9 | 4.0 | 3.9 | 3.9 | 4.0 | 3.7 | 3.6 | 4.3 |
| depth (mm) | Day 644 | 4.1 | 4.1 | 3.9 | 3.9 | 3.9 | 4.0 | 4.0 | 4.1 | 3.8 | 3.6 | 4.3 |
| shellshape (L/W) | Day 644 | 4.2 | 4.1 | 4.1 | 4.0 | 3.9 | 4.1 | 4.1 | 4.1 | 3.8 | 3.6 | 4.4 |
| cupshapeA (W/D) | Day 644 | 4.2 | 4.2 | 4.0 | 4.0 | 4.1 | 4.0 | 4.0 | 4.0 | 3.7 | 3.6 | 4.4 |
| cupshapeB (L/D) | Day 644 | 4.0 | 4.0 | 3.9 | 3.7 | 3.9 | 3.9 | 3.8 | 3.9 | 3.7 | 3.6 | 4.2 |
| weight (g) | Day 950 | 4.1 | 4.1 | 4.0 | 3.9 | 4.0 | 3.9 | 4.0 | 4.0 | 3.8 | 3.5 | 4.3 |
| length (mm) | Day 950 | 4.0 | 4.0 | 3.9 | 3.9 | 4.0 | 3.8 | 3.9 | 4.1 | 3.8 | 3.6 | 4.2 |
| width (mm) | Day 950 | 4.1 | 4.1 | 4.0 | 3.9 | 3.9 | 4.0 | 4.0 | 4.1 | 3.8 | 3.6 | 4.3 |
| depth (mm) | Day 950 | 4.1 | 4.2 | 3.9 | 3.9 | 3.9 | 3.9 | 4.0 | 4.0 | 3.8 | 3.6 | 4.3 |
| shellshape (L/W) | Day 950 | 4.2 | 4.2 | 4.1 | 3.9 | 4.1 | 4.1 | 4.1 | 4.1 | 3.7 | 3.5 | 4.4 |
| cupshapeA (W/D) | Day 950 | 4.1 | 4.1 | 3.8 | 4.0 | 3.9 | 4.0 | 4.0 | 4.0 | 3.8 | 3.5 | 4.3 |
| cupshapeB (L/D) | Day 950 | 4.2 | 4.1 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.7 | 3.7 | 4.4 |

*Individual Wet Live Weight*

We identified and mapped two QTLs and one potential QTL for individual live wet weight. We mapped one QTL accounting for 8.7% of the total phenotypic variation for Day 140 individual wet live weight to LG6 near the microsatellite marker Cg130 (38.99 cM), which is flanked by the AFLP markers E33M59f211 (35.41 cM) and E32M238f108 (45.52 cM), using both interval mapping (IM) and multiple QTL model (MQM) analyses with either the regression or mixture model algorithms and the fixed effect Site, which was included in all analyses. Figure 5.1 shows the estimated LOD scores by chromosomal position (cM) across all linkage groups using the robust regression algorithm and IM analysis and a genome-wide LOD significance threshold of 4.2 ($\alpha = 0.05$).

Instead of showing the LOD plots for all linkage groups as in Figure 5.1, henceforth we simplify our findings by only presenting LOD plots for the analyzed method and algorithm that resulted in the greatest log-likelihood for linkage groups with significant or potential QTLs. Figure 5.2 plots the MQM analysis results for LG6 from the mixture model algorithm with the microsatellite marker Cg130 (LG5) as an analysis cofactor and a chromosome-wide LOD significance threshold of 3.9 ($\alpha = 0.01$).

We also mapped one QTL accounting for 10.1% of the total phenotypic variation for Day 358 individual wet live weight corrected for hatchery and nursery effects using the design covariate Day 140 individual wet live weight to LG10 near the AFLP marker E35M59f141 (56.40 cM), which is flanked by the microsatellite and AFLP markers GL10 (53.43 cM) and E36M59f134 (56.83 cM), using the MQM analyses with either the regression or mixture model algorithms, the microsatellite marker GL10 (LG10) as an analysis cofactor and a genome-wide LOD significance threshold of 4.3 ($\alpha = 0.05$). Figure 5.3 plots the MQM analysis results from the mixture model algorithm. Although no interval exceeded the genome-wide LOD significance threshold with any IM analyses (LOD = 3.67), one interval on LG10 did exceed the chromosome-wide threshold of 3.6 ($\alpha = 0.01$).

No additional QTL could be detected utilizing a genome-wide LOD significance threshold ($\alpha = 0.05$) for individual wet live weight as the oyster aged (average age of 644 days and 950 days). However, we mapped one potential QTL accounting for 10.4% of the phenotypic variation for Day 950 individual wet live weight corrected for hatchery and nursery effects using the design covariate Day 140 individual wet live weight to LG4's terminal end near the microsatellite marker Cg198 (72.37 cM), which is flanked on one side by the microsatellite marker Cg049 (56.75 cM), using MQM analysis with either the regression or mixture model algorithms and the microsatellite marker Cg198 (LG4) as an analysis cofactor. Figure 5.4 plots the MQM analysis results using the mixture

model algorithm. Although the observed LOD score of 4.17 does not exceed the genome-wide significant threshold of 4.3 ($\alpha = 0.05$), it does exceed the chromosome-wide threshold of 3.9 ($\alpha = 0.01$). No significant LOD score was associated with any IM analysis.

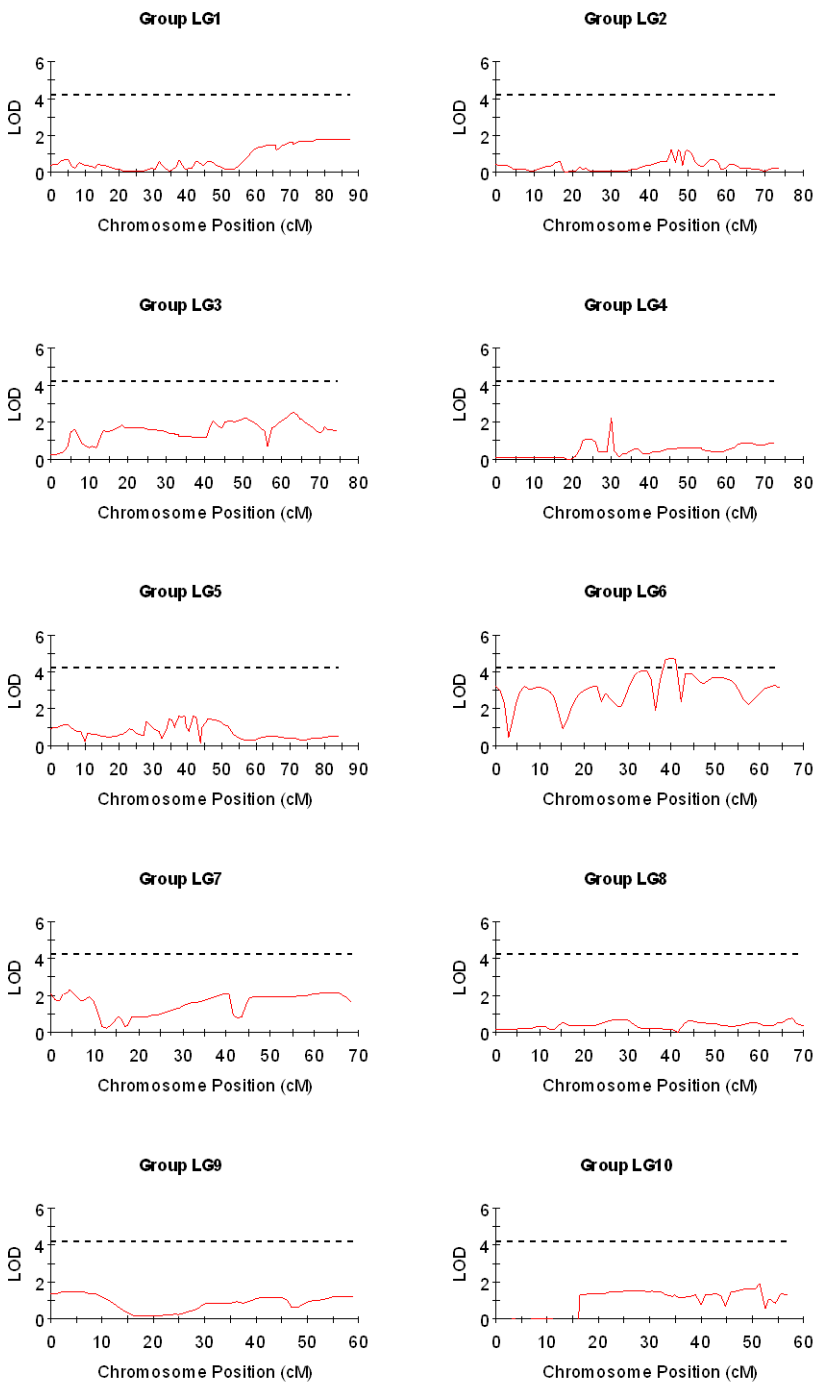


**Figure 5.1** Identification and mapping of Day 140 individual wet live weight QTL. LOD scores were estimated using the robust regression algorithm and IM analysis. One QTL is observed on LG6 using a genome-wide LOD significance threshold of 4.2 ($\alpha = 0.05$), which is depicted as the dotted line.

