2009

Assessing genetic variation within and among native populations and hatchery stocks of Crassostrea ariakensis using microsatellite markers

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ASSESSING GENETIC VARIATION WITHIN AND AMONG NATIVE POPULATIONS AND HATCHERY STOCKS OF *CRASSOSTREA ARIAKENSIS* USING MICROSATELLITE MARKERS

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

By

Jie Xiao

2009

APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

the requirement for the degree of

Doctor of Philosophy

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Approved, by the Committee, October 2008

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ACKNOWLEDGEMENTS

I am very grateful to my major advisor Dr. Kimberly Reece for her guidance, patience, support and encouragement during the past several years. I thank Dr. Jan Cordes who provided tremendous help throughout the course of this research, and whose critical review on this dissertation and the counterpart manuscripts contributed countless suggestions for improvement. I would also like to thank Dr. Ximing Guo, not only for his generosity in providing many valuable samples for this research, but also for his introducing me into this scientific world nine years ago. I sincerely appreciate the support and help from Dr. Standish Allen during the hybridization experiment and for providing hatchery samples, and the constant assistance from Dr. Mark Luckenbach in the completion of this dissertation.

Additional thanks go to all current and previous members of Dr. Recce's lab, and the neighboring lab (Dr. Burreson's), notably Gail Scott, Jessica Moss, Sharon Furiness, Kelly Johnson, Ryan Carnegie, Jens Carlsson, Bill Jones, Hamish Small, and Corinne Audemard, for their assistance, help and warmness during my time at VIMS. I also wish to thank Ms. Liz Walker and Ashley New, who contributed much help and many long hours maintaining oysters and raising the larvae at Kauffman Center.

Finally, I gratefully acknowledge all the individuals involved in collecting samples for this study from China, Japan and South Korea, especially Haiyan Wang, Guofan Zhang, Xiao Liu, Huayong Que, Aiming Wang, Junya Higano, Hyesuk An, and others. Successful completion of this project would have been impossible without their help and assistance.

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LIST OF ABBREVIATIONS

θ_{ST}	Weir and Cockerham's (1984) F_{ST}
A	Allelic richness
ABC	Aquaculture Genetics and Breeding Technology Center
AMOVA	Analyses of molecular variance
COI	Mitochondrial cytochrome oxidase subunit I
D_{CE}	Cavalli-Sforza and Edwards' (1967) genetic distances
F	Fertilization ratio
FCA	Factorial correspondence analysis
F_{IS}	Inbreeding coefficient
F_{ST}	Fixation index
H_E	Expected heterozygosity
H_O	Observed heterozygosity
H_S	Gene diversity
HWE	Hardy-Weinberg equilibrium
IBD	Isolation by distance
ICES	International Council for the Exploration of the Seas
mtDNA	Mitochondrial DNA
n	Sample size
N_e	Effective population size
NJ tree	Neighbor-joining tree
OsHV-1	Oyster herpes-like virus type 1

PCR	Polymerase chain reaction
r	Pearson's coefficiency
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
Rs	Survival rate
s	slope of linear regression
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat region
VIMS	Virginia Institute of Marine Science

ABSTRACT

In response to the dramatic decline of eastern oyster (*Crassostrea virginica*) populations in the Chesapeake Bay, introduction of the non-native Asian oyster *Crassostrea ariakensis* has been proposed. Currently several hatchery stocks of *C. ariakensis*, derived from a few wild populations along the coast of Japan and China, are being maintained in U.S. hatcheries in the Pacific northwest and on the east coast. In recent years, as the risks of the proposed introduction are being assessed, these hatchery animals have been widely used for various research or comparative studies on this species' ecology, biology and disease tolerance, although to date only reproductively sterile triploid individuals have been used for field trials. Genetic concerns have been raised regarding the proposed introduction. There is a need to assess genetic variation within and among native *C. ariakensis*, determine genetic relationships between existing U.S. hatchery stocks and native populations, and monitor the amount of genetic variation in hatchery populations. In addition, recent studies have revealed that it is very difficult, and indeed often impossible to discriminate among the many species of *Crassostrea* sympatric with *C. ariakensis* using morphological features. In fact, a newly described species, *Crassostrea hongkongensis*, is commonly found to coexist with *C. ariakensis* in southern China, and, as determined by molecular genetic identification of samples collected for broodstocks and research purposes, these two species are often confused.

The genetic research presented here includes a comprehensive study of genetic variability within and among the hatchery stocks in the U.S. and wild populations in Asia using a novel set of microsatellite markers developed specifically for *C. ariakensis*. In addition, a laboratory hybridization trial was conducted for *C. ariakensis* and *C. hongkongensis*, as there are questions regarding the species status of these two taxa.

In the hybridization trials, semi gametic incompatibility was observed between *C. ariakensis* and *C. hongkongesis*, indicating a partial reproductive isolation between these two taxa. In addition, an order of magnitude higher genetic divergence was observed between these two taxa compared with that found within each taxon based on analyses with three microsatellite markers. The results from the molecular marker analyses, coupled with additional genetic data indicating low transferability of *C. ariakensis* microsatellite loci to *C. hongkongensis* and detection of no natural hybrids in samples comprised of thousands of oysters, were consistent with previous sequence phylogeny studies and support the distinct species status of *C. ariakensis* and *C. hongkongesis*.

Analysis of *C. ariakensis* wild populations from the coast of Japan, South Korea, and China based on polymorphisms at eight microsatellite loci found a small but significant genetic differentiation among them, which could be characterized by a genetic pattern of isolation by distance. Eight genetically differentiated populations were further identified across the distribution range of *C. ariakensis* confirmed to date.

Genetic differentiation among five hatchery stocks in the U.S. was five-fold larger than that observed among wild populations in Asia. In addition, significant reduction in genetic diversity compared to wild source populations was observed in these five hatchery stocks, indicating a genetic bottleneck in the stocks. Two mature stocks (TUI and WCA), isolated from their wild source population over 30 yrs, showed greater reduction in allelic diversity (60%) and a significant decrease in heterozygosities (11% -

26%) compared to their wild source population, whereas three recent stocks (NCA, SCA99 and SCA00) showed less severe allelic diversity reduction (18% - 30%) and non-significant change in levels of heterozygosities. These microsatellite markers have proven useful for genetically tracking the origins of *C. ariakensis* that might be introduced to Chesapeake Bay, particularly for animals originating from hatchery stocks.

PREFACE:

RATIONALE FOR AND OBJECTIVES OF POPULATION GENETIC STUDIES ON *CRASSOSTREA ARIAKENSIS*

RATIONALE

Crassostrea ariakensis is an Asian oyster species whose name 'Suminoegaki' in Japanese refers to the Suminoe River, where the aquaculture of this species has a long history (Langdon and Robinson 1996). In the 1970s, it was accidentally introduced to the west coast of the United States along with a shipment of *Crassostrea gigas* and *Crassostrea sikamea* seed oysters (Breese and Malouf 1977). Research on this species has been limited, and it did not draw much attention from American scientists and policymakers until the 1990s, when an alternative to the native oyster species *Crassostrea virginica* found along the US east coast was sought to help restore the oyster industry and improve water quality in the Chesapeake Bay.

Populations of the native oyster *C. virginica* in the Chesapeake Bay have declined dramatically over the last century, due to long-term over-fishing, disease, and habitat degradation (NRC 2004). In the early 1990s there was a growing interest in the possibility of introducing a non-native oyster to the region (Mann et al. 1991), and in 1995 the Virginia House of Delegates passed joint resolution NO. 450, which requested that the Virginia Institute of Marine Science (VIMS) "...develop a strategic plan for molluscan shellfish research and begin the process of seeking the necessary approvals for in-water testing of non-native oyster species..." Initial efforts focused on the Pacific oyster *C. gigas*, which has been introduced around the world. Early trials in the region with this species were not promising, however, and focus shifted to *C. ariakensis* (Calvo et al. 1999, 2001), leading to a call for more research on the species, including studies to

explore the "ecological, genetic and disease relations of the species in its natural range and environment" (ICES 2005).

Though various studies have been conducted in the U.S. on the disease tolerance, biology and ecology of *C. ariakensis* using diploid and triploid animals from the hatcheries (Calvo et al. 2001, Grabowski et al. 2004, Bishop and Hooper 2005, Hudson et al. 2005, Moss et al. 2006, Alexander et al. 2008, Kingsley-Smith and Luckenbach 2008, McGhee et al. 2008, Paynter et al. 2008, Tamburri et al. 2008), little work has been done on the genetic variability and genetic population structure within and among natural populations in Asia and hatchery stocks in the U.S. This data could provide important information for stock management and on the genetic background of those animals being used in research. In addition, notorious problems with oyster morphological plasticity and nomenclature has complicated species designations and the identification of *C. ariakensis* in its native region (Zhou and Allen 2003, Boudry et al. 2003, Reece et al. 2008, Cordes et al. 2008) and may also affect the sampling for intra-specific genetic studies on *C. ariakensis*. Specifically, a new species, *Crassostrea hongkongensis* (Lam and Morton 2003), was described quite recently and reported to be sympatric with *C. ariakensis* in southern China (Wang et al. 2004, Zhang et al. 2005, Reece et al. 2008). There is little information on differences in biology, physiology or ecology between these two taxa except for several mitochondrial gene sequence phylogenies which support the two distinct species hypothesis (Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008). In 1999, *C. hongkongensis* was mistakenly imported into U.S. along with *C. ariakensis* for propagation of hatchery broodstocks for *C. ariakensis*, though most F_1 progeny were found to be *C. ariakensis* (Zhang et al. 2005). In addition to the reported gene-based

sequence phylogenies, laboratory hybridization studies would provide additional evidence of species distinctness, given that speciation is a complex process and there can be variation among genes in their reflection of evolutionary processes and speciation.

My doctoral research sought to first study the taxonomic relationship between *C. ariakensis* and *C. hongkongensis* from both a reproductive isolation aspect and from a population genetic perspective. Subsequently, oysters confirmed to be true *C. ariakensis* were chosen for the intra-species population genetic studies, and a set of highly polymorphic microsatellite markers was developed specifically for this species as a genetic tool. These markers were used to characterize the genetic structure, diversity, and gene flow among *C. ariakensis* populations in their native region, and to compare natural populations in Asia with hatchery stocks introduced into the U.S. This work provides genetic information critical to: 1) assess the feasibility of a *C. ariakensis* introduction to the Chesapeake Bay region, 2) minimize the genetic bottlenecking of introduced stocks, and 3) provides genetic tools for long-term tracking and management of any *C. ariakensis* introductions.