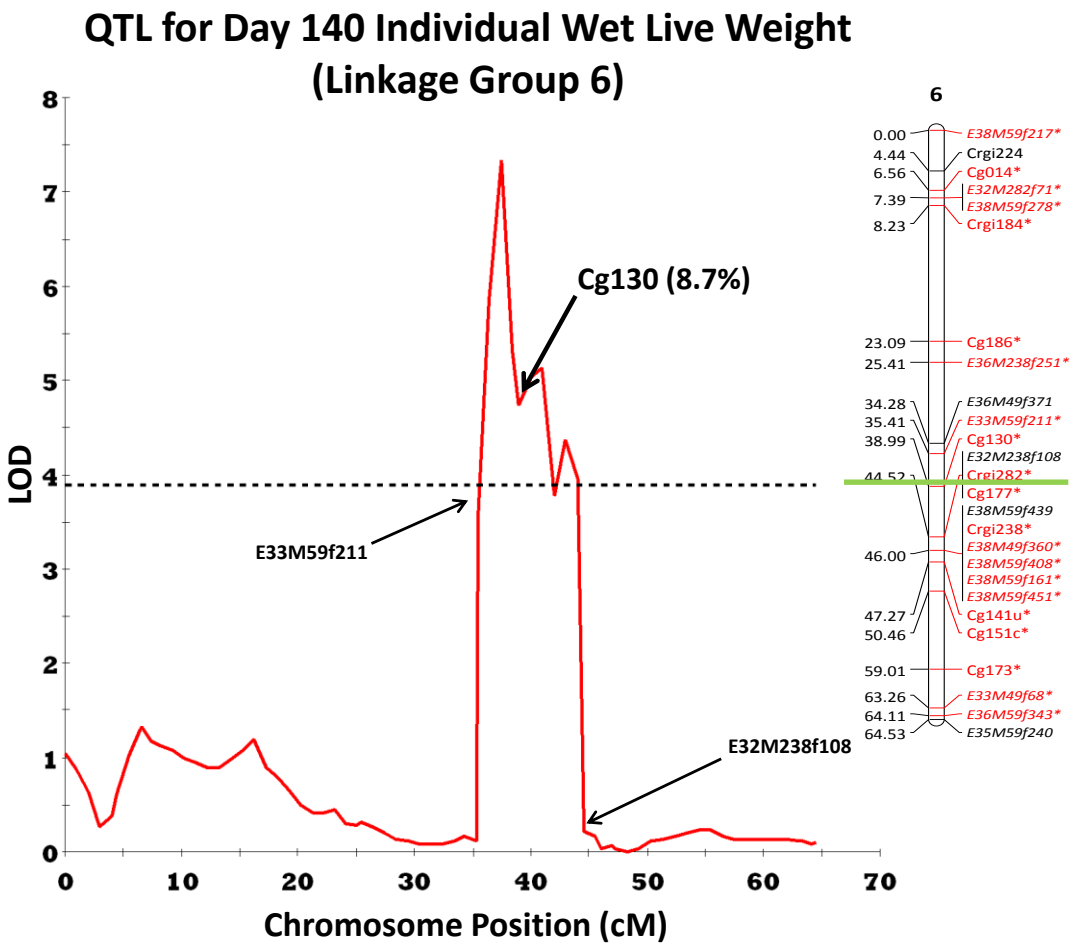


**Figure 5.2** Identification and mapping of Day 140 individual wet live weight QTL on LG6. Identified QTL accounts for 8.7% of the total phenotypic variation and maps near the microsatellite marker Cg130 (green bar located on linkage map at 38.99 cM), which is flanked by the AFLP markers E33M59f211 (35.41 cM) and E32M238f108 (45.52 cM). A chromosome-wide significance threshold of 3.9 ($\alpha$ = 0.01) is depicted.

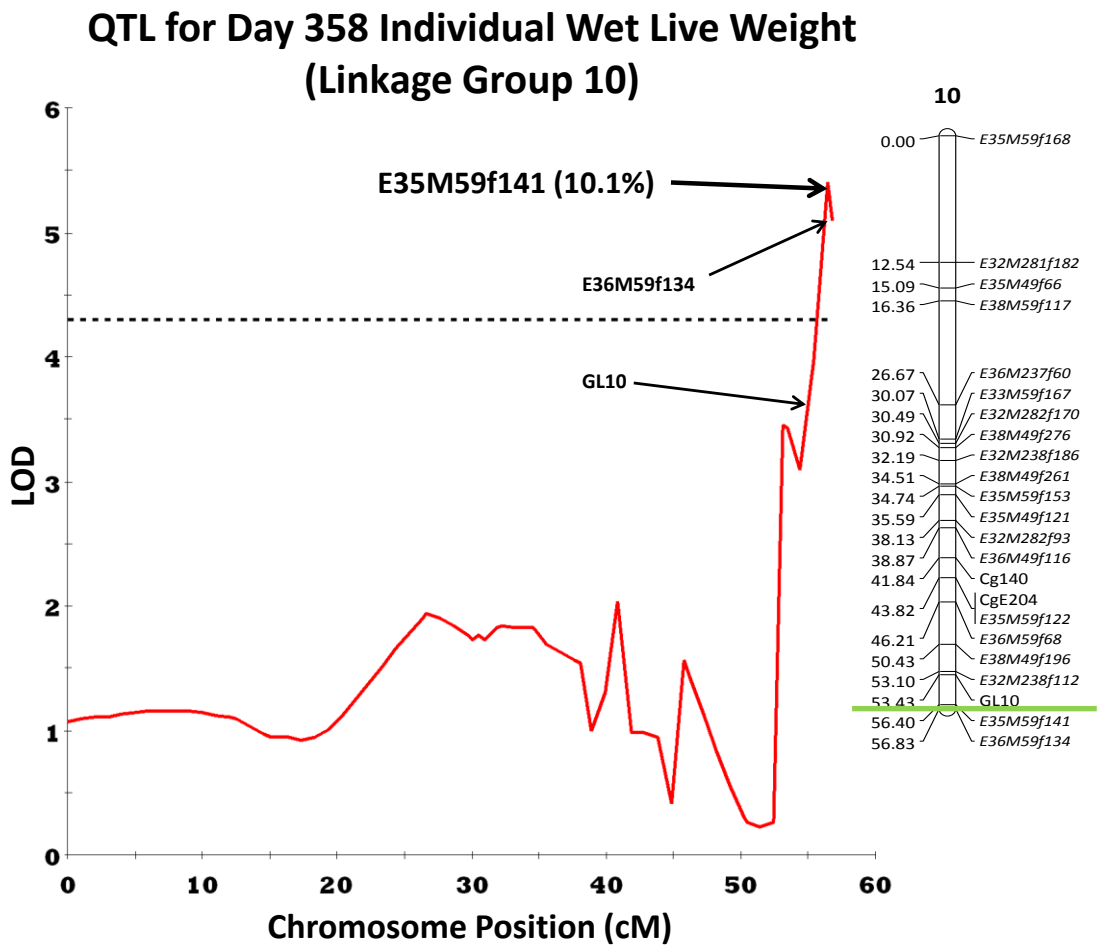


**Figure 5.3** Identification and mapping of Day 358 individual wet live weight QTL on LG10. Identified QTL accounts for 10.1% of the total phenotypic variation and maps near the AFLP marker E35M59f141 (green bar located on linkage map at 56.40 cM), which is flanked by the microsatellite and AFLP markers GL10 (53.43 cM) and E36M59f134 (56.83 cM). A genome-wide significance threshold of 4.3 ($\alpha$ = 0.05) is depicted.

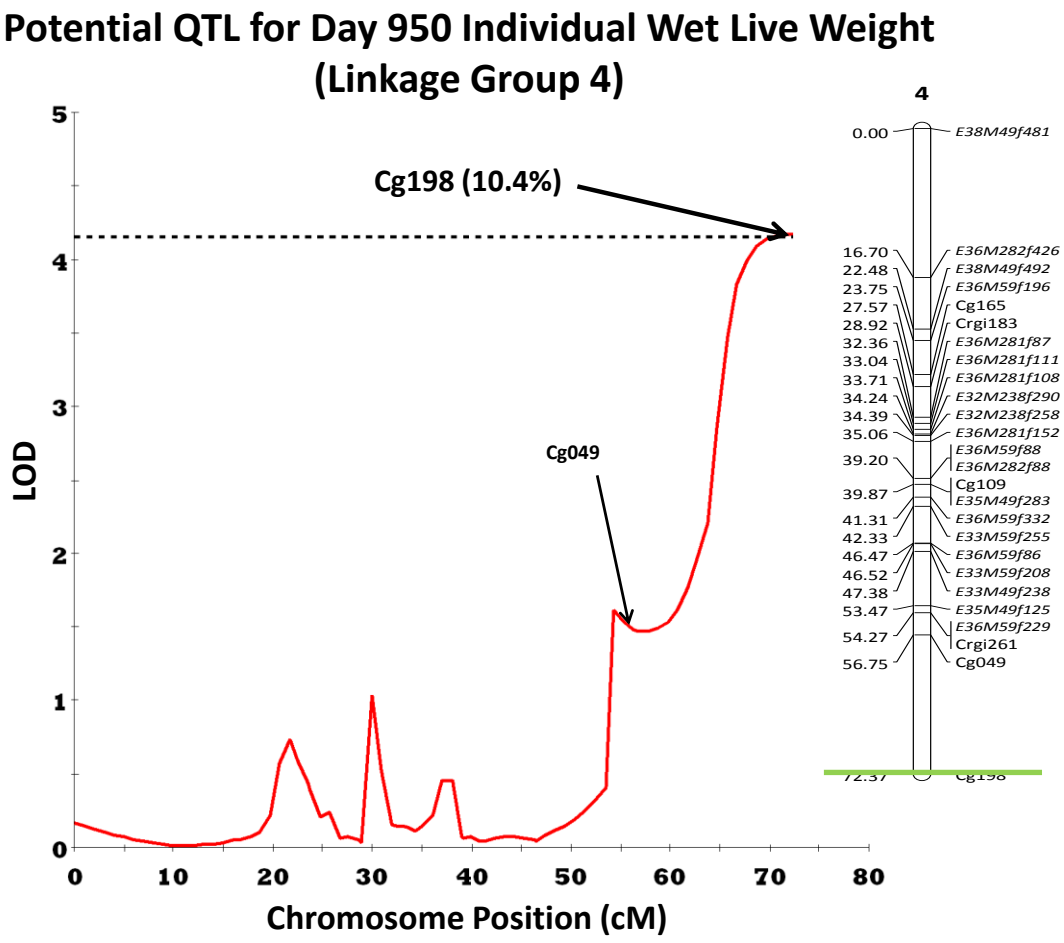


**Figure 5.4** Identification and mapping of a potential Day 950 individual wet live weight QTL on LG4. Identified QTL accounts for 10.4% of the total phenotypic variation and maps near the microsatellite marker Cg198 (green bar located on linkage map at 72.37 cM), which is flanked on one side by the microsatellite marker Cg049 (56.75 cM). The depicted LOD of 4.17 falls under the genome-wide significant threshold of 4.3 ($\alpha = 0.05$) but exceeds the chromosome-wide threshold of 3.9 ($\alpha = 0.01$).

*Shell Length*

We identified and mapped a total of 5 QTLs for shell length measured from the anterior hinge to the posterior shell margin. We mapped one QTL accounting for 4.6% of the total phenotypic variation for Day 140 shell length corrected for hatchery and nursery effects using the design covariate Day 140 individual wet live weight to LG6 near the region identified as significant for Day 140 wet live weight (38.99 cM) using both the IM and MQM analyses with the mixture model algorithm, the microsatellite marker Cg130 (LG6) as an analysis cofactor and a genome-wide LOD significance threshold of 4.3 ($\alpha = 0.05$). Figure 5.5 plots the MQM analysis results from the mixture model algorithm and a chromosome-wide LOD significance threshold of 3.9 ($\alpha = 0.01$). No significant LOD score was associated with use of the regression algorithm.

We also mapped one QTL accounting for 8.0% of the total phenotypic variation for Day 358 shell length to LG3 near the AFLP marker E33M49f145 (40.20 cM), which is flanked by AFLP markers E32M282f175 (39.09 cM) and E33M59f127 (43.33 cM), using the MQM analyses with either the regression or mixture model algorithms and the AFLP markers E35M59f139 (LG1) and E33M49f145 (LG3) as analysis cofactors and a genome-wide LOD significance threshold of 4.2 ($\alpha = 0.05$). Figure 5.6 plots the MQM analysis results from the mixture model

algorithm and a chromosome-wide LOD significance threshold of 3.9 (α = 0.01). No significant LOD score was associated with any IM analysis.

We mapped two QTLs for Day 644 shell length to LG4 using both the IM and MQM analyses with either the regression or mixture model algorithms and the AFLP markers E36M49f89 (LG2) and E36M281f111 (LG4) as analysis cofactors and a genome-wide LOD significance threshold of 4.3 (α = 0.05). Figure 5.7 plots the MQM analysis results from the mixture model algorithm and a chromosome-wide LOD significance threshold of 4.0 (α = 0.01) where the first QTL accounts for 23.7% of the total phenotypic variation and maps near the AFLP markers E36M59f88 and E36M282f88 (39.20 cM), which are flanked by AFLP and microsatellite marker E36M281f152 (35.06 cM) and Cg109 (39.87 cM). The second QTL accounts for 15.7% of the total phenotypic variation and maps near the AFLP marker E33M49f238 (47.38 cM), which is flanked by the AFLP markers E33M59f208 (46.52 cM) and E35M49f125 (53.47 cM).

We mapped one QTL accounting for 9.7% of the total phenotypic variation for Day 950 shell length to LG5 near the AFLP marker E32M282f392 (23.00 cM), which is flanked by AFLP markers E32M282f120 (20.98 cM) and E33M59f199 (24.01 cM), using both the IM and MQM analyses with the regression algorithm and the AFLP marker E32M282f392 (LG5) as an analysis cofactor and a genome-wide LOD significance threshold of 4.3 (α = 0.05). Figure 5.8 plots the MQM

analysis results from the regression algorithm and a chromosome-wide LOD significance threshold of 3.9 (α = 0.01). No significant LOD score was associated with use of the mixture model algorithm.

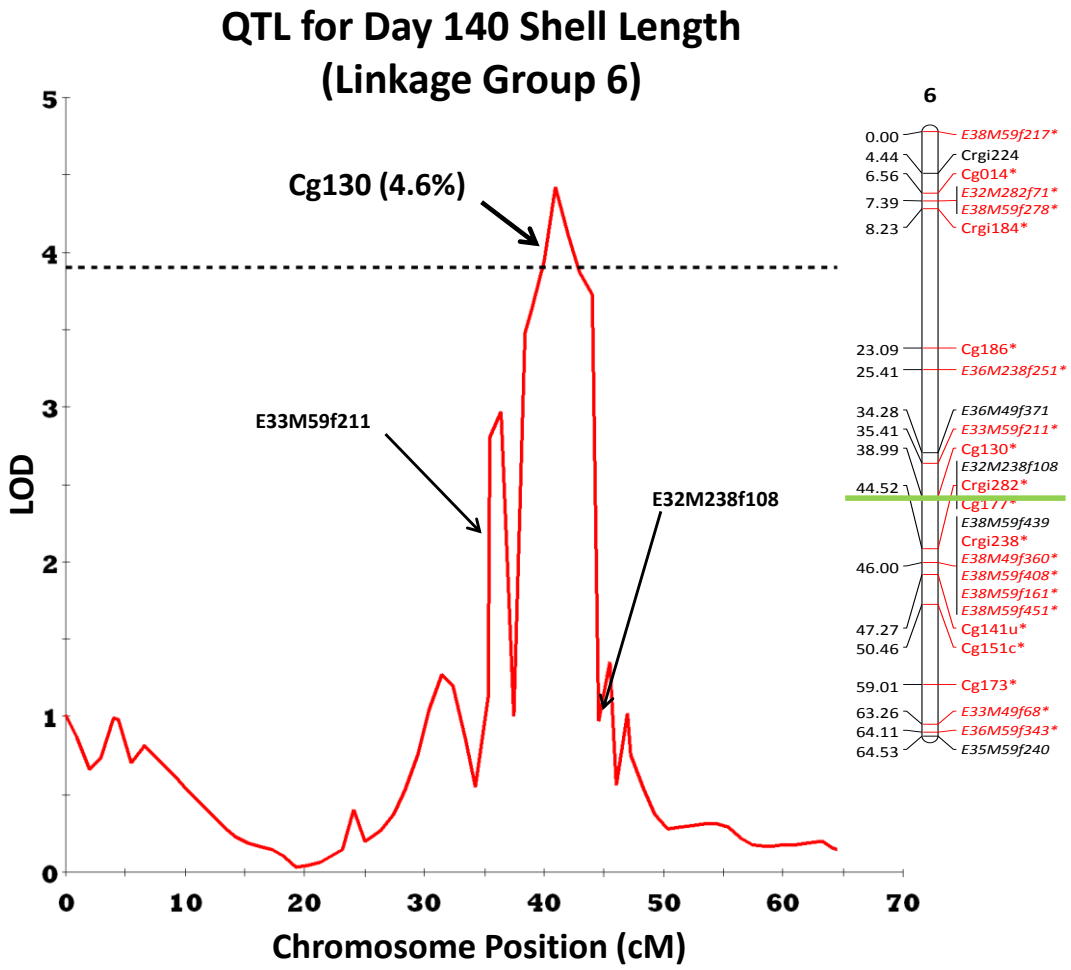


**Figure 5.5** Identification and mapping of Day 140 shell length QTL on LG6. Identified QTL accounts for 4.6% of the total phenotypic variation and maps near the microsatellite marker Cg130 (green bar located on linkage map at 38.99 cM), which is flanked by the AFLP markers E33M59f211 (35.41 cM) and E32M238f108 (45.52 cM). This is the same genomic region identified as significant for Day 140 individual wet live weight. A chromosome-wide significance threshold of 3.9 (α = 0.01) is depicted.

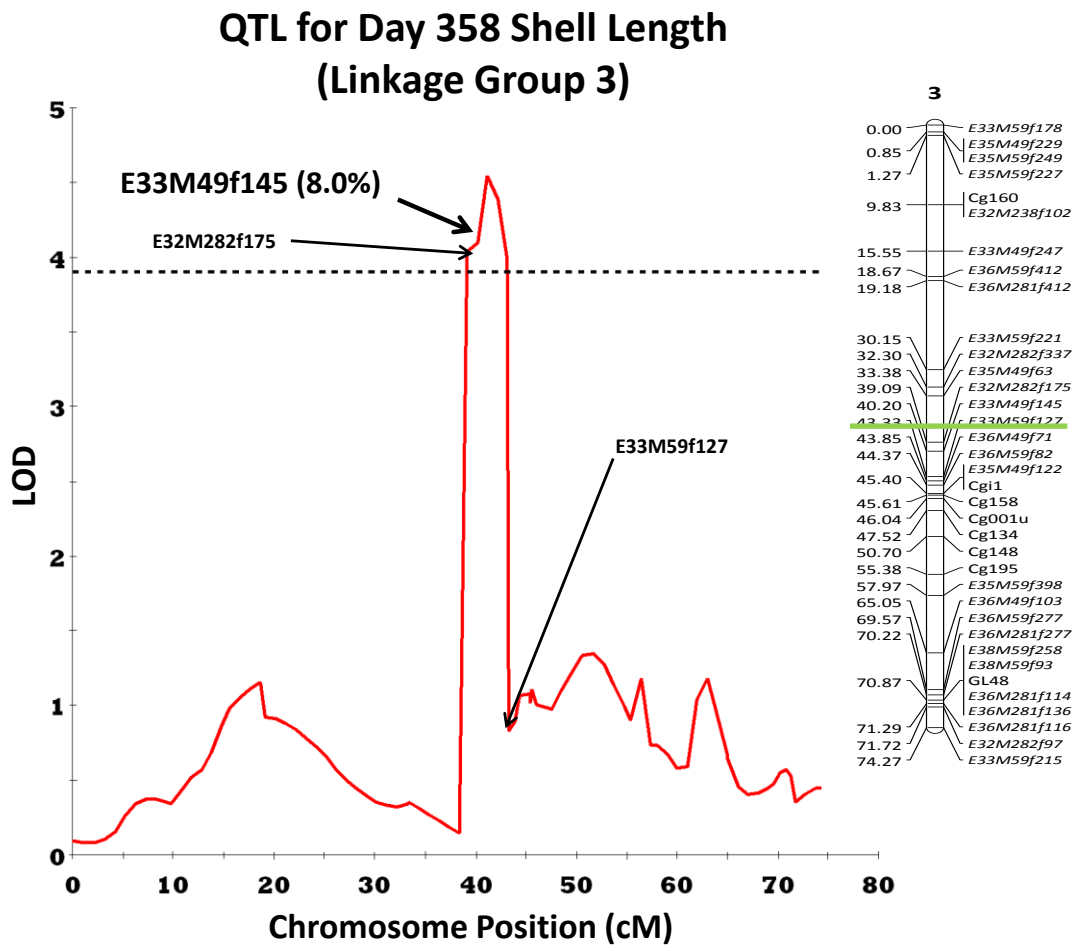


**Figure 5.6** Identification and mapping of Day 358 shell length QTL on LG3. Identified QTL accounts for 8.0% of the total phenotypic variation and maps near the AFLP marker E33M49f145 (green bar located on linkage map at 40.20 cM), which is flanked by AFLP markers E32M282f175 (39.09 cM) and E33M59f127 (43.33 cM). A chromosome-wide significance threshold of 3.9 ($\alpha = 0.01$) is depicted.