OBJECTIVES

1. Develop a suite of microsatellite markers for population genetic studies.
    a) Optimize primers and PCR parameters for successful amplification of microsatellite regions.
    b) Test the markers on family crosses to identify null allele problems.
    c) Redesign primers and retest as necessary to minimize null allele problems with these markers.
2. Study the taxonomic relationship between *C. ariakensis* and the newly described species *C. hongkongensis* (Lam and Morton 2003)
    a) Determine the ability of *C. ariakensis* microsatellite primers to amplify homologous loci in *C. hongkongensis.*
    b) Do reciprocal hybridization studies between these two species, and evaluate fertilization and viability of hybrids.
3. Use the microsatellite markers to study genetic variability among wild populations of *C. ariakensis* in its native region.
    a) Provide basic genetic information on wild *C. ariakensis* for future reference and for introduction activity.
    b) Investigate the evolutionary history and gene flow among those populations.
4. Assess the genetic make-up of *C. ariakensis* hatchery stocks in the U.S. using the developed microsatellite markers.
    a) Look at the genetic variation within and among US hatchery stocks.

b) Compare hatchery stocks to native populations and specifically to source populations, where possible.

5. Assess the ability of these markers to discriminate among different populations and stocks of *C. ariakensis* and identify the source of individuals by assignment testing.

CHAPTER 1

MICROSATELLITE MARKER DEVELOPMENT FOR *C. ARIAKENSIS*

INTRODUCTION

Microsatellites are simple sequence repeat regions (SSRs) in genomic DNA. Since microsatellite sequences were first found in eukaryotic genomes 20 years ago (Tautz and Renz 1984, Tautz 1989), they have been used as powerful genetic markers for disease studies, genome mapping, population genetics studies, and for examination of patterns of evolution and mutations in the repeat regions themselves (Jarne and Lagoda 1996, Chambers and MacAvoy 2000). In general, microsatellite loci are located in noncoding regions, although some microsatellite markers have been found associated with coding regions (e.g. EST-linked), typically in introns or flanking untranslated regions (Vasemägi et al. 2005, Carlsson and Reece 2007, Dreyer et al. 2007). Microsatellites are normally considered to be evolutionarily neutral DNA markers; however, various functions of certain SSRs have been identified, such as chromatin organization, regulation of DNA metabolic processes, and regulation of gene activity (reviewed by Li et al. 2002). Microsatellite mutation rates are generally high ($10^{-2} - 10^{-6}$ events per locus per generation, Li et al. 2002) and often occur as changes in the number of repeat units. Putative mutation mechanisms include slippage during DNA replication and recombination between DNA strands (reviewed by Li et al. 2002).

With several advantages compared to other common molecular markers (Liu and Cordes 2004), microsatellite markers have been utilized in many marine fish population genetic studies, parentage and kinship analyses, and genome mapping efforts (reviewed by O'Connell and Wright 1997, Liu and Cordes 2004). Since the first attempt to clone and study satellite DNA in *C. gigas* (Clabby et al. 1996), microsatellite markers have

been developed for a number of commercially important *Crassostrea* species over the past ten years. These markers have been successfully applied to examine the geographic structure of wild oyster populations (Huvet et al. 2000b, Launey et al. 2002, Rose et al. 2006), perform parentage analyses (Huvet et al. 2001, Boudry et al. 2002), study inbreeding effects (Bierne et al. 1998, Yu and Guo 2005) and for linkage mapping (Yu and Guo 2003, Hubert and Hedgecock 2004). Microsatellites developed for various *Crassostrea* species, including *C. gigas* (Huvet et al. 2000a, Li et al. 2003, Sekino et al. 2003, Hedgecock et al. 2004) and *C. virginica* (Brown et al. 2000, Reece et al. 2004, Carlsson et al. 2006, Rose et al. 2006), are of limited utility for genetic studies in *C. ariakensis* due to low cross-species transferability (Hedgecock et al 2004). Specifically, microsatellite markers developed for *C. gigas* showed extensive heterozygote deficiency when they were applied to *C. ariakensis* populations (Zhang et al. 2005), which confounded the detection of finer population genetic structure in wild populations and founder effects in hatchery stocks. In this chapter, results of developing novel microsatellite primer pairs specifically for *C. ariakensis* and the performance of these markers in *C. ariakensis* family crosses and their transferability to a sister taxon *C. hongkongensis* (Reece et al. 2008) are reported. This work was recently published in Xiao et al. 2008.

MATERIALS AND METHODS

Microsatellite-enriched library construction, primer design, testing and optimizing

Four partial *C. ariakensis* genomic DNA libraries enriched for microsatellite sequences containing AAG, AAAG, AAAT, and TAGA repeat motifs were constructed by Genetic Identification Services (GIS, Chatsworth, CA) following the methods described in Jones et al. (2002). Resulting recombinant clones were selected at random and sequenced on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA) using ABI Prism® Taq dye terminator cycle sequencing methodology. Initial polymerase chain reaction (PCR) primers were designed for flanking regions of microsatellite-containing sequences using DesignerPCR v1.03 (Research Genetics Inc., Huntsville, AL). Primers were tested and PCR conditions optimized using whole genomic DNA extracted from a panel of eight *C. ariakensis* from Beihai, Guangxi Province, China, using the DNeasy Kit (Qiagen Inc., Valencia, CA). Loci were tested for null alleles by genotyping two parents and ten offspring for each of eight *C. ariakensis* full-sibling family crosses (96 individuals total) produced by the Aquaculture Genetics and Breeding Technology Center (ABC) hatchery at the Virginia Institute of Marine Science (VIMS) using Japanese-derived and northern Chinese broodstocks. Primers were redesigned for loci exhibiting null alleles, stutter bands, or secondary amplification products using MacVector®8.1.2 (MacVector Inc., Cary, NC).

Loci were amplified in 5 ul reactions containing 0.6 ug/ul BSA, 1X PCR buffer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 0.05 U/ul Taq polymerase (Invitrogen, Carlsbad, CA), 0.2 – 0.5 ul template DNA, 0.025 pmol/ul forward primer with a T3 tail (5'-

AATTAACCCTCACTAAAGGG-3') at the 5' end, 0.1 pmol/ul reverse primer, and 0.1 pmol/ul universal T3 tailed primer labeled with one of four fluorescent dyes (Table 1.1) at the 5' end. All primers were synthesized by Invitrogen. PCR cycling was performed on MJ Research thermocyclers (BioRad Inc., Hercules, CA) with the following program: initial denaturation at 95°C for 4 min, then 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min (see Table 1.1 for temperature), extension at 72°C for 2 min, and a final extension at 72°C for 20 min. Products were visualized on an ABI 3130 Genetic Analyzer (Applied Biosystems) using 36cm capillary arrays and POP7™ polymer with GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems). Image analysis and allele scoring were done using GeneMarker® (SoftGenetics, LLC State College, PA). Estimates of observed (H_O) and expected (H_E) heterozygosities and deviations from Hardy-Weinberg Equilibrium (HWE) were calculated using GENEPOP 3.1b (Raymond and Rousset 1995). Tests for genotypic disequilibrium were calculated by GENETIX (Belkhir et al. 1996-2004) with 10,000 permutations based on 32 individuals from a wild population in the Yellow River basin, Shandong Province, China.

Microsatellite allele sequencing

Problems such as null alleles and amplification of multiple loci were detected when nine primer pairs (*Car5*, *Car10*, *CarE12*, *CarG1*, *CarH7*, *Car130*, *Car112*, *Car115*, *CarG122*) were used to amplify the eight family crosses. It was believed that these problems could be overcome by primer redesign (Reece et al. 2004). Consequently, alleles amplified by these nine primer pairs were cloned and sequenced in order to find a conservative flanking region for primer redesign. Standard PCR amplification without

fluorescence-labeled T3 universal primer was conducted for each locus with DNA isolated from two to four individual *C. ariakensis* oysters. PCR was conducted using the same cycling protocols and reagent concentrations as described above for amplification of fluorescence labeled products, except that the primer concentrations were 0.1 pmol/ul for both forward and reverse primers, and no universal T3 tailed primer was used. Products were separated on 3% agarose gels and recovered from the gels using QIAquick® Gel Extraction Kits (Qiagen, Inc.). Products were then cloned into the pCR®4-TOPO® vectors, and transformed into competent *E. coli* cells using the TOPO® TA Cloning Kit (Invitrogen) following the manufacturer's protocol. Bacterial clones were screened for inserts by PCR amplification using the M13 vector primers. Products with the inserts were cleaned using shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I; Amersham Biosciences, Piscataway, NJ), and then sequenced bi-directionally with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Each of the 5 ul sequencing reactions contained 0.875 ul sequencing buffer, 0.25 ul BigDye reagent and 0.32 ul of 100 pmol/ul forward or reverse M13 primer. Sequencing reaction products were separated on an ABI 3130 after precipitation with the ethanol/sodium acetate method (Applied Biosystems) and were re-suspended in Hi-Di formamide. Sequences from each amplicon were aligned with the original sequence obtained from the library using the ClustalW algorithm in the software package MacVector® 8.1.2 with the default parameters, and adjusted manually by eye.

Cross amplification of developed microsatellite markers

Cross amplification efficiency of these *C. ariakensis* microsatellite markers in *C. hongkongensis* was tested using 24 individuals from Beihai, Guangxi Province, China (B_Ch) and 44 individuals from Yamen River, Zhuhai, Guangdong Province, China (C_Ch) (both collected in 1999), under the same PCR conditions as *C. ariakensis* amplifications. These 68 individuals were originally sampled as *C. ariakensis* / *C. rivularis* but later confirmed to be *C. hongkongensis* in a previous study (Zhang et al. 2005, also see Chapter 2).

RESULTS

Optimized microsatellite markers for *C. ariakensis*

Microsatellite-containing sequences were found in 64 of 100 clones screened. Primers were designed and tested for 34 loci after eliminating duplicate and high GC content sequences, and those with prohibitively short flanking regions. Tests on the panel and family crosses produced 11 polymorphic microsatellite markers (Table 1.1). Genotyping of the eight families revealed multiple null alleles or binding at multiple sites using initial primer pairs at nine of the 11 loci (see the section Polymorphisms detected in the flanking regions of microsatellite loci for details). Segregation in the families conformed to expected Mendelian patterns at each locus after primer redesign with the following exceptions: *Car11-70*, *Car130-08*, *CarH7-ca* and *CarE12-0a* (Table 1.1). Null alleles at these loci ranged from one to three. Overall, two to 36 alleles per locus were observed (Table 1.1), and average H_O and H_E in the wild population were 0.743 (±0.279) and 0.783 (±0.218), respectively. After Bonferroni correction (Rice 1989), only one of 55 pairwise tests of linkage disequilibrium (*Car115-a0* vs. *CarG4-60*) and two of 11 tests for deviations from HWE (*Car5-0a* and *CarH7-ca*) were found to be significant in the wild population. Deviations from HWE may be due to additional null alleles observed in the wild population, but not found during the family screens, or sampling error associated with small sample sizes.

Polymorphisms detected in the flanking regions of microsatellite loci

Sequencing of PCR products produced by nine primer pairs detected a total of 69 different alleles from 15 loci. Fourteen of them are listed in Table 1.2, while locus *CarE12* c is not listed since only one allele was amplified and sequenced. The sequencing confirmed that four of the initially designed primer pairs (*Car5*, *Car10*, *CarE12* and *CarG1*) were amplifying two or three different loci, which was initially indicated by allele segregation analysis in the eight family crosses. Another primer pair (*CarH7*) was found to bind multiple loci by sequencing analysis, though this was not detected in the segregation analysis. Redesigned primers were able to successfully bind to a single locus for four of these loci (*CarE12-0a*, *CarG1b-0b*, *CarH7-ca* and *Car5-0a*), but not for the others. Within the 2370 bp of flanking regions sequenced, 17 indels were detected with approximately 1 indel every 100 bp and an average size of 7 bp. Of these indels, 94% were less than 10 bp in length, but a large deletion of 44 bp was observed at locus *CarG1-a*. A large number (86) of single nucleotide substitutions were also observed in these flanking regions with about 3.6 substitutions per 100 bp sequenced. With each indel weighted the same as a single substitution, the percentages of polymorphic positions in these flanking regions ranged from 0.0% - 15.6% with an overall average polymorphism of 4.3%.

In general, sequencing of products by these nine primer pairs resulted in detection of 15 different loci and high numbers of polymorphisms at microsatellite flanking regions (4.3%). Redesigned primers were able to successfully bind to a single locus for 4 loci (*CarE12-0a*, *CarG1b-0b*, *CarH7-ca* and *Car5-0a*) and reduce the null alleles detected in families at another five loci (*Car11-70*, *Car119-6a*, *Car115-a0*, *CarG4-60* and *Car130-*

08) with or without sequencing analysis. These nine loci, along with three loci using the originally designed primers, were optimized and developed for amplification of DNA from *C. ariakensis* samples.

Transferability of *C. ariakensis* markers to *C. hongkongensis*

Three of these eleven markers were positively amplified in most (94.1% - 100%) of the 68 *C. hongkongensis* individuals from two samples (B_Ch and C_Ch) tested (Table 1.3). This low transferability (27%) of *C. ariakensis* markers to *C. hongkongensis* probably reflects the high levels of genomic heterogeneity often seen among *Crassostrea* species (Hedgecock et al. 2004) and supports the two species hypothesis (Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008). A total of 32 alleles were amplified at these three loci in two *C. hongkongensis* populations, and these alleles had similar sizes to those amplified in *C. ariakensis*. The allele frequencies were in HWE in both populations at all three amplified loci after Bonferroni correction ($\alpha = 0.01$, $K = 2$).

DISCUSSION

Apparently, the relatively low efficiency of this microsatellite development process was at least partially due to the high level of polymorphism (4.3%) found in the flanking region sequences, as shown by the sequence analysis of multiple alleles at 15 loci. A similar proportion of polymorphism (4.5%) was observed when *C. virginica* microsatellite alleles were sequenced (Reece et al. 2004). Hedgecock et al. (2004) indicated a high prevalence (1.2%) of SNPs in primer-binding regions of *C. gigas* microsatellite loci, and this could be an underestimation since only one SNP was assumed for each null allele. The results of the present study support the previous observations that intra-species genome sequence polymorphism is a common phenomenon in marine bivalves.

Another factor that affected the efficiency of microsatellite marker development in this study was duplicated DNA sequences. Amplification of multiple loci by each of four primer pairs (*Car10*, *Car5*, *CarE12*, *CarG1*) was demonstrated by the allele segregation observed in family crosses, where more than two alleles were amplified for one individual parent and there was independent segregation in progeny. Sequence analysis confirmed that these four primer pairs were indeed binding to more than one locus, and indicated multiple binding at another locus (*CarH7*), a phenomenon that was not detected in the segregation analysis. The overall proportion of multiple loci amplified by the original 34 primer pairs was approximately 26.5% (Xiao et al. unpublished data), and this percentage could be higher since some duplicated sequences had been eliminated before primer design (see RESULTS). Loci amplified by the same primer pair were very

likely from duplicated DNA sequences (Zhang and Rosenberg 2007) since the chance of random loci being amplified by the same primer pair is quite low (8.27×10^{-25}) for primers 20 bp in length (the average length of primers used in this study). Gene and genome duplications are widely reported in human, fly and yeast genomes (Sankoff 2001), and recently duplicated microsatellite loci have been used to study the evolutionary history of duplicated genes or genomes in a variety of organisms (David et al. 2003, Zhang and Rosenberg 2003). Reports of duplicated microsatellite loci in oysters are rare, and for this study most primers that amplified multiple loci were eliminated during the marker development process. However, Gaffney (2002) found that many microsatellites, particularly those with tri- and tetra-nucleotide motifs, were embedded in or associated with repetitive flanking regions after analyzing a 0.7 Mb genomic sequence database from *C. virginica* and a similar sized genomic region of other bivalve species. DNA duplication and the polymorphisms in the flanking regions illustrate not only the difficulties associated with microsatellite development, but also the complexity and variance within the genome sequences of marine bivalves.

In summary, eleven novel microsatellite markers were developed from this study for *C. ariakensis* and optimized to control for the presence of null alleles, making them available to study genetic variability among and between wild and hatchery populations of *C. ariakensis* (Chapter 3 and Chapter 4). In addition, three of the markers should be applicable to population genetic studies in *C. hongkongensis,* or for further studies on inter-species genetic divergence between *C. ariakensis* and *C. hongkongensis* (Chapter 2).

Table 1.1 Primer sequences, repeat motifs, annealing temperatures (Ta), and locus characteristics for 11 microsatellite markers developed for *Crassostrea ariakensis*. Significant departures of observed heterozygosity (H_O) from Hardy-Weinberg Equilibrium after Bonferroni correction are shown in boldface. [1] GenBank accession numbers EU241318-EU241328. [2] Based on analyses of two parents and ten offspring from eight family crosses (96 individuals total). [3] Allele sizes including primers and the extra 20 bp of the universal T3 primer. [4] Based on 32 individuals from a wild population.

Locus [1]	Label	Primer sequence	Repeat motif	Ta (°C)	No. null alleles [2]	Allele size range (bp) [2, 3, 4]	No. alleles [2, 4]	H_E [4]	H_O [4]	P [4]
CarG110	NED	F: 5'-AACTCTCTGTCCACTTCTCTG-'3 R: 5'-ACACGCCATCAACACTATT-'3	$(TTC)_n$	56	0	263-287	10	0.803	0.875	0.935
Car11-70	FAM	F: 5'-ACAAGCCAAGGGGAATAC-3' R: 5'-GAAACGGACGACTGAAAT-'3	$(AGAT)_n$	53	1	127-216	17	0.906	1.000	0.783
CarG4-60	VIC	F: 5'-CGGAGAATTTATCCCGAATTTA-'3 R: 5'-CATCCAGTTTAAGCCTACAACT-'3	$(TCT)_n$	53	0	130-195	26	0.941	0.936	0.360
Car119-6a	PET	F: 5'-ACCAGTGTTCCGAAAAATCT-'3 R: 5'-GTTTACGGAGCCCCATGTTA-'3	$(TATC)_n$	52	0	104-190	14	0.865	0.875	0.448
CarG1-0b	NED	F: 5'-TGGCGAGAGTAAGTCATCA-'3 R: 5'-GAAATTGTTTGGTAACTTAGTTC-'3	$(CTT)_n$	52	0	187-214	14	0.694	0.688	0.666
Car115-a0	PET	F: 5'-GAGGGACAATTGGCTTTACG-'3 R: 5'-ACGCTTAGTCTTGGTCCATAG-'3	$(TATC)_n(TA)_n$	56	0	166-266	22	0.875	0.875	0.485
CarG122	FAM	F: 5'-TACCTCTTCCCTTCCTAACTG-'3 R: 5'-GAAACCACTCCTGTTGTAAAAT-'3	$(CCT)_nG(TTC)_n$	56	0	262-265	2	0.305	0.313	1.000
Car130-08	VIC	F: 5'-AGACATGAAGCATGAGGTTATA-'3 R: 5'-CTGGAAGAAATAGGGTTTCA-'3	$(GATA)_n$	53	2	156-219	21	0.920	0.906	0.111
Car5-0a	NED	F: 5'-GTAACCTTTTTTTCGTCTCAAG-'3 R: 5'-CCTCTCAGGCACACCTCAAT-'3	$(TAGA)_n$	52	0	131-293	36	0.952	**0.807**	**0.000**
CarH7-ca	PET	F: 5'-TCCAGGGTTTATCTACATGTG-'3 R: 5'-CTGTGACACTGGAGTCTGCTG-'3	$(ATTT)_n$	58	2-3	210-222	4	0.438	**0.111**	**0.001**
CarE12-0a	VIC	F: 5'-GGTAAACTTGACCAGCATCTGA-'3 R: 5'-CTGGCGAAACGTTGAATAG-'3	$(TAGA)_n$	54	3	226-364	18	0.914	0.786	0.008

Table 1.2 Polymorphisms in the flanking regions of 14 *C. ariakensis* microsatellite loci identified by sequencing alleles amplified by 9 primer pairs.