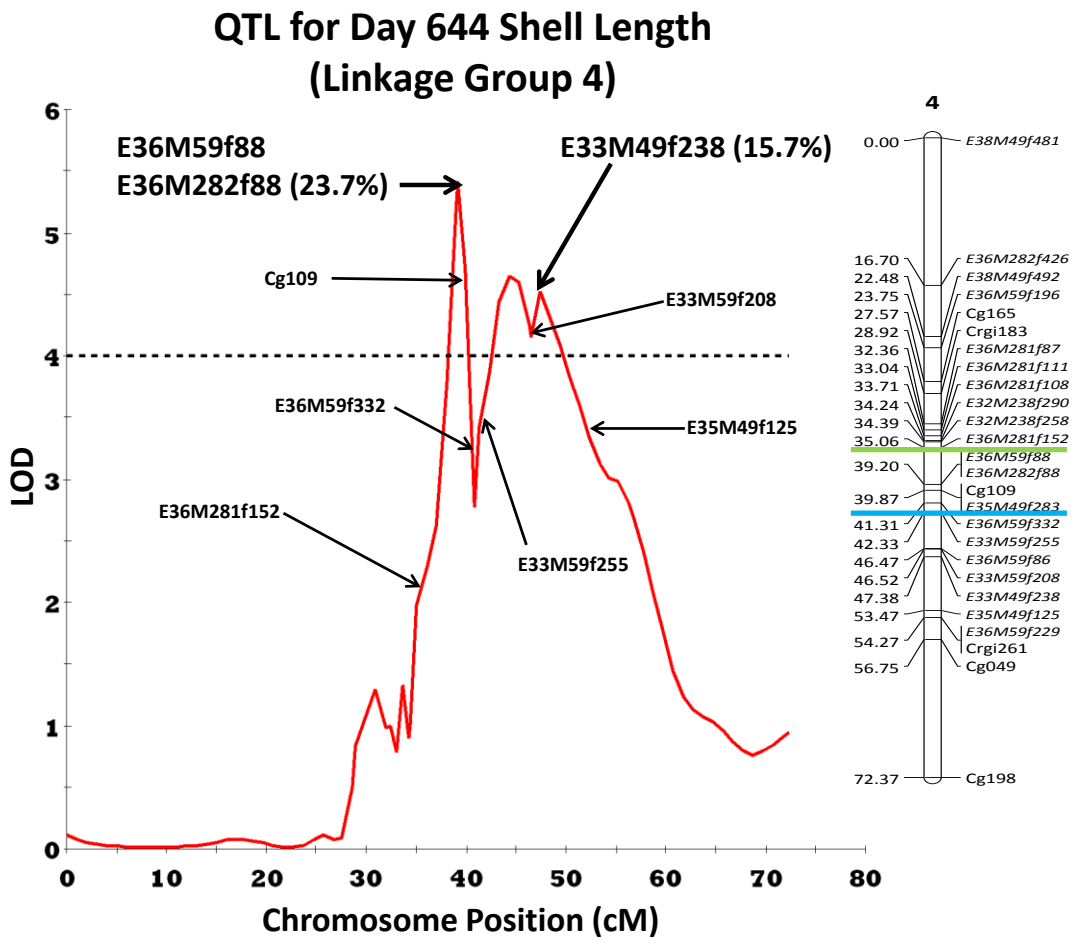


**Figure 5.7** Identification and mapping of two Day 644 shell length QTLs on LG4. The first QTL accounts for 23.7% of the total phenotypic variation and maps near the AFLP markers E36M59f88 and E36M282f88 (green bar located on linkage map at 39.20 cM), which are flanked by AFLP and microsatellite marker E36M281f152 (35.06 cM) and Cg109 (39.87 cM). The second accounts for 15.7% of the total phenotypic variation and maps near the AFLP marker E33M49f238 (blue bar located on linkage map at 47.38 cM), which is flanked by the AFLP markers E33M59f208 (46.52 cM) and E35M49f125 (53.47 cM). A chromosome-wide significance threshold of 4.0 ($\alpha = 0.01$) is depicted.

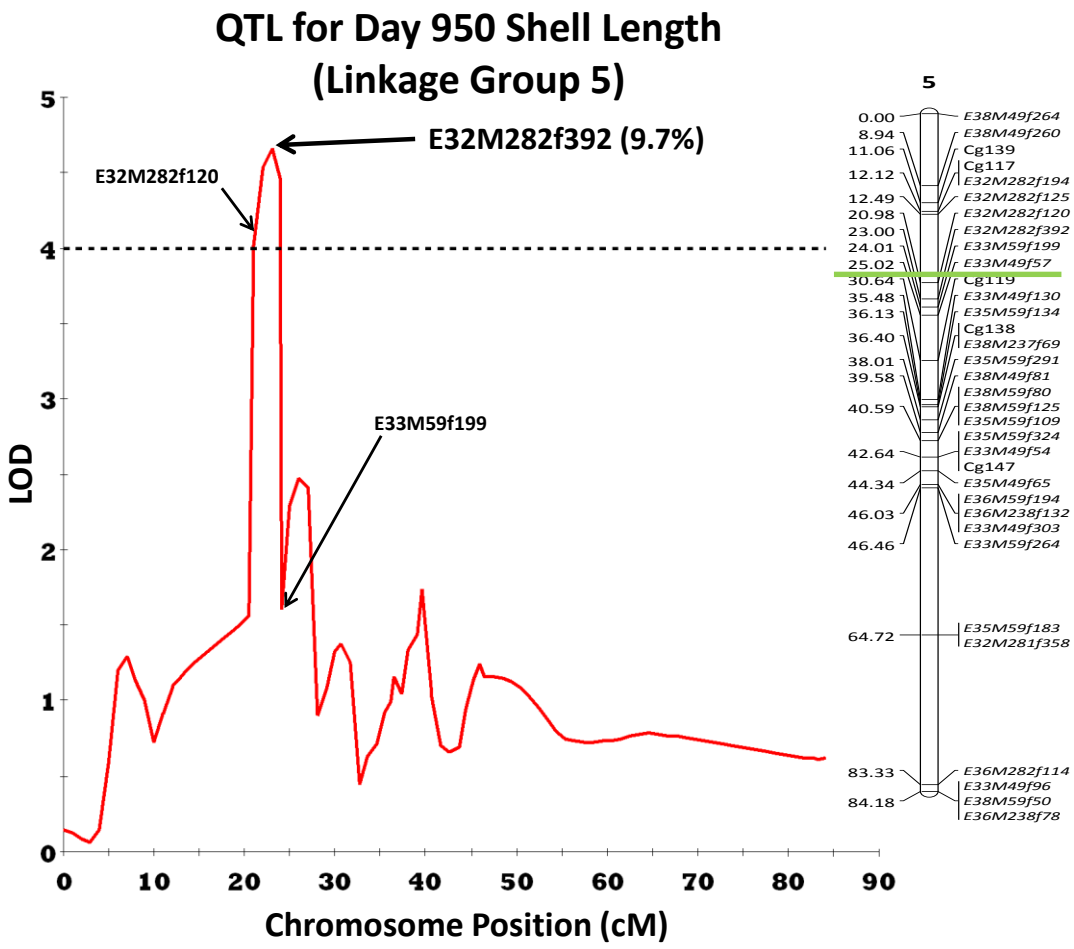


**Figure 5.8** Identification and mapping of Day 950 shell length QTL. Identified QTL accounts for 9.7% of the total phenotypic variation and is located on LG5 near the AFLP marker E32M282f392 (green bar located on linkage map at 23.00 cM), which is flanked by AFLP markers E32M282f120 (20.98 cM) and E33M59f199 (24.01 cM). A chromosome-wide significance threshold of 3.9 ($\alpha$ = 0.01) is depicted.

*Shell Width*

We identified and mapped two QTLs and one potential QTL for shell width measured perpendicular to shell length between the widest dorsal-ventral margins. We mapped one QTL accounting for 7.4% of the total phenotypic variation for Day 140 shell width corrected for hatchery and nursery effects using the design covariate Day 140 individual wet live weight to LG4 near the AFLP marker E35M49f125 (53.47 cM), which is flanked by AFLP and microsatellite markers E33M49f238 (47.38 cM) and E36M59f299 and Crgi261 (54.27 cM) (Figure 5.9), and one potential QTL accounting for 2.1% of the total phenotypic variation for Day 140 shell width corrected for hatchery and nursery effects using the design covariate Day 140 individual wet live weight to LG7 near E33M59f137 (46.49 cM), which is flanked by the AFLP markers E35M59f268 (44.93 cM) and E36M49f92 (60.54 cM) (Figure 5.10), using both IM and MQM analyses with either the regression or mixture model algorithms and the AFLP markers E35M49f125 (LG4) and E33M59f137 (LG7) as analysis cofactors and a genome-wide LOD significance threshold of 4.3 ($\alpha = 0.05$). Figure 5.9 plots the MQM analysis results from the mixture model algorithm and a chromosome-wide LOD significance threshold of 3.8 ($\alpha = 0.01$), while Figure 5.10 plots the same MQM analysis and an LOD significance threshold of 3.92, which fell just under chromosome-wide LOD significance threshold of 4.0 for LG7 ($\alpha = 0.01$).

We mapped one QTL accounting for 10.1% of the total phenotypic variation for Day 358 shell width to LG10 near the AFLP marker E36M59f68 (46.21 cM), which is flanked by the AFLP and microsatellite markers E35M59f122 and CgE204 (43.82 cM) and E38M49f196 (50.43 cM), using both the IM and MQM analyses with either the regression or mixture model algorithms, the AFLP marker E36M59f68 (LG10) as an analysis cofactor and a genome-wide LOD significance threshold of 4.2 ($\alpha = 0.05$). Figure 5.11 plots the MQM analysis results from the mixture model algorithm and a chromosome-wide LOD significance threshold of 3.4 ($\alpha = 0.01$).

No additional QTL could be verified utilizing either the data's genome-wide ($\alpha = 0.05$) or chromosome-wide LOD significance threshold ($\alpha = 0.01$) for shell width as the oysters aged (Day 644 or 950).

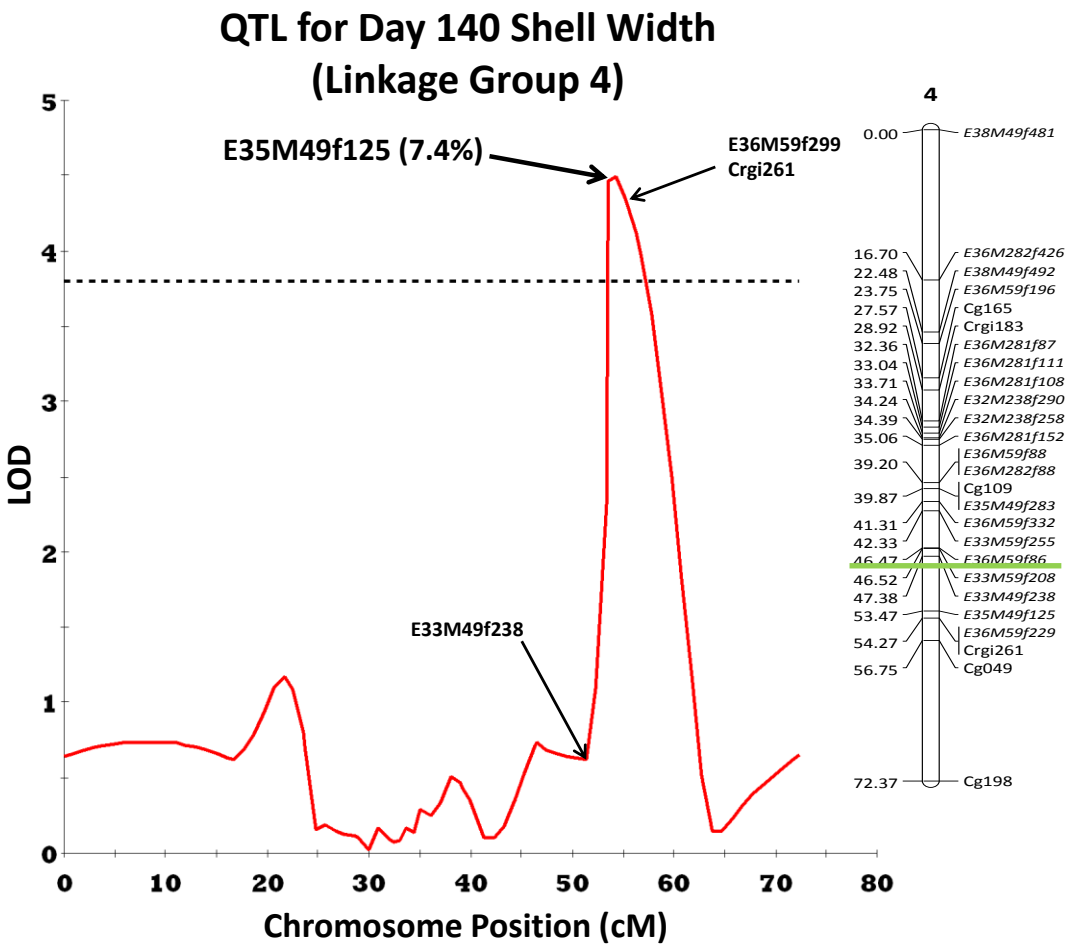


**Figure 5.9** Identification and mapping of Day 140 shell width QTL. Identified QTL accounts for 7.4% of the total phenotypic variation and is located on LG4 near the AFLP marker E35M49f125 (green bar located on linkage map at 53.47 cM), which is flanked by AFLP and microsatellite markers E33M49f238 (47.38 cM) and E36M59f299 and Crgi261 (54.27 cM). A chromosome-wide significance threshold of 3.8 ($\alpha = 0.01$) is depicted.