Locus	# of alleles sequenced	Flanking region length sequenced (bp)	# of indels (length)	# of substitutions	% polymorphism	Primers
Car5-0a	5	39	1 (1 bp)	1	5.1	Car5 F, Car5 R
Car5-b	3	38	1 (8 bp)	0	2.6	Car5 F, Car5 R
Car10-a	7	158	2 (1 of 8 bp; 1 of 13 bp)	11	8.2	Car10 F, Car10 R
Car10-b	5	164	1 (8 bp)	10	6.7	Car10 F, Car10 R
Car112	8	173	2 (1 of 3 bp; 1 of 2 bp)	11	7.5	Car112 F, Car112 R
Car115-a0	4	161	1 (2 bp)	1	1.2	Car115 F, Car115 R
Car130-08	6	65	1 (3)	2	4.6	Car130 F, Car130 R
CarE12-0a	8	64	2 (1 of 9 bp; 1 of 1bp)	8	15.6	CarE12 F, CarE12 R
CarE12-b	3	77	0	0	0.0	CarE12 F, CarE12 R
CarG1-a	3	463	4 (1 of 5 bp; 1 of 3 bp; 1 of 7 bp; 1 of 44 bp)	9	2.8	CarG1 F, CarG1 R
CarG1-0b	4	172	1 (5 bp)	5	3.5	CarG1 F, CarG1 R
CarG122	4	150	1 (1 bp)	12	8.7	CarG122 F, CarG122 R
CarH7-ca	4	324	0	13	4.0	CarH7 F, CarH7 R
CarH7-b	4	322	0	3	0.9	CarH7 F, CarH7 R
Overall	68	2370	17	86	4.3	

Table 1.3 Cross amplification of eleven *C. ariakensis* microsatellite markers in 68 *C. hongkongensis* individuals from Beihai, China (B_Ch, n = 24) and Zhuhai, China (C_Ch, n = 44). NA indicates no amplifications were successful for these loci. H_O is observed heterozygosity, H_E is expected heterozygosity and P is probabilities of HWE tests. P values in bold are significant departure from HWE after Bonferroni correction ($\alpha = 0.01$, K = 2).

Locus	# of alleles	Allele size range (bp)	H_O (B_Ch, C_Ch)	H_E (B_Ch, C_Ch)	P (B_Ch, C_Ch)
CarG110	NA	-	-	-	-
Car11-70	NA	-	-	-	-
CarG4-60	5	145 - 164	0.286, 0.364	0.254, 0.483	1.000, 0.066
Car119-6a	NA	-	-	-	-
CarG1-0b	NA	-	-	-	-
Car115-a0	NA	-	-	-	-
CarG122	3	253 - 262	0.292, 0.068	0.259, 0.066	1.000, 1.000
Car130-08	24	156 - 234	0.682, 0.825	0.904, 0.916	0.008, 0.091
Car5-0a	NA	-	-	-	-
CarH7-ca	NA	-	-	-	-
CarE12-0a	NA	-	-	-	-

CHAPTER 2

HYBRIDIZATION AND GENETIC DIVERGENCE BETWEEN *C. ARIAKENSIS* AND *C. HONGKONGENSIS*

INTRODUCTION

Nomenclature of *C. ariakensis* has followed a tortuous path that involves a history of confusion between use of the names *C. ariakensis* and *C. rivularis* in different countries (reviewed by Zhou and Allen 2003). Although the designation *C. rivularis* (Gould 1861) has precedence, *C. ariakensis* (Fujita 1913) has been widely used in Japan, and *C. rivularis* was synonymized with *C. ariakensis* by Torigoe (1981). The name *C. rivularis* was traditionally used in China, referring to the 'jin jiang' oyster in Chinese (Zhang and Lou 1956, Xu 1997), which means "close to river" oyster (Zhou and Allen 2003). Local fishermen often discriminate between two types of 'jin jiang' oysters they are culturing based on slight differences in the color of adductor muscle, called 'red meat' and 'white meat' in Chinese (Guo et al. 1999, Zhou and Allen 2003, Wang et al. 2004).

Recently, Lam and Morton (2003) designated a new name — *C. hongkongensis* — for a type of cupped oyster popularly cultured by local farmers in Hong Kong and later found to coexist with *C. ariakensis* along the coast of southern China, from Fujian Province to the Guangdong and Guangxi provinces (Wang et al. 2004, Reece et al. 2008). Debates concerning these two species, including species designations, their genetic relationship, and their morphological and biological differences, are still ongoing (Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008).

Currently the name *C. rivularis* is being replaced by *C. ariakensis* and *C. hongkongensis* in China based on genetic sequences deposited in GenBank (Wang et al. 2004). *C. ariakensis* now refers to the oyster species originally described in Ariake Bay,

southern Japan. Based on the monophylotype from this population and molecular data, additional natural populations of this species have been found along the coast of South Korea and northern and southern China (Wang et al. 2004, Zhang et al. 2005, Reece et al. 2008, Wang et al. 2008a, Yoon et al. 2008). In contrast, the name *C. hongkongensis* refers to the species originally described from Hong Kong and later found along the coast of southern China (Lam and Morton 2003, Wang et al. 2004).

In general, shell morphology and anatomy of sympatric *Crassostrea* species are often considered to be indistinguishable (Littlewood 1994, Hedgecock et al. 1999, Boudry et al. 2003, Lam and Morton 2003), although slight differences in anatomy have been reported between *C. hongkongensis* and *C. ariakensis* (Wang et al. 2004). Most classifications of *C. hongkongensis* have been based on gene sequence phylogenies (Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008). Little is known about the ecology, morphology and physiology of *C. hongkongensis*, or differences between this species and *C. ariakensis.*

Given complex speciation processes and inter- and intra-species interactions, categorizing species separations using more than one approach may be more appropriate and convincing. Although several species concepts have been advanced, none of them adequately define this basic unit for all types of organisms found in nature (Avise 1994). Cracraft (1983) advanced the phylogenetic species concept (PSC) — a monophyletic group composed of "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent". Although it has been widely used in the last several decades, facilitated by popularization of automatic sequencing methods and the blossoming number of molecular markers, researchers still struggle with

how to harmonize gene trees with other hypotheses of organism speciation (Avise 1994). Discrepancies occur between gene trees and species trees, especially when using sequences from only one gene or locus to generate species phylogenies. In addition, trees generated from multicopy gene sequences might include some paralogous as well as orthologous copies, confusing any species hypotheses and emphasizing the need for caution when interpreting results from molecular phylogenetic analyses. Mayr's (1942) biological species concept (BSC) – that species are "groups of actually or potentially interbreeding natural populations which are reproductively isolated from such other groups" – is the most influential concept, and remains popular now. Confirming the species status of *C. hongkongensis* and *C. ariakensis* under a combination of these two species concepts may help clarify their taxonomic relationship.

Although more and more people have been using DNA sequence-based phylogenies to distinguish closely related species, there are still many debates on the utility of sequence-based taxonomy. Phylogenetic trees of *Crassostrea* species are quite different using different genetic datasets, such as those generated based on COI (Ó Foighil et al. 1998, Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008), 16S, 18S, 28S rDNA (Lapègue et al. 2002, Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004) and other nuclear DNA regions (López-Flores et al. 2004, Reece et al. 2008). In addition, DNA based taxonomy may not agree with species designations based on investigations into reproductive isolation, as in the case of *C. angulata* and *C. gigas*. Studies of COI sequences and microsatellite markers clearly demonstrated genetic differences between these two taxa, although monophyly of the clades was not always observed, and the genetic distance between them is very low

compared to that observed between other *Crassostrea* species (Ó Foighil et al. 1998, Boudry et al. 1998, Huvet et al. 2000b, Boudry et al. 2003, Reece et al. 2008). Nonetheless, a series of hybridization experiments showed no evidence of a reproductive barrier between these two taxa, and natural hybrids were found in a contact zone (Huvet et al. 2001, 2002, 2004). In contrast, the genetic distances between *C. ariakensis* and *C. hongkongensis* are larger than those observed between *C. gigas* and *C. angulata* (Lam and Morton 2003), and to date no natural hybrids have been observed in contact zones (Zhang et al. 2005, Wang et al. 2008b, Reece et al. 2008). There was, however, no direct information on the reproductive isolation between *C. ariakensis* and *C. hongkongensis* before this study. Another drawback of sequence phylogenetic studies arises because they usually include a limited number of genes and survey a limited number of individuals. At present, three mitochondrial genes (COI, 16S, 28S) and only one nuclear region (ITS-1) have been used to distinguish *C. ariakensis* and *C. hongkongensis* (Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008). In Reece et al. (2008) ITS-1 sequences of *C. hongkongensis* did not form a monophyletic clade, despite the relatively large number of individuals of several Asian *Crassostrea* species sequenced (Reece et al. 2008). Clearly, a survey of more nuclear genes and more individuals would help to confirm these are separate species, given the high intra-specific nucleotide diversity in oysters (Hedgecock et al. 2004), different polymorphic levels at different genes, and the close relationship between *C. ariakensis* and *C. hongkongensis* as sister taxa (Reece et al. 2008).

In this study, laboratory hybridization experiments were conducted and the genetic differences among populations of *C. ariakensis* and *C. hongkongensis* were

compared using three newly developed microsatellite markers, in order to study the taxonomic status between these two species.

MATERIALS AND METHODS

Samples

Live oysters, presumably including both *C. ariakensis* (A) and *C. hongkongensis* (H), were brought back from Beihai, Guangxi Province in China in November 2006. They were held in a quarantine room at the VIMS Kauffman Center at 23 ppt salinity and 17°C until preparation for spawning, when the salinity was progressively decreased to 20 ppt and the temperature increased to approximately 25°C. Dead and/or sacrificed oysters were identified to species using molecular markers (Cordes et al. 2008; also see Molecular identification of species below). Due to the low occurrence of *C. ariakensis* in this sample based on the initial species screening (around 1% after screening 165 individuals), a *C. ariakensis* sample (about 50 individuals) from the SCA hatchery broodstock (derived from sources in Beihai, Guangxi Province, southern China), which had been held and spawned at the ABC since 1999, served as the source for *C. ariakensis* parents used in these reciprocal hybridization experiments.

Hybridization experiment

Spawning Ripened oysters of each species were selected, dissected, and identified as either male or female through microscopy. The gonad was stripped into filtered seawater using a sterile knife. Four to ten females and males were stripped for each cross, with the exact number dependent upon the gamete quality and density. Those with highest gamete viability were selected for hybridizing. A piece of gill and/or adductor muscle tissue from

each oyster was preserved in 100% EtOH for subsequent DNA extraction and species identification.

Hybridization Sperm and oocyte solution from a single dam or sire were mixed in a rough final ratio of 1:10 oocyte to sperm. A higher number of sperm was sometimes added in cases of low sperm viability. The mating scheme followed the diagram in Figure 2.1, with four crosses using two male and two female *C. ariakensis* and *C. hongkongensis* (4 individuals total) and three replicates of each cross (total 12 treatments). Abbreviations for crosses are as follows: A = *C. ariakensis*, H = *C. hongkongensis* and females were listed first. Formation of the 1st and 2nd polar bodies was followed by microscopy to determine fertilization success. The fertilization ratio (F) was estimated for each cross based on the count of larvae that passed the 2nd polar stage at Day 2 divided by the total number of eggs used for each cross.

Post-insemination culture Larvae were cultured in hung 5-gallon buckets and sieved approximately every two days by mesh screens with progressively increased sizes. All crosses were reared at 20 ppt salinity and 23 – 26°C. Only live and normal growing larvae were saved for continued culturing. During each sieve, numbers of remaining larvae were estimated and shell length was measured for 10 individuals per treatment. Larvae were fed with mixed algae food (cultured in Kauffman Center) daily. Survival rate (Rs) was estimated as a proportion of live larvae counts determined at every sieving compared to the initial number of larvae produced after fertilization.

Molecular identification of species

Genetic markers were used in this study to confirm the species of individuals used for spawning, determine the hybrid status of larvae, and control for potential contaminations that had commonly occurred in previous hybridization experiments (Gaffney and Allen 1993). Parental oyster DNA was extracted from the preserved tissue using the Qiagen DNeasy Kit. The species of each individual was determined by ITS-1 and COI PCR-RFLP analysis as described by Cordes et al. (2008). ITS-1 and COI amplicons were digested with *Hae* III and *Dde* I enzymes, respectively, based on their ability to discriminate among *Crassostrea* species (Cordes et al. 2008). For the larvae, however, a different DNA extraction procedure, PCR reaction, and restriction enzyme were used due to the small amount of available DNA from each individual and the complicated process of identifying the hybrid status of individuals. A small volume (~2 ml) of concentrated larval culture solution was sampled for each treatment and preserved in 100% EtOH. Individual larvae were then picked out using a 10 ul pipet under a dissecting microscope, and the whole genomic DNA was extracted using the Chelex method modified from Launey and Hedgecock (2001). Basically, an individual larva was transferred into a 1.5 ml centrifuge tube containing 60 ul 5% Chelex®100 resin (Bio-Rad) and 3 ul proteinase K (Qiagen Inc.). After vortexing for several seconds, the mixture was incubated at 55°C for 2 hr and 100°C for 10 min. The solution was then centrifuged at 300 R/min for 5 min, and the supernatant was used as DNA template. Two ul of template DNA were used for each reaction in a total volume of 15 ul to amplify the ITS-1 region. PCR amplification of the ITS-1 region followed the protocol in Cordes et al. (2008). Products were then electrophoresed on a 1.5% agarose gel at 90 v for approximately 30

min, and the positive amplifications were subjected to RFLP assays. Due to the high number of fragments in the ITS-1 RFLP pattern of these hybrids, the commonly used enzyme *Hae* III (Cordes et al. 2008) was not suitable for hybrid larval identification in this study. Another restriction endonuclease *Sau96* I (New England Biolabs® Inc., Ipswich, MA) was selected for the RFLP assay based on its clear and simple digestion patterns to distinguish *C. ariakensis* and *C. hongkongensis* and its ability to clearly discriminate hybrids (J.F. Cordes, personal communication). Restriction digestions were conducted following the manufacturer's protocol. The digested fragments were separated on a 3% agarose gel and visualized under UV light after ethidium bromide staining. The RFLP pattern of hybrid larvae was not the simple combination of bands from parental species as expected. Extra bands were observed in hybrids (Figure 2.5), which is most likely from the heteroduplex DNA molecules formed during PCR reaction (Camara et al. 2008).

Microsatellite amplifications

Samples from sixteen wild populations of *C. ariakensis* from various locations of Japan, South Korea and China (see Chapter 3 for details) and two populations of *C. hongkongensis* from Yamen River, Zhuhai, Guangdong Province (C_Ch) and Beihai, Guangxi Province (B_Ch), China were amplified using three microsatellite markers (*CarG4-60*, *CarG122* and *Car130-08*) following the protocol described in Chapter 1. The whole genotype dataset, which included 605 individuals of *C. ariakensis* and 68 *C. hongkongensis*, was input into the program GENETIX (Belkhir et al. 1996-2004) to estimate genetic differentiations (F_{ST}, Weir and Cockerham 1984) among populations and

conduct the FCA analyses. A neighbor-joining (NJ) tree was also constructed to visualize the genetic differentiations using the software package PHYLIP 3.67 (Felsenstein 1989) based on Cavalli-Sforza & Edwards' (1967) chord distance (D_{CE}). Robustness of each node was evaluated by bootstrapping over alleles with 10,000 iterations.

RESULTS

Two hybridization trials, designated as "Spawn 1" and "Spawn 2", were done during the periods of June 12, 2007 – July 6, 2007 and August 14, 2007 – September 3, 2007, respectively, with six replicates for each cross and a total of 24 treatments. Unfortunately, no larvae from either pure or hybrid crosses survived to spat stage. Fertilization success and growth in the early developmental stages, however, were recorded for each cross in order to find any evidence of differential reproductive success between these two species and their hybrids. Because of contamination found in treatments AA6 and HA6 from Spawn 2 (Table 2.1, see below for details), this replicate was excluded from all the analyses on fertilization, survival and growth rates.

Fertilization ratios

Fertilization ratios for all 24 treatments are shown in Figure 2.2. Mean values were highly variable both among the four types of crosses and between spawns, with Spawn 2 having a significantly lower fertilization success ($P = 0.004$, nested ANOVA) for the HA and HH crosses compared to that observed in Spawn 1. The two pure AA crosses (ranged from 83.0% - 98.2%) had relatively higher fertilization percentages compared to the HH and hybrid crosses. Percent fertilization success for the other hybrid (HA) crosses (mean of 61.8±13.0% for Spawn 1, 14.9±1.3% for Spawn 2) did not differ significantly from the pure HH crosses (60.5±25.1% for Spawn 1, 32.8±19.4% for Spawn 2) for both spawn trials (ANOVA $P = 0.94$ and 0.16, respectively). Five of the six replicates at the AH crosses showed zero fertilization, and the only live larva found in

sub-samples from Spawn 2 exhibited extremely retarded development (four-cell stage at Day 2) and did not survive beyond this point.

Survival

As described earlier, no larvae survived to the eye-spot stage, even in the control groups (AA and HH). As shown in Figure 2.3, most animals from both trials (96.5% – 100% in Spawn 1 and 92.8% – 100% in Spawn 2) died within three weeks. However, Spawn 2 had relatively lower mortalities (mean ranges 46.5% – 65.9%) at Day 7 than Spawn 1 (72.2% – 93.2%). In Spawn 1, the AA crosses had the best survival rates, followed by HA and HH; while in Spawn 2, the HH crosses performed best, and the hybrid HA crosses showed lower survival than AA before Day 7, but performed better after that point. AH crosses did not produce any viable larvae due to unsuccessful fertilizations.

Growth

Unfertilized eggs of *C. ariakensis* and *C. hongkongensis* were both approximately 50 um in diameter, and all larvae aborted development before the eye-spot stage. Some larvae in Spawn 1 passed the D-hinged stage and developed into veliger larvae, but almost none did so in Spawn 2. Figure 2.4 shows quite similar overall growth patterns among the three surviving crosses (AA, HA and HH). In both trials, larvae from the AA crosses had the largest sizes, followed by HA and HH. However, in Spawn 1, larvae demonstrated larger size differences across the three groups, where HH larvae did not show substantial growth after Day 7 (60 – 85 um at Day 7, 60 – 80 um at Day 14), but the

other two groups increased about 20% in mean size from Day 7 to 14, and reached sizes to 100 – 120 um by the end of the trial. In contrast, larvae in Spawn 2 did not demonstrate substantial growth after Day 3, and only reached sizes of 85 – 100 um (AA), 85 – 95 um (HA), and 80 – 100 um (HH) by the end of this trial.

Species IDs

All parents (total 24 oysters) used in the two spawns were positively identified after spawning as members of their initial species designation. ITS-1 RFLP patterns digested with *Sau96 I* enzyme could clearly distinguish among *C. ariakensis*, *C. hongkongensis*, and their hybrid progeny, as shown in Figure 2.5. In addition, species identifications were done for 99 larvae (26 from Spawn 1, 73 from Spawn 2; Table 2.1). Apparent contamination occurred only in replicate 6 from Spawn 2, where eight unexpected genotypes were found. One larva from the pure AA cross had a hybrid genotype, while seven larvae from the HA hybrid cross showed a pure *C. ariakensis* genotype, indicating contamination occurred in these two treatments. Whether contamination happened before fertilization or after fertilization is unclear. Regardless, this replicate was excluded from all fertilization, growth and survival analyses.

Microsatellite analysis

Using three microsatellite markers, the two *C. hongkongensis* samples were clearly differentiated from the 16 *C. ariakensis* samples. F_{ST} values between these two groups (0.330 – 0.402) (Table 2.2) were more than ten times larger than those observed within the samples of *C. hongkongensis* (0.028) and *C. ariakensis* (-0.005 – 0.026). The

overall F_{ST} between *C. ariakensis* and *C. hongkongensis* was 0.328 ($P < 0.001$). Patterns of genetic relationships between the species were visualized by a 3D factorial correspondence analysis (FCA) (Figure 2.6) and an unrooted NJ tree (Figure 2.7). Three axes in the FCA explained 45.2%, 9.1% and 6.7% of total variance observed, respectively. Clearly a large proportion of the genetic variance was explained by differences between the two groups of oysters. In the NJ tree, the eighteen samples formed two distinct clades, with one comprised of the 16 *C. ariakensis* samples and the other comprised of the two *C. hongkongensis* samples. Both analyses indicate large genetic divergence between species, which is congruent with the F_{ST} analysis. Genetic relationships among the 16 *C. ariakensis* samples shown here is different from that shown in Chapter 3, probably due to the low number of loci (3) used here. For details on the genetic relationships within *C. ariakensis* populations and hatchery stocks, the reader should refer to Chapter 3.