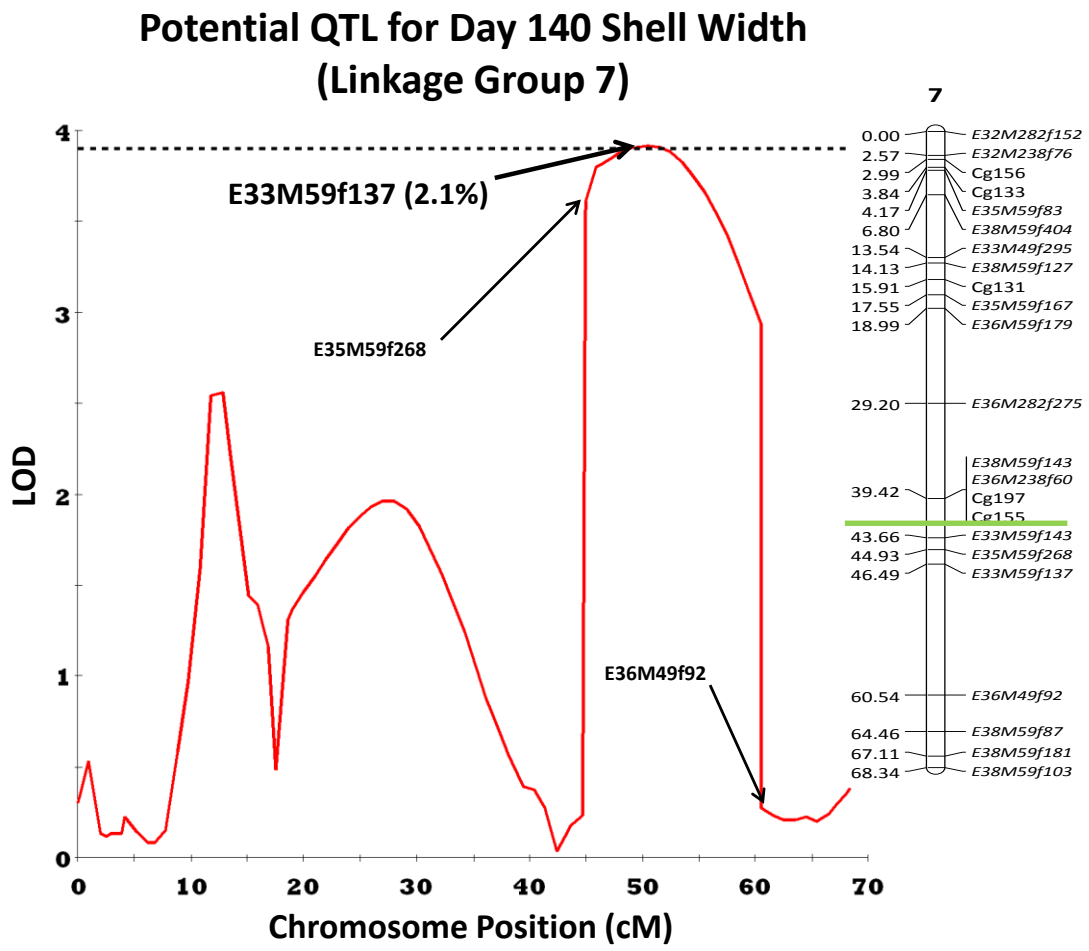


**Figure 5.10** Identification and mapping of a potential Day 140 shell width QTL on LG7. Identified QTL accounts for 2.1% of the total phenotypic variation and is located near the AFLP marker E33M59f137 (green bar located on linkage map at 46.49 cM), which is flanked by the AFLP markers E35M59f268 (44.93 cM) and E36M49f92 (60.54 cM). The depicted LOD significance threshold of 3.92 falls under the chromosome-wide threshold of 4.0 ($\alpha = 0.01$).

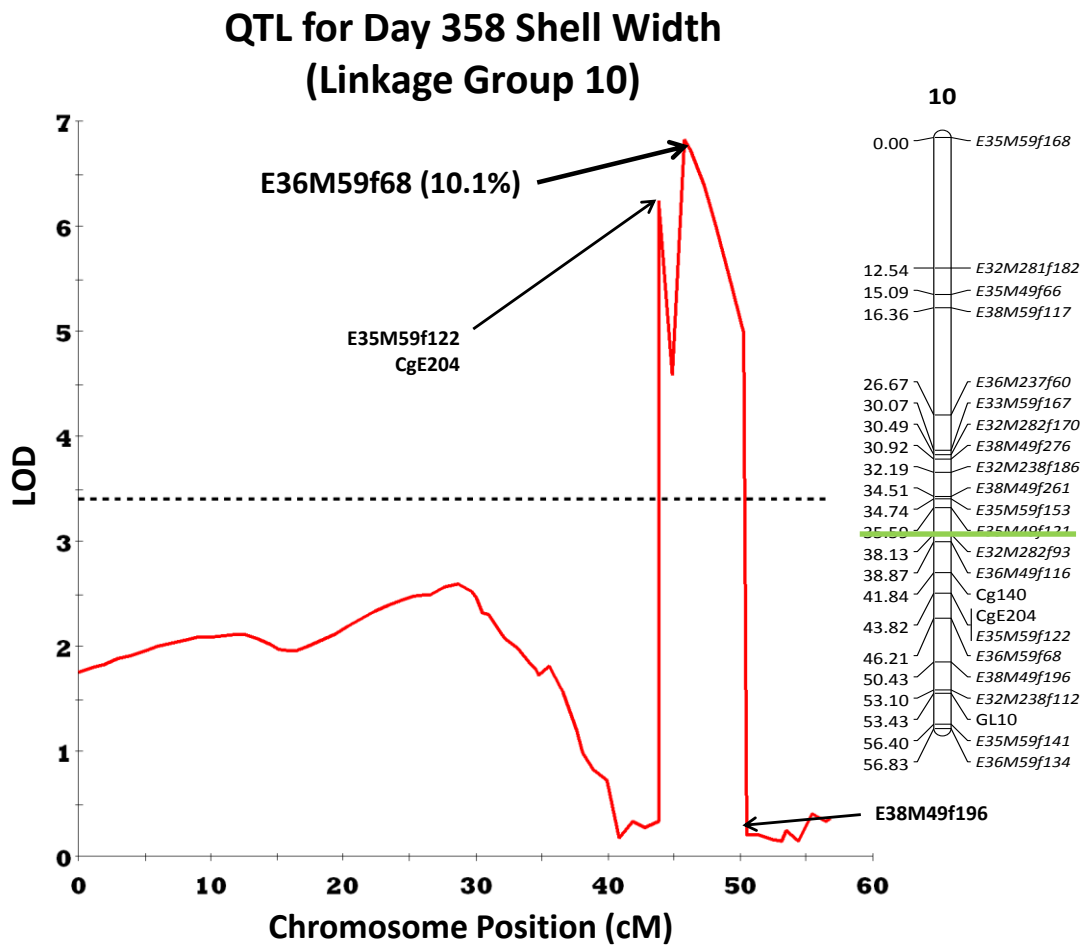


**Figure 5.11** Identification and mapping of a Day 358 shell width QTL on LG10. Identified QTL accounts for 10.1% of the total phenotypic variation and is located near the AFLP marker E36M59f68 (green bar located on linkage map at 46.21 cM), which is flanked by the AFLP and microsatellite markers E35M59f122 and CgE204 (43.82 cM) and E38M49f196 (50.43 cM)). A chromosome-wide significance threshold of 3.4 ($\alpha = 0.01$) is depicted.

*Shell Depth*

We identified and mapped three QTLs and three potential QTL for shell depth measured as the greatest vertical distance between the two valves. We mapped two QTLs for Day 140 shell depth corrected for hatchery and nursery effects using the design covariate Day 140 individual wet live weight (Figure 5.12) using both the IM and MQM analyses with either the regression or mixture model algorithms, the microsatellite and AFLP markers Crgi257 (LG1) and E35M59f268 (LG7) as analysis cofactors and a genome-wide LOD significance threshold of 4.2 ($\alpha = 0.05$). One QTL accounts for 10.8% of the total phenotypic variation and maps to LG1 located near the microsatellite marker Crgi257 (44.69 cM), which is flanked by AFLP markers E32M281f319 (40.38 cM) and E35M59f226 (45.54 cM). The other QTL accounts for 8.5% of the total phenotypic variation and maps to LG7 near the AFLP marker E35M59f268 (44.93 cM), which is flanked by the AFLP markers E33M59f143 (43.66 cM) and E33M59f137 (46.49 cM).

We mapped one potential QTL accounting for 3.6% of the total phenotypic variation for Day 358 shell depth to LG4 between the microsatellite markers Cg049 (56.75 cM) and Cg198 (72.37 cM) using both the IM and MQM analyses with the mixture model algorithm and the microsatellite marker Cg049 (LG4) as an analysis cofactor and a genome-wide LOD significance threshold of 4.2 ($\alpha = 0.05$). Figure 5.13 plots the MQM analysis results from the mixture model

algorithm and a chromosome-wide LOD significance threshold of 3.9 (α = 0.01). No significant LOD score was associated with the regression algorithm and no individual mapped marker was deemed significant with any analysis or algorithm method.

We mapped one QTL accounting for 9.2% of the total phenotypic variation for Day 644 shell depth to LG10 near the AFLP marker E32M282f93 (38.13 cM), which is flanked by the AFLP markers E35M49f121 (35.59 cM) and E36M49f116 (38.87 cM), using both the IM and MQM analyses with the mixture model algorithm and the AFLP markers E32M282f120 (LG5) and E36M49f116 (LG10) as analysis cofactors and a genome-wide LOD significance threshold of 4.3 (α = 0.05). Figure 5.14 plots the MQM analysis results from the mixture model algorithm and a chromosome-wide LOD significance threshold of 3.6 (α = 0.01). Although no significant genome-wide LOD score was associated with use of the regression algorithm, LOD scores did exceed the chromosome-wide threshold of 3.6 (α = 0.01) for LG10.

We also mapped one potential QTL accounting for 18.4% of the total phenotypic variation for Day 644 Shell Depth to LG5 near the AFLP marker E32M282f120 (20.98 cM), which is flanked by the AFLP markers E32M282f125 (12.49 cM) and E32M282f392 (23.00 cM), using the same analysis as the previously described for Day 644 Shell Depth QTL (Figure 5.15). However, the observed LOD score of

3.49 did not exceed the chromosome-wide LOD significance threshold of 3.6 (α = 0.01).

No additional QTL were detected utilizing either a genome-wide (α = 0.05) or chromosome-wide LOD significance threshold (α = 0.01) for shell depth as the oysters aged (Day 950). However, we mapped one potential QTL accounting for 33.1% of the total phenotypic variation for Day 950 shell depth to LG5 near the AFLP marker E32M282f392 (23.00 cM), which is flanked by the AFLP markers E32M282f120 (20.98 cM) and E33M59f199 (24.01 cM), using the MQM analyses with the mixture model algorithm and the AFLP markers E33M49f247 (LG3) and E32M282f392 (LG5) as analysis cofactors. Although the observed LOD of 3.92 did fall under the genome-wide significant threshold of 4.3 (α = 0.05), it exceeded the chromosome-wide threshold of 3.9 (α = 0.01) (Figure 5.16). No significant LOD score was associated with any IM analysis or use of the regression algorithm.

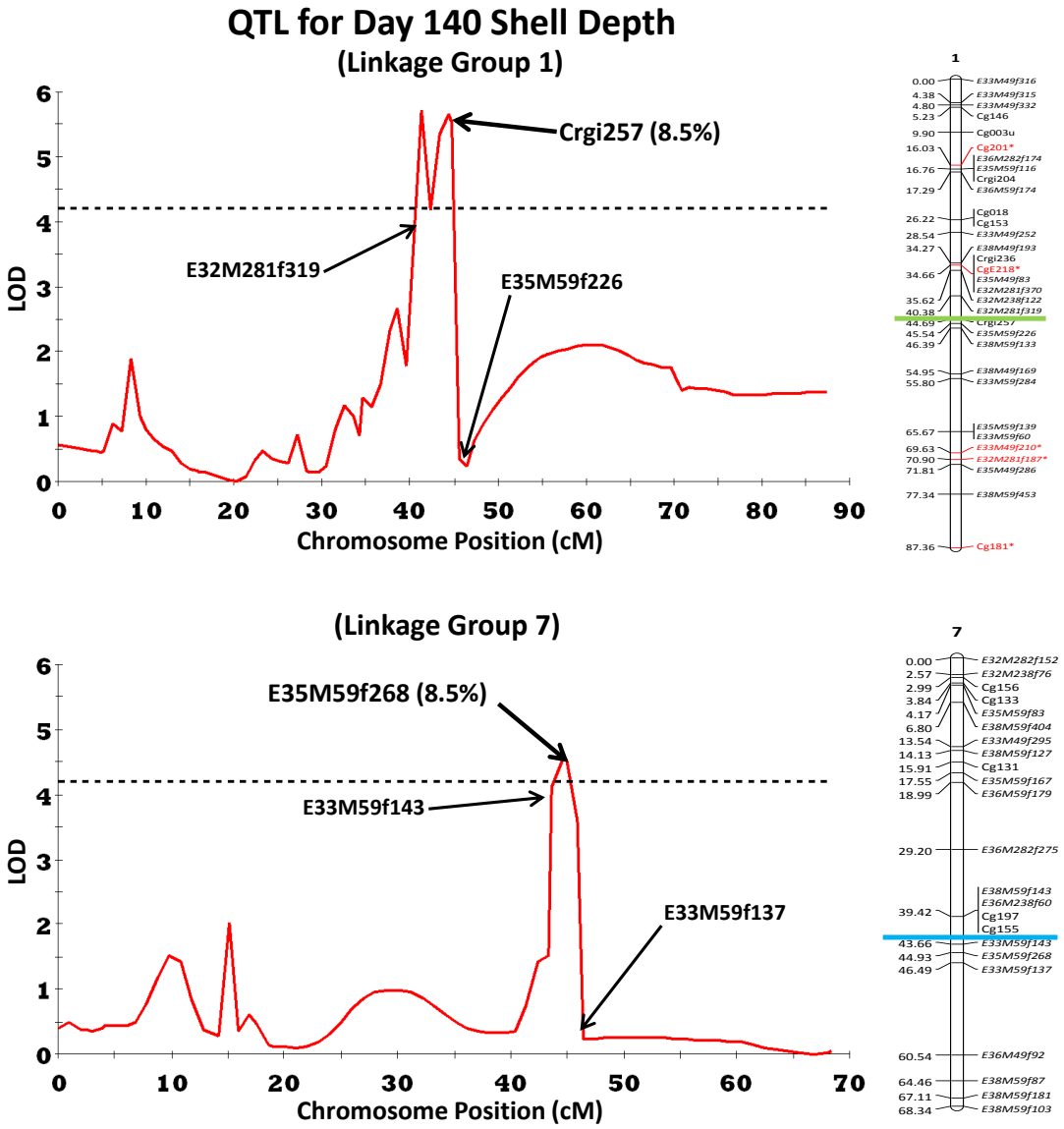


**Figure 5.12** Identification and mapping of two Day 140 shell depth QTL. The first identified QTL accounts for 10.8% of the total phenotypic variation and is located on LG1 near the microsatellite marker Crgi257 (green bar on LG1 linkage map at 44.69 cM), which is flanked by AFLP markers E32M281f319 (40.38 cM) and E35M59f226 (45.54 cM). The second accounts for 8.5% of the total phenotypic variation and maps to LG7 near the AFLP marker E35M59f268 (blue bar on LG7 linkage map at 44.93 cM), which is flanked by the AFLP markers E33M59f143 (43.66 cM) and E33M59f137 (46.49 cM). A genome-wide significance threshold of 4.2 ($\alpha = 0.05$) is depicted.

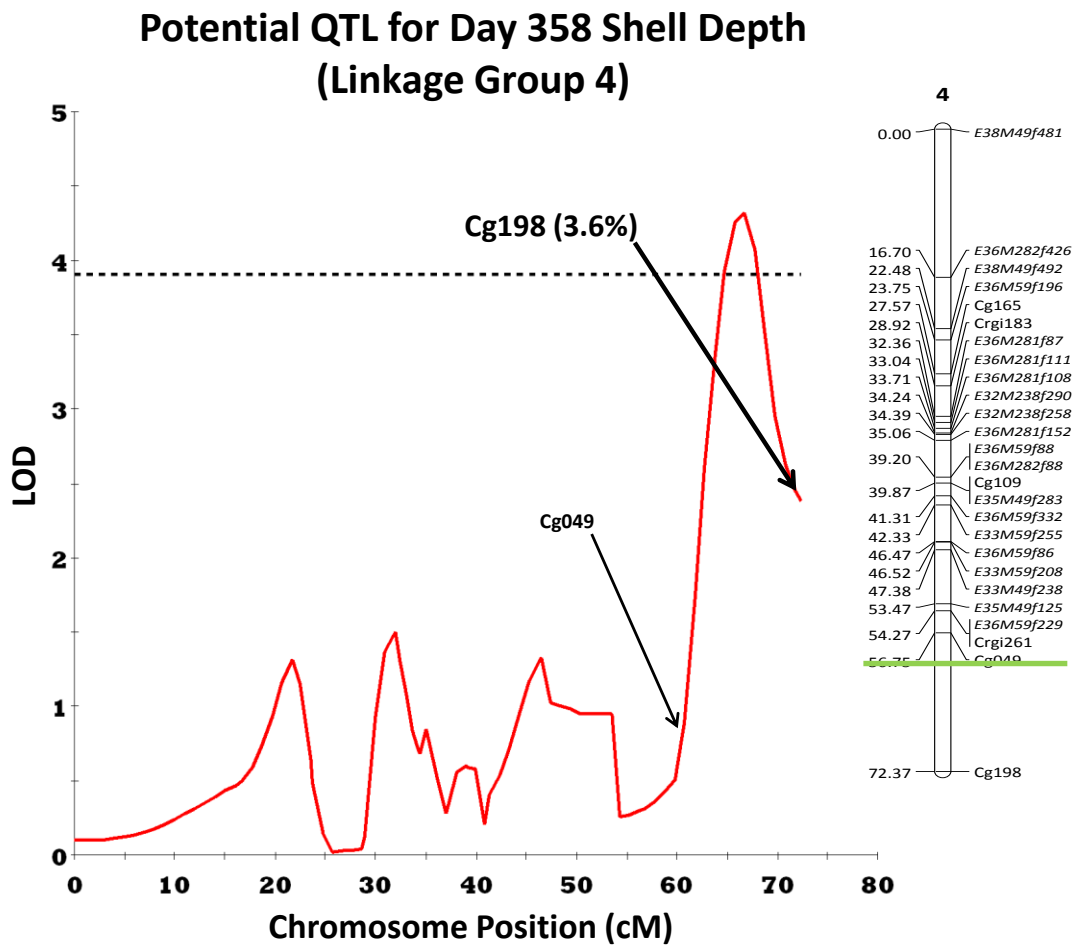


**Figure 5.13** Identification and mapping of a potential Day 358 shell depth QTL on LG4. Identified QTL accounts for 3.6% of the total phenotypic variation (green bar located on linkage map at 66.75 cM) and is flanked by the microsatellite markers Cg049 (56.75 cM) and Cg198 (72.37 cM). The depicted chromosome-wide LOD significance threshold ($\alpha = 0.01$) is 3.9 and although the LOD score clearly exceeds the genome-wide significance threshold, no individual mapped marker was significant.