DISCUSSION

Results from the population genetic analysis support the hypothesis that *C. ariakensis* and *C. hongkongensis* are separate species under the phylogenetic species concept. As shown in both the NJ tree and 3-D factorial correspondence analyses, levels of differentiation based on F_{ST} and D_{CE} measures of genetic distance between wild populations of *C. ariakensis* and *C. hongkongensis* were much larger than those observed within each of the two species, consistent with the results from Zhang et al (2005). Moreover, attempted cross amplification of *C. ariakensis* microsatellite markers failed at eight of eleven loci in *C. hongkongensis* (Chapter 1), indicating high genomic heterogeneity between these two species. All these results were congruent with the reported molecular data from several mitochondrial genes (Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008) and one nuclear region (ITS-1, Reece et al. 2008), and support the two-species hypothesis (Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008). The genetic divergence between *C. ariakensis* and *C. hongkongensis* (32.79%, based on F_{ST} value) estimated by microsatellite polymorphisms in the present study was much smaller than that estimated from PCR-RFLP (93.4 – 100%, based on F_{ST} values) (Zhang et al. 2005), but higher than nucleotide divergence (13.6 – 15.1%) estimated from mitochondrial and nuclear DNA sequences (Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004). This is probably due to different polymorphic levels of these markers and indicates the difficulty in comparing results from different types of markers. Using the same type of marker (microsatellite markers), the overall genetic differentiation (F_{ST}) between these *C. ariakensis* and *C. hongkongensis*

was an order of magnitude higher than that found between *C. gigas* and *C. angulata* (Huvet et al. 2000), two taxa with questionable species status but showing strong genetic structure between them (Ó Foighil et al. 1998, Boudry et al. 1998, Huvet et al. 2000b, 2001, 2002, 2004). Estimated from several mitochondrial and one nuclear gene sequences, the genetic divergence between *C. ariakensis* and *C. hongkongensis* was comparable to the genetic divergence at the inter-species level reported among several other *Crassostrea* species (Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008). Therefore, genetic data from both this population genetic study and reported sequence phylogenies consistently support distinct species status between *C. ariakensis* and *C. hongkongensis* (Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008).

In terms of the biological species concept, surveys of wild samples as well as hatchery progeny using various molecular markers did not reveal any natural hybrids between *C. ariakensis* and *C. hongkongensis*. Zhang et al (2005) found previously that oysters initially identified as *C. ariakensis/C. rivularis* from a southern Chinese location had alternate homozygous genotypes at the ITS-1 locus, which were called S-type and N-type. The S-type *C. ariakensis/C. rivularis* was later confirmed to be *C. hongkongensis*, while the N-type was true *C. ariakensis* (Reece et al. 2008, Xiao unpublished data). No heterozygotes (hybrids) were detected in wild samples in this study (Zhang et al. 2005). In addition, after spawning was done at the VIMS hatchery using broodstock unknowingly comprised of both species, almost all progeny had the *C. ariakensis* genotype, and no hybrid individuals were observed (Zhang et al. 2005). Likewise, Reece et al (2008), and more extensive surveys conducted in her laboratory using the COI and

ITS-1 RFLP keys of Cordes et al. (2008), did not detect any natural hybrids among nearly 1000 wild individuals that included both *C. ariakensis* and *C. hongkongensis* from the coast of southern China (K.S. Reece, pers. comm.). Surveys of various locations in China by colleagues from HSRL (Haskin Shellfish Research Lab, Rutgers University) using other molecular markers did not detect any natural hybrids of these two putative species either (Wang et al. 2008b), adding additional support to the hypothesis of strong reproductive isolation between *C. ariakensis* and *C. hongkongensis* in the natural environment. Consequently, laboratory hybridization experiments were conducted as part of the present research in order to provide direct information on whether there is reproductive isolation between these two species.

Mechanisms of species isolation in marine taxa are variable and still unclear (Palumbi 1992, 1994). Laboratory hybridizations among *Crassostrea* species have been used for taxonomic studies (Banks et al. 1994, Huvet et al. 2001, 2002) and for seeking breeding stocks with novel genetic types (Allen et al. 1993, Allen and Gaffney1993). Successful hybridizations have been reported between various *Crassostrea* species for centuries, but credibility of these reports is undermined by the now recognized difficulties of oyster identification and classification, and by the experimental designs themselves (reviewed by Gaffney and Allen 1993). Hybridization trials, which included genetic confirmation of parents and progeny, have shown that inter-specific hybrids through the umbo stage can be obtained in the laboratory between several species including *C. gigas* × *C. ariakensis* (Allen and Gaffney 1993, Que and Allen 2002), *C. virginica* × *C. ariakensis* and *C. virginica* × *C. gigas* (Allen et al. 1993, Lyu and Allen 1999, Bushek et al. 2008). However, viable hybrids that survived past-settlement were

only found between *C. sikamea* and *C. gigas* (Hedgecock et al. 1993, Banks et al. 1994). Occurrence of natural hybrids between *Crassostrea* species is rare. With the exception of *C. gigas* and *C. angulata*, whose taxonomic relationship is still in question (Huvet et al. 2001, 2002, 2004, Leitão et al. 2007, Reece et al. 2008), only one individual of *C. hongkongensis* × *C. sikamea* (Reece et al. 2008) and one of *C. ariakensis* × *C. sikamea* (Wang et al. 2008b) were found in southern China. Gaffney and Allen (1993) indicated little prezygotic obstruction to mating, but a strong postzygotic barrier may exist in this genus, where there is little correlation between genetic distances and the ability to hybridize.

In the current study there was an almost complete failure of fertilization between *C. ariakensis* females with *C. hongkongensis* males, which indicates a gametic incompatibility between these two species, while low to moderate fertilization in the reciprocal crosses suggested this gametic incompatibility is unidirectional. This one-way gametic incompatibility resulted in at least a partial reproductive isolation and indicated a prezygotic barrier between *C. ariakensis* and *C. hongkongensis*. Similar one way gametic incompatibility was seen in a previous study of reciprocal crosses between *C. gigas* and *C. sikamea*, where a failure of acrosome reaction was speculated as the block of sperm-egg interaction (Banks et al. 1994). Artificial chemical stimulation of the acrosome reaction between *C. gigas* eggs and *C. sikamea* sperm suggested that this block occurs before acrosome reaction in the series of fertilization reactions (Palumbi 1992, Banks et al. 1994). Microscopic observations during spawning in the current research found that *C. hongkongensis* sperm bounced around the *C. ariakensis* eggs instead of sticking on the eggs' surface, as was observed in the control groups and the HA hybrid groups (data not

show), suggesting a recognition block between *C. hongkongensis* eggs and *C. ariakensis* sperm.

Though studies on molecular mechanisms of reproductive isolation in oysters are lacking, extensive research on prezygotic barriers have found that various proteins are involved in the reproductive isolation of free-spawning marine organisms including a species-specific lysine in abalones, bindin in sea urchins, and M7 lysin in mussels (Nei and Zhang 1998, Swanson and Vacquier 2002, Geyer and Palumbi 2003, Riginos and McDonald 2003, Riginos et al. 2006). Nei and Zhang (1998) indicated that "reproductive isolation between different species appears to be caused by the incompatibility of alleles at two or more loci that control mating, spermiogenesis and development". Thus one-way gametic incompatibility probably represents different receptivity of eggs and sperm among species. Asymmetrical fertilization, including the extreme case of one-way gametic incompatibility, has been documented in marine invertebrates including *Crassostrea* oysters (Allen and Gaffney 1993, Palumbi 1994). Though the underlying biological mechanisms are still unknown, "changes in this receptivity have been hypothesized to be important to rapid species formation" (Palumbi 1994).

Even with one-way gametic incompatibility, however, one might expect introgression to occur in the other direction, and at least F_1 hybrids might be found in the contact zone. Nevertheless, no *C. ariakensis*/*C. hongkongensis* hybrids have been detected to date in the natural habitat of these two species (Zhang et al. 2005, Reece et al. 2008, Wang et al. 2008b) or among the hatchery progeny derived from a source population containing almost equal numbers of the two species (Zhang et al. 2005). All these facts lead to the speculation that there might be additional mechanisms reinforcing

reproductive isolation. During the second spawn conducted for the current study, gonad condition varied markedly between these two species, which had been conditioned in the same tank for over six months. Both female and male *C. hongkongensis* had fully mature gonads, while *C. ariakensis* still demonstrated some gonadal immaturity. This suggests that different environmental conditions may be required for gonad maturation in the two species, hence differences in habitat requirements or spawning season could lead to reproductive isolation in nature. Unfortunately, there is no clear information on the ecology and biology of *C. ariakensis* in its native region so far. Earlier reports of the gametogenic cycle of *C. ariakensis* in its native region are suspect due to nomenclature confusions (Zhou and Allen 2003, Wang et al. 2004, Reece et al. 2008). Studies of *C. ariakensis* hatchery stocks on the west coast of the U.S. revealed late gonad maturity in this foreign environment, and currently the success of spawning and larval recruitment for this species in the west coast of U.S. relies entirely on artificial hatchery techniques (Breese and Malouf 1977, Perdue and Erickson 1984, Langdon and Robinson, 1996). Dependence on estuaries for natural larval growth and recruitment, however, has been suggested for *C. ariakensis*, and the optimal salinity for setting of *C. ariakensis* larvae was determined to be 15 to 20 ppt (Langdon and Robinson 1996). In comparison, it has been reported that *C. hongkongensis* requires temperatures > 27°C (optimal at 29°C) and salinities of ≤ 15 ‰ (optimal at 7 ‰) for spawning (Mok 1973, cited by Lam and Morton 2003). In addition, as discussed before, 97% of the progeny propagated in the VIMS hatchery from a source population comprised of approximately 50% of each of the two species were identified as *C. ariakensis* genotypes (Zhang et al. 2005), suggesting that the hatchery environment favored reproduction of *C. ariakensis*. Thus, different optimal

environmental conditions for spawning and larval growth may be required by these two species, resulting in habitat and/or seasonal isolation.