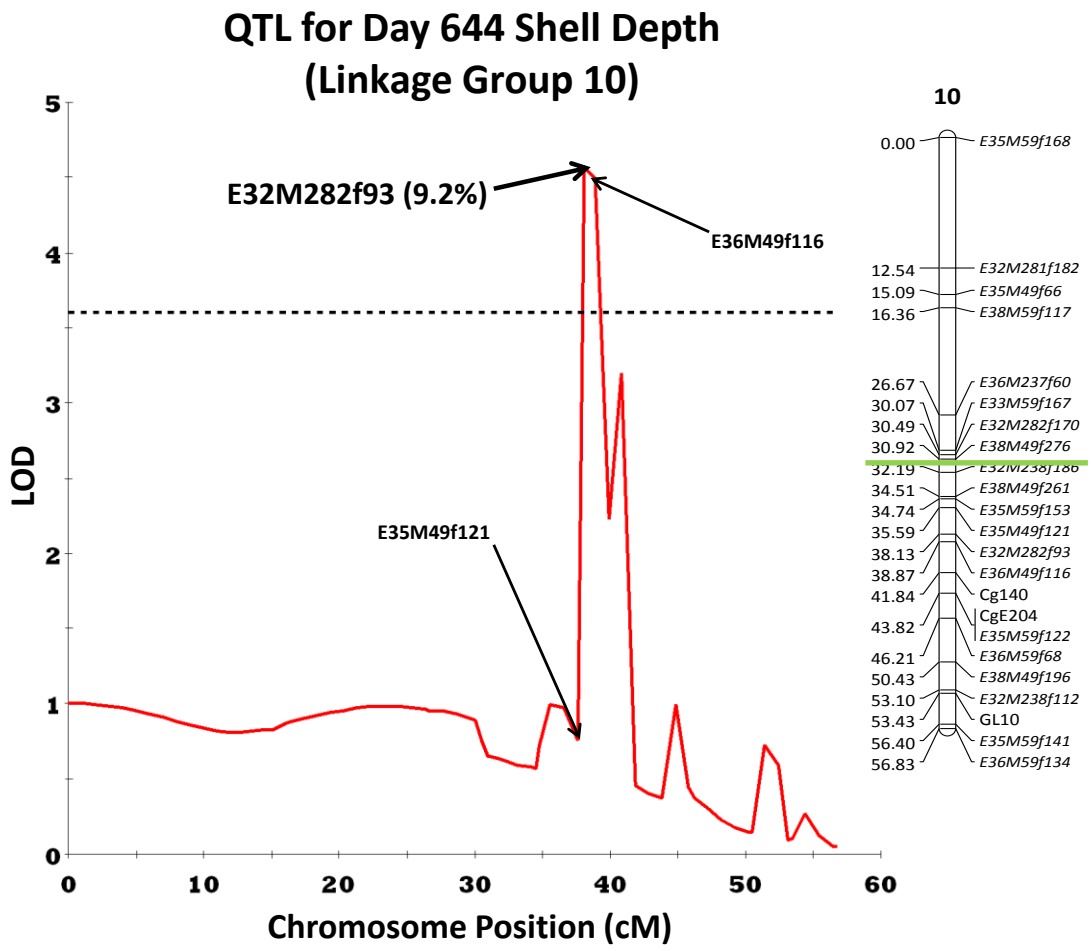


**Figure 5.14** Identification and mapping of a Day 644 shell depth QTL on LG10. Identified QTL accounts for 9.2% of the total phenotypic variation and is located near the AFLP marker E32M282f93 (green bar located on linkage map at 38.13 cM), which is flanked by the AFLP markers E35M49f121 (35.59 cM) and E36M49f116 (38.87 cM). A chromosome-wide significance threshold of 3.6 ($\alpha$ = 0.01) is depicted.

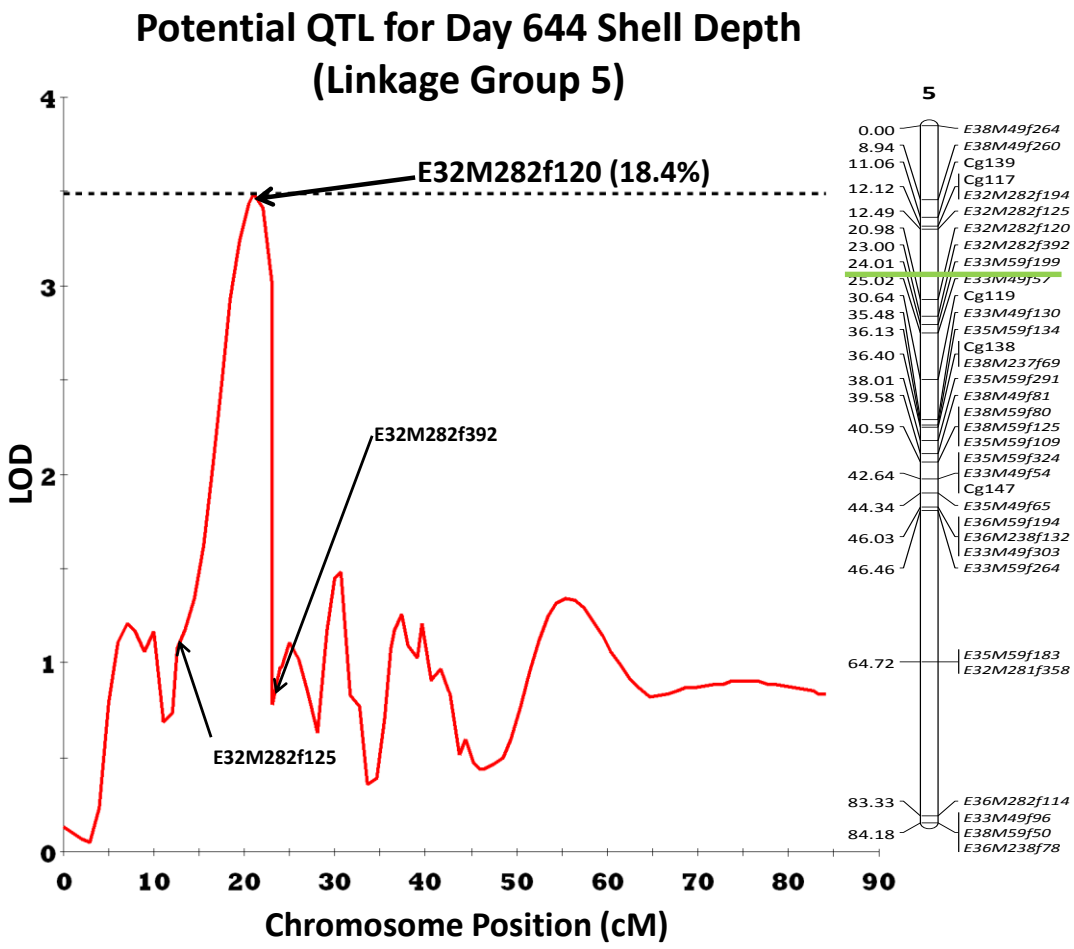


**Figure 5.15** Identification and mapping of a potential Day 644 shell depth QTL on LG5. Identified QTL accounts for 18.4% of the total phenotypic variation and maps near the AFLP marker E32M282f120 (green bar located on linkage map at 20.98 cM), which is flanked by the AFLP markers E32M282f125 (12.49 cM) and E32M282f392 (23.00 cM). The depicted LOD significance threshold of 3.49 falls under the chromosome-wide significance threshold of 3.6 ($\alpha = 0.01$).

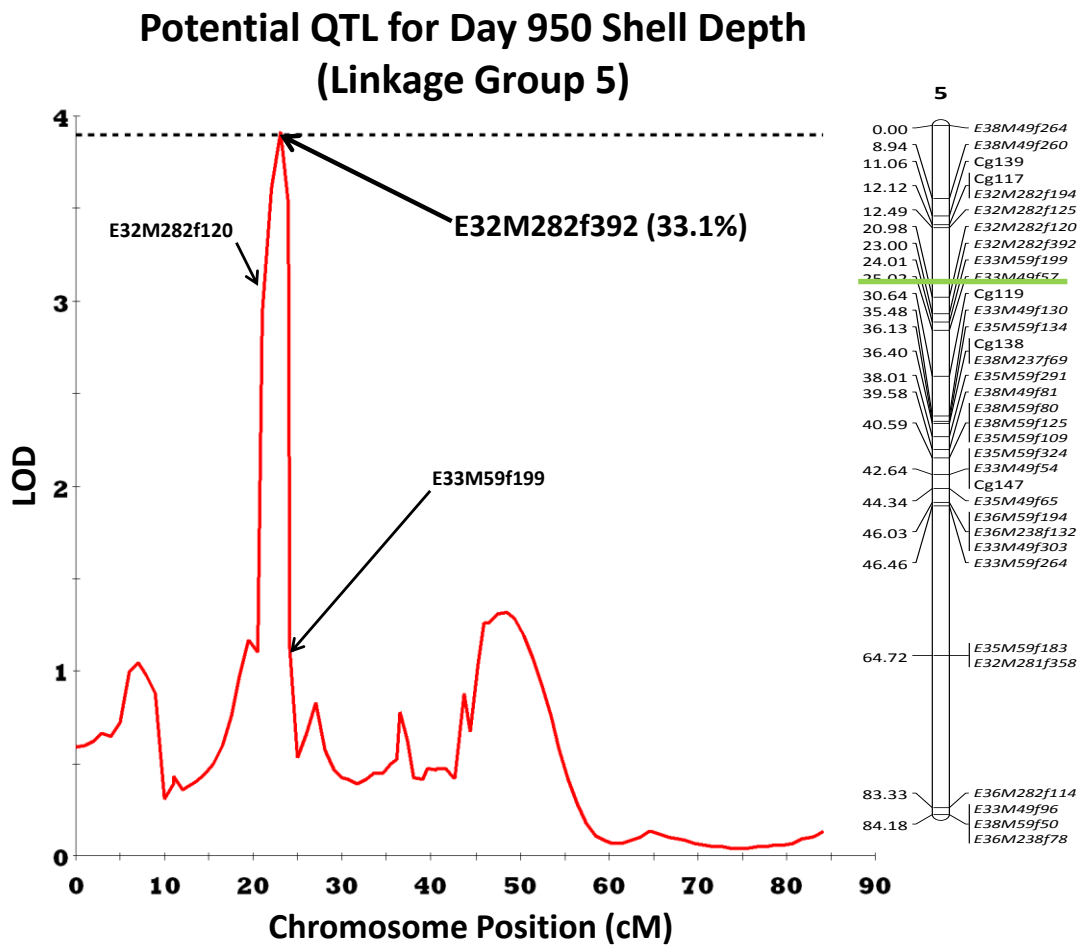


**Figure 5.16** Identification and mapping of a potential Day 950 shell depth QTL on LG5. Identified QTL accounts for 33.1% of the total phenotypic variation and maps near the AFLP marker E32M282f392 (green bar located on linkage map at 23.00 cM), which is flanked by the AFLP markers E32M282f120 (20.98 cM) and E33M59f199 (24.01 cM). A chromosome-wide significance threshold of 3.9 ($\alpha$ = 0.01) is depicted.

*Shell Shape and Cup Shape*

Although the same genomic regions that had been previously identified by mapped QTLs associated with the component morphometric measures utilized by the shape index were again observable in the shell shape (Length/Width) or cup shape (Width/Depth or Length/Depth) LOD profiles, no LOD exceeded either the data's genome-wide ($\alpha = 0.05$) or chromosome-wide LOD significance threshold ($\alpha = 0.01$) for any morphometric shape index, with or without model adjustment for either the fixed effect Site or design covariate Day 140 live wet weight at any time point. We observed no evidence for the existence of a QTL specific to any shape index not previously identified as part of the individual component morphometric measure from which it was constructed.

**Discussion**

QTL analyses can identify genomic regions responsible for part of the phenotypic variation associated with a quantitative trait. If the quantitative trait investigated is also of an economic interest, then the identification and mapping of QTL associated with that trait may be of value for breeding programs simply by demonstrating alleles accounting for a quantifiable proportion of the total phenotypic variation are segregating within the breeding population. This variation may be easily amendable to selective pressures through traditional methods (Andersson 2001; Spelman *et al.* 1996), especially for the developing U.S. West Coast Pacific oyster breeding and genetic improvement programs where selection is still in its infancy (Langdon *et al.* 2003) and the fixation of major genes known to influence harvest yield can have an immediate impact (Muir 1996; Muir 2005; Muir& Stick 1996).