In summary, the results from this study support the hypothesis that *C. ariakensis* and *C. hongkongensis* are distinct species (Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008), based both on genetic divergence and observations of some level of reproductive isolation. Large genetic differentiation between the two taxa was observed, which was an order of magnitude greater than that found among individuals within each of the two taxa, and is congruent with previous research (Zhang et al. 2005) and sequence phylogenies (Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008). In addition, a semi-prezygotic reproductive isolation was found between the two taxa. Different habitat or spawning conditions for *C. ariakensis* and *C. hongkongensis* may reinforce the reproductive isolation. Nevertheless, it was difficult to characterize the postzygotic differences in growth and survival among the hybrid cross HA and the pure control crosses HH and AA. Larvae sizes and survival may have been affected by gamete quality, since low quality sperm and oocytes could result in inactive zygotes that show high mortality and retarded development. Furthermore, hatchery culling might artificially select traits of fast growth and larger size for the hatchery oysters (Taris et al. 2006). Because of the different sources of *C. ariakensis* (domesticated since 1999) and *C. hongkongensis* (from wild stocks) used in the experiment for spawning, the slight advantage of AA larvae in terms of both size and survival in Spawn 1 might represent improved performance of a domesticated stock in the laboratory environment. Finally, there could be many different explanations for the larvae in the current hybridization study failing to survive through early development, including a non-optimal laboratory

environment and low quality of gametes, since even the progeny of the pure control crosses did not survive. As the reasons for this are beyond the scope of the major research objectives of this dissertation, however, they were not thoroughly investigated. The focus of the discussion above centered on comparisons among the crosses in their performance at either the fertilization or post-fertilization stages, and the possible biological implications of these results to the hypothesis that *C. ariakensis* and *C. hongkongensis* are distinct species (Lam and Morton 3003, Wang et al. 2004, Reece et al. 2008). Further investigation will be needed to determine whether the HA hybrids are viable like *C. sikamea* and *C. gigas* (♀×♂) hybrids (Hedgecock et al. 1993, Banks et al. 1994), or inviable like hybrids among most other *Crassostrea* species (Allen et al. 1993, Gaffney and Allen 1993, Lyu and Allen 1999).

Table 2.1 Number of larvae in all 24 treatments identified as either parental types or hybrids by PCR-RFLP analysis of the ITS-1 region. Unexpected genotypes in treatments are indicated in parentheses. '-' indicates case with no individuals were tested due either to insufficient viable larvae or larval lysis during ethanol fixation. See Figure 2.1 for detailed descriptions on abbreviations of four crosses.

Trial	Replicate	AA	AH	HA	HH
	1	9	-	-	-
Spawn 1	2	-	-	14	-
	3	-	-	3	-
	4	10	-	10	10
Spawn 2	5	-	-	10	10
	6	3 (1 hybrid)	-	10 (7 *C. ariakensis*)	10

Table 2.2 Pairwise F_{ST} (Weir and Cockerham 1984) values based on three microsatellite loci between 16 wild *C. ariakensis* samples (IR99, IR05, KR04, SR04, KI04, YR99, YR06, BZ05, BZ06, NT05, NT06, TT06, HC05, HC06, BH99 and BH05) and 2 *C. hongkongensis* samples (B_Ch and C_Ch). Significant values are shown in **boldface** after Bonferroni correction for multiple tests (α = 0.05 and K = 17). Sample abbreviations for *C. ariakensis* are as indicated in Table 3.1 and abbreviations for *C. hongkongensis* populations are described in MATERIALS AND METHODS of this chapter.

	IR99	IR05	KR04	SR04	KI04	YR99	YR06	BZ05	BZ06	NT05	NT06	TT06	HC05	HC06	BH99	BH05	B_Ch	C_Ch
IR99	-	-0.0006	0.0209	0.0115	0.0071	0.0103	0.0126	0.0090	0.0145	0.0071	0.0120	0.0074	0.0135	0.0098	0.0192	0.0177	0.3473	0.3562
IR05	0.5203	-	0.0232	0.0159	0.0032	0.0085	0.0157	0.0124	0.0155	0.0058	0.0114	0.0073	0.0131	0.0088	0.0242	0.0214	0.3474	0.3578
KR04	**0.0005**	0.0001	-	-0.0027	0.0245	0.0128	0.0100	0.0234	0.0047	0.0118	0.0033	0.0118	0.0073	0.0107	0.0121	0.0092	0.3904	0.4021
SR04	0.0291	0.0064	0.6594	-	0.0066	-0.0013	0.0024	-0.0014	-0.0007	0.0013	-0.0048	0.0023	-0.0008	0.0056	0.0115	0.0099	0.3850	0.3944
KI04	0.1061	0.2379	0.0048	0.1676	-	-0.0024	0.0108	-0.0010	0.0168	0.0042	0.0168	0.0061	0.0064	0.0044	0.0258	0.0233	0.3524	0.3610
YR99	0.0168	0.0285	0.0103	0.5350	0.6028	-	0.0023	-0.0025	0.0036	-0.0016	0.0020	0.0061	0.0045	0.0055	0.0212	0.0208	0.3596	0.3693
YR06	0.0009	**0.0001**	0.0049	0.2603	0.0332	0.2187	-	0.0009	0.0020	0.0020	0.0018	0.0066	0.0077	0.0095	0.0174	0.0219	0.3663	0.3733
BZ05	0.1017	0.0503	0.0061	0.5095	0.4474	0.5744	0.3885	-	0.0018	0.0024	0.0034	-0.0008	0.0068	0.0094	0.0187	0.0235	0.3828	0.3888
BZ06	0.0012	0.0006	0.1028	0.4903	0.0153	0.1552	0.2104	0.3384	-	0.0058	-0.0038	0.0048	0.0086	0.0123	0.0175	0.0171	0.3755	0.3885
NT05	0.0223	0.0353	0.0035	0.3499	0.1710	0.6496	0.1963	0.2933	0.0432	-	0.0011	0.0001	-0.0009	-0.0017	0.0093	0.0125	0.3422	0.3550
NT06	0.0025	0.0035	0.1549	0.8614	0.0138	0.2444	0.2073	0.2365	0.9247	0.2804	-	0.0029	0.0052	0.0093	0.0137	0.0135	0.3715	0.3813
TT06	0.0231	0.0216	0.0091	0.2844	0.1214	0.0704	0.0273	0.4979	0.0788	0.4276	0.1518	-	0.0032	0.0019	0.0129	0.0114	0.3476	0.3605
HC05	0.0006	**0.0005**	0.0241	0.5433	0.0926	0.0865	0.0054	0.1072	0.0085	0.6029	0.0366	0.1213	-	-0.0011	0.0046	0.0060	0.3539	0.3642
HC06	0.0057	0.0066	0.0081	0.1113	0.1571	0.0632	0.0031	0.0651	0.0029	0.7260	0.0071	0.2261	0.6387	-	0.0092	0.0113	0.3302	0.3429
BH99	0.0010	**0.0001**	0.0147	0.0466	0.0036	0.0017	**0.0004**	0.0245	**0.0005**	0.0180	0.0039	0.0086	0.1039	0.0232	-	0.0033	0.3834	0.3937
BH05	**0.0005**	**0.0001**	0.0272	0.0527	0.0034	**0.0003**	**0.0000**	0.0060	0.0006	0.0014	0.0012	0.0070	0.0373	0.0027	0.2205	-	0.3568	0.3689
B_Ch	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	-	0.0279
C_Ch	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	0.0135	-

Figure 2.1 Diagram of crosses between *C. ariakensis* (A) and *C. hongkongensis* (H) for each of the two hybridization trials. Each cross consists of a single dam and sire.

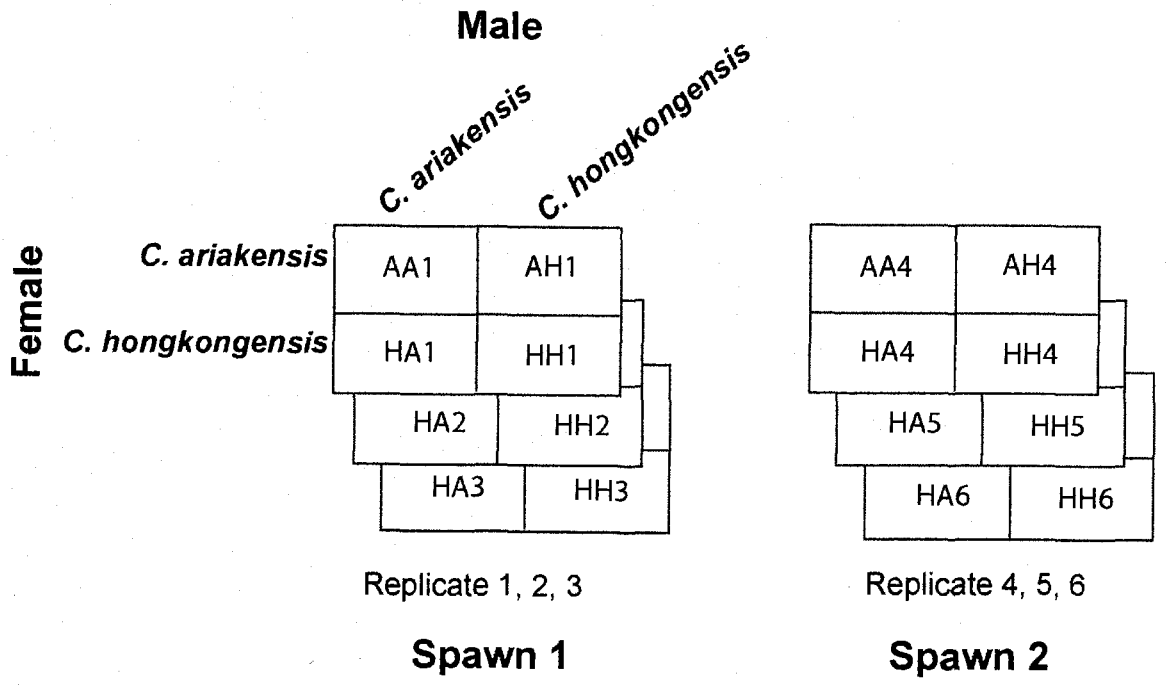

Male
C. ariakensis
C. hongkongensis
Female
C. ariakensis
C. hongkongensis
AA1
AH1
HA1
HH1
HA2
HH2
HA3
HH3
AA4
AH4
HA4
HH4
HA5
HH5
HA6
HH6
Replicate 1, 2, 3
Replicate 4, 5, 6
Spawn 1
Spawn 2

Figure 2.2 Fertilization ratio (%) for four crosses between *C. ariakensis* (A) and *C. hongkongensis* (H) (see Figure 2.1 for cross abbreviations). Black columns are mean values across three replicates of Spawn 1, and white are mean values over all replicates (except replicate 6) at Spawn 2. Error bars indicate the standard deviation for each mean.