We utilized an integrated mapping strategy based on crosses between phenotypically divergent parents from differentiated outbred families (Wu *et al.* 2002) to maximize the within-family genetic variance. Any growth-related QTL detected from the strategy of crossing two Molluscan Broodstock Program (MBP) Cohort 14 third-generation families selected for harvest yield provides strong evidence that alleles accounting for a significant portion of the phenotypic variation have not been fixed and are still segregating within the breeding

population. By not using inbred families to create the segregating populations, we can reduce the likelihood that either inbreeding depression or heterosis influenced the underlying genetic distribution (Freyer *et al.* 2009) but we cannot differentiate between the type or proportion of total phenotypic variation associated with different types of gene action (additive or dominance) using a single outcrossed mapping family (Van Ooijen 2009).

The distribution of any segregating quantitative trait in a QTL mapping population is a mixture of normal distributions within each of the genotypic marker classes. However, if the overall phenotypic distribution of the quantitative trait appears normal as well, it does not mean there aren't QTL present, but could instead mean that multiple smaller QTL are contributing to the phenotypic effect making the distribution appear normal (Doerge *et al.* 1997). By detecting several different genomic regions, each accounting for approximately equal proportions of the phenotypic variance instead of identifying a single QTL that influences the same trait as the oyster aged, supports the existence of multiple small QTL (Leamy *et al.* 2010; Mackay 2010).

Our detection of QTLs using multiple algorithms (regression and mixture model) and different analysis methods (IM and MQM) does lend support to our results even if multiple unresolved QTLs are also influencing the trait (Haley *et al.* 1994; Knott *et al.* 1996; Knott *et al.* 1998). Furthermore, use of the MQM analysis, which

is equivalent to composite interval mapping (Zeng 1993, 1994), increased our QTL resolution by controlling residual noise caused from existing QTLs in the model. Our use of Day 140 live wet weight as a design covariate in the biologically relevant models, including the non-weight-based Day 140 morphometric measures (length, width and depth) and subsequent weight analyses as the oyster aged covariate, increased our precision by correcting for within-family hatchery and nursery effects caused by husbandry practices. However, the LOD score associated with any individual marker remained unchanged if the covariate was incorporated into any model other than these specific biologically relevant models and was therefore, not used in those analyses.

We were able to identify and map a total of 12 QTL and 5 potential QTL. Identified QTL were not equally distributed among the genome and appeared to favor specific linkage groups as well as specific regions (hotspots) on those linkage groups. Linkage Group 4 had the most mapped regions with a total of 3 QTL and 2 potential QTL, favoring the genomic region between 39 cM and the terminal end located at 72 cM. Although both potential QTLs (Day 358 shell depth and Day 950 individual wet live weight) mapped to the same general region and may indicate a single gene, all other LG4 QTL (Day 140 shell width and 2 for Day 644 shell length) mapped to very different but adjoining genomic regions separated by at least 5 cM. Linkage Group 10 had a total of 3 QTL (Day 358 individual live wet weight and shell width and Day 644 shell depth) favoring the

genomic region between 38 cM and the terminal end located at 53 cM, with each identified QTL separated by at least 8 cM. Linkage Group 5 also had one QTL (Day 950 shell length) and 2 potential QTL (Day 644 and Day 950 shell depth). However, these QTLs mapped to a very small genomic region on LG5 between 21 and 23 cM, which may indicate a common gene. Linkage Group 6 had what appears to be a single QTL near 39 cM affecting both Day 140 individual wet live weight and shell length and LG 7 mapped a QTL (Day 140 shell depth) and a potential QTL (Day 140 shell width) to what is also likely to be a common QTL located between 45 and 46 cM. Both LG1 and LG3 mapped a single QTL (Day 140 shell depth and Day 358 shell length, respectively) and no QTL or potential QTL were associated with LG2, LG8 or LG9. These candidate genomic hotspots may be of particular interest for more fine-scale mapping efforts.

Although pleiotropy (Mangin *et al.* 1998) or the existence of several closely related major genes or fractionated genes (multiple closely linked small effect QTL) residing within a common genomic region (Studer& Doebley 2011) is a simple explanation for multiple QTLs also being associating with that region, genomic hotspots have also been attributed to polymorphisms in a genomic regulatory or transcriptional factor, which are capable of affecting multiple traits (Breitling *et al.* 2008; Keurentjes *et al.* 2007; Wu *et al.* 2008), as well as linked epistatic complexes (Eckardt 2008). Effects attributed to multiple QTLs will be overestimated if they are linked in coupled phase (Liu 1998), creating “ghost”

QTLs that falsely contribute phenotypic variation to a genomic region and therefore, being mistaken as a hotpot. However, our use of marker cofactors in the MQM analysis helps eliminate this possibility (Arends *et al.* 2010).

Summarizing by trait, we identified 2 QTL and one potential QTL for individual wet live weight, 5 QTL for shell length as measured from the anterior hinge to the posterior shell margin, 2 QTL and one potential QTL for shell width as measured perpendicular to shell length between the widest dorsal-ventral margins, and 3 QTL and 3 potential QTL for shell depth measured as the greatest vertical distance between the two valves. Moderate phenotypic correlations among traits existed (data not shown) possibly indicate underlying genetic correlations, especially at the Day 140 evaluation where a common genomic region on LG6 was identified affecting both individual wet live weight and shell length and on LG7 a common genomic region was identified for both shell depth and shell width. No QTL or potential QTL could be detected with any morphometric shape index evaluated, largely in part to the greatly reduced phenotypic variation associated with these measures.

We detected the most QTL, 5 (individual wet live weight, shell length, shell width and 2 for shell depth) and one potential QTL (shell width), with the Day 140 measurement point. We detected fewer QTLs as the oyster aged with 3 QTL (individual wet live weight, shell length and shell width) and one potential QTL

(shell depth) with the Day 358 measurement point, 3 QTL (shell depth and 2 for shell length) and one potential QTL (shell depth) with the Day 644 measurement point and one QTL (shell length) and 2 potential QTL (individual wet live weight and shell depth) with the Day 950 measurement point. Oysters, like most bivalves, are known for their phenotypic plasticity (Galtsoff 1964), inflating the phenotypic distribution as oysters age. This additional source of variation and the fact that field mortality decreased our sample size by as much as 27.3%, in part explains our detection of more QTL at the earlier life ages. It is also likely that the increased power and precision achieved by blocking our experimental design for the fixed effect time of plant out and covariate accounting for potential hatchery, nursery and initial growout effects prior to plant out contributed to our ability to associate more QTL with the Day 140 measurement.

There are a number of uncontrollable experimental factors influencing mapping power when searching for QTL including genome size, number of genes controlling the trait, genomic position and distribution of the gene's genetic effects as well as the heritability of the trait (Liu 1998). Other factors are controllable such as mapping population type and size, analysis methodology, linkage map density and missing data proportion. We utilized the most complete linkage map published to date with an average genomic coverage of 91.39% and an average distance between markers of 2.62 cM as well as the most advanced QTL analysis method available for an outcross design (Van Ooijen 2009). Our

integrated mapping strategy based on crosses between phenotypically divergent parents from differentiated outbred families did result in identification and mapping of at least one QTL for each trait analyzed at the majority of the time points evaluated, in spite of funding limiting the mapping population size.

Because growth traits tend to be highly heritable in most species, including oysters (Degremont *et al.* 2007; Toro *et al.* 1995; Wang *et al.* 2010), our discovery of multiple growth-related QTL capable of affecting harvest yield in a mapping family created by crossing two third-generation families selected for yield indicates that traditional selection methods focusing on existing genetic variation can result in substantial improvements of harvest yield. We propose evaluating additional mapping families, which were created and measured as part of this study, to provide better understanding of how these QTL interact among morphometric traits and oyster age.

## Conclusion

We identified 12 QTLs and 5 potential QTLs segregating in an outcrossed mapping population for multiple growth-related morphometric measures at different field ages. Therefore, alleles accounting for a significant proportion of the total phenotypic variation remain segregating within the broodstock of West Coast Pacific oyster selective breeding programs and may be amendable to traditional selective methods.

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# Chapter 6
# Summary of Conclusions

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We utilized molecular genetics to answer specific questions in the fields of both population and quantitative genetics of Olympia and Pacific oysters. We evaluated remnant populations of Olympia oysters covering much of the species' contemporary range, which had been drastically reduced in extent and population size from historical densities, for genetic structure among populations. This research was motivated by the fact that numerous restoration efforts were proceeding without any understanding of the genetic structure among extant populations, which could be substantial as a consequence of limited dispersal, local adaptation and/or anthropogenic impacts. Before any population analysis could be attempted, however, we needed to develop genetic markers specific to the Olympia oyster.

We isolated and characterized 19 polymorphic microsatellite markers for the Olympia oyster population genetic analyses. We used 10 of these to survey 2,712 individuals from 25 extant Olympia oyster populations between the northern tip of Vancouver Island BC and Elkhorn Slough CA. We found evidence of genetic population differentiation at both the regional and local scale indicating restricted gene flow among many remnant populations, including some

neighboring geographic localities. Our results have resulted in Olympia oyster restoration managers re-thinking their original policy of using any available broodstock source to only using broodstock associated with the restoration site if available or if not, broodstock originating from a nearby location where gene flow is evident based on genetic analysis.

We also wanted to determine whether major genes influencing the quantitative trait yield at harvest remained segregating in the broodstock associated with West Coast Pacific oyster breeding and genetic improvement programs. Knowledge of segregating alleles that account for an observable proportion of the total phenotypic variation in a mapping population created from broodstock selectively bred over three generations for the improvement of yield may be important to managers of Pacific oyster breeding programs. We used molecular tools to identify and map quantitative trait loci (QTL) associated with several growth-related morphometric measures.

First, we created a full-sib mapping family using phenotypically divergent parents from differentiated outbred families to maximize the within-family genetic variance, which we used to create an integrated genetic linkage map utilizing both microsatellite and AFLP markers. Our map, the most informative reported to date, consisted of 65 microsatellite (18 of which were previously unmapped) and 212 AFLP markers, spanning 710.48 cM across 10 linkage groups

and an average genomic coverage of 91.39%. We then used the map to identify a total of 12 QTLs and 5 highly probable QTLs segregating in our F1 mapping population for growth-related traits, including individual wet live weight, shell length, shell width and shell depth at four post-fertilization time points: plant-out (average age of 140 days), first year interim (average age of 358 days), second year interim (average age of 644 days) and harvest (average age of 950 days). Our results prove that alleles accounting for a significant proportion of the total phenotypic variation associated with growth-related traits that influence harvest yield remain segregating within the broodstock of the West Coast Pacific oyster breeding programs. This knowledge may help managers of these programs when deciding what selective criteria are important when creating their selective models.

# Chapter 7
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