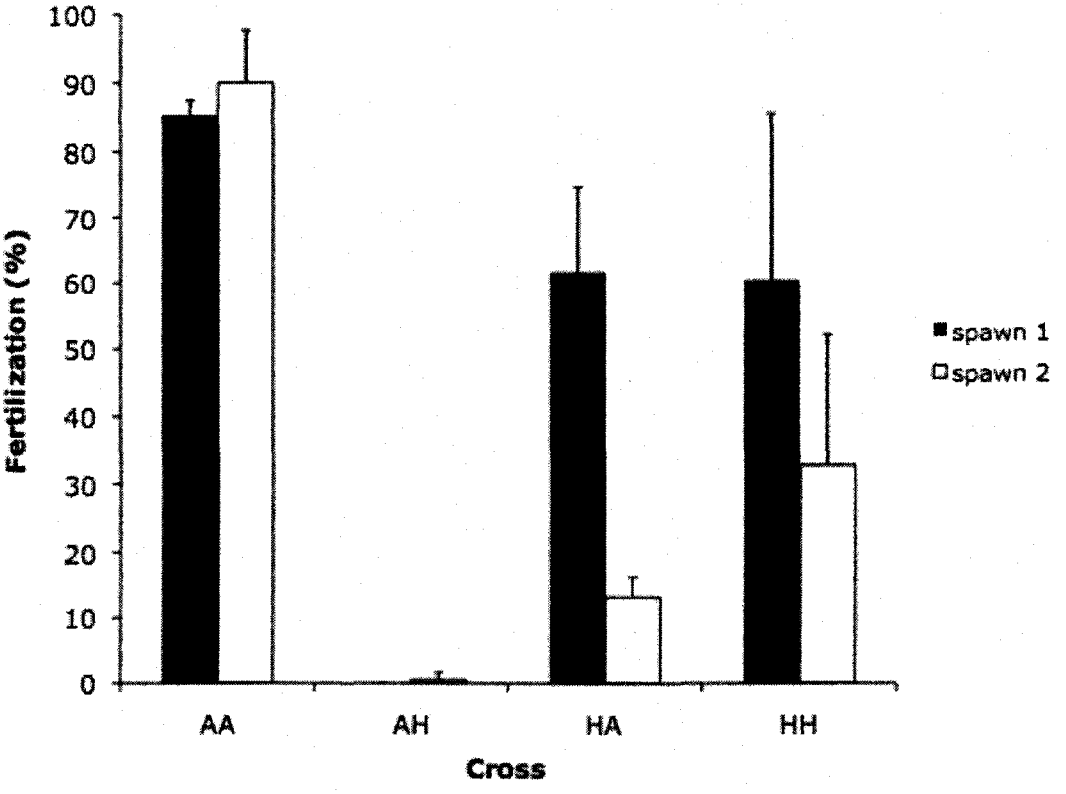
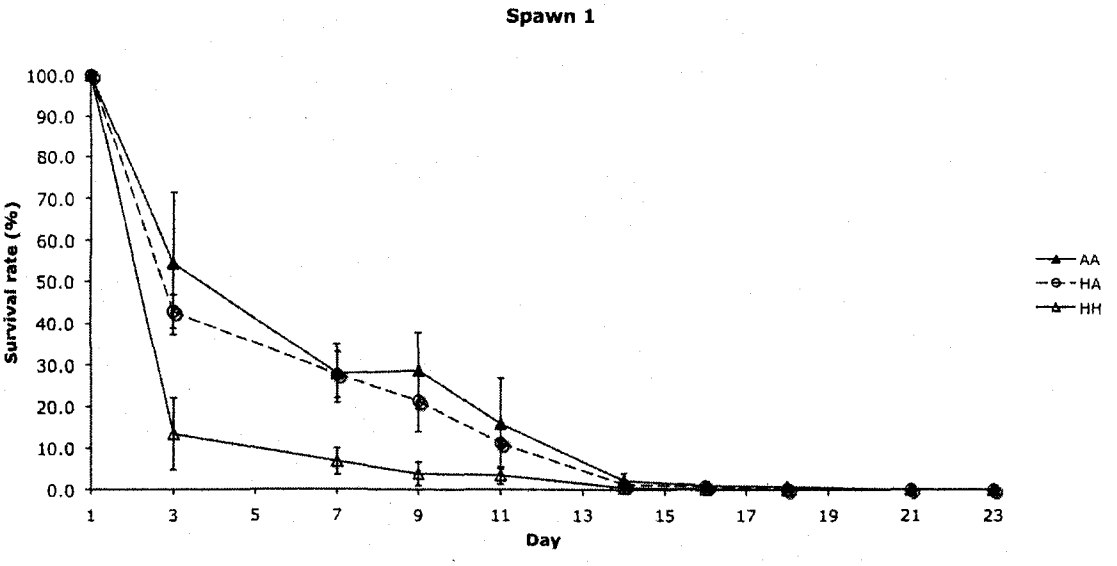

100
90
80
70
60
50
40
30
20
10
0
Fertilization (%)
AA
AH
HA
HH
Cross
spawn 1
spawn 2

Figure 2.3 Mean survival rate over all replicates (except replicate 6) of two hybridization trials throughout the experimental period (23 days for Spawn 1, and 19 days for Spawn 2). Error bar is the standard deviation of each mean. Survival rates for AH crosses are not shown here since no viable larvae were produced in this group.


Spawn 1
Survival rate (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
1
3
5
7
9
11
13
15
17
19
21
23
Day
AA
HA
HH

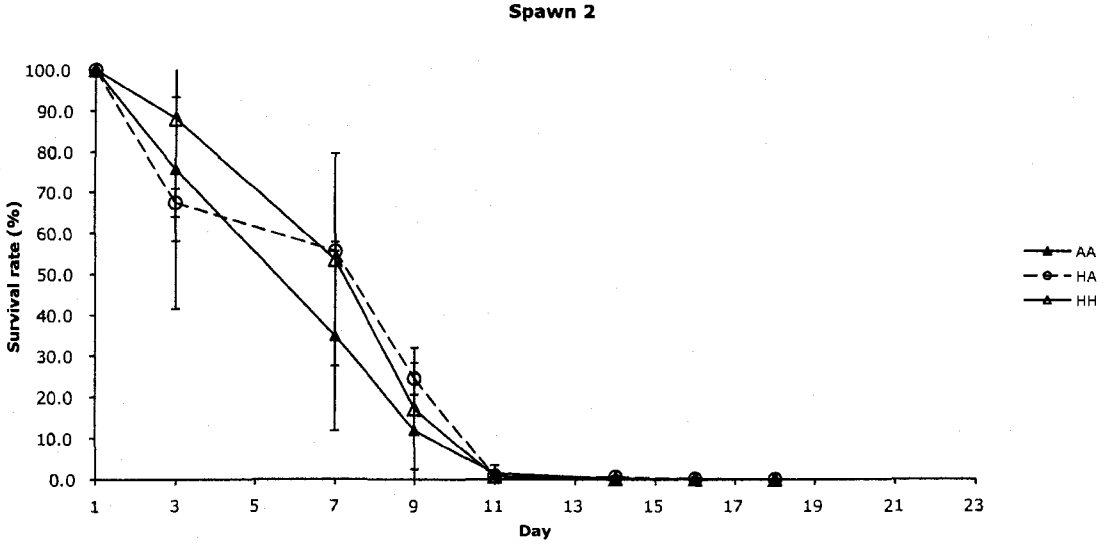
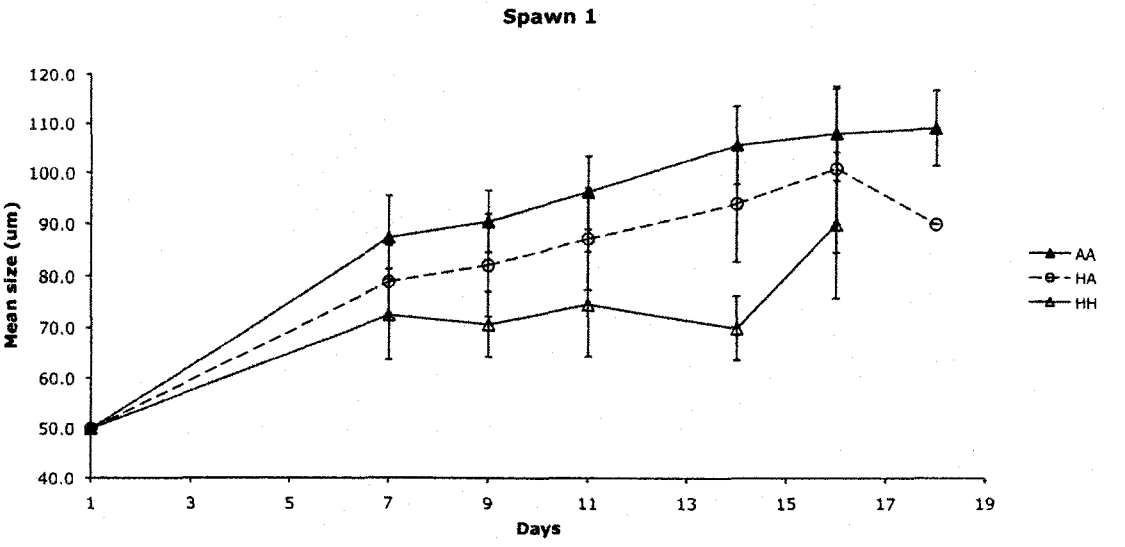

Spawn 2
Survival rate (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
1
3
5
7
9
11
13
15
17
19
21
23
Day
AA
HA
HH

Figure 2.4 Mean larvae sizes over all replicates (except replicate 6) of each cross throughout the experimental period. Error bar indicates the standard deviation of each mean. Growth of the AH cross was not shown here because no viable larval were produced in this cross.


Spawn 1
Mean size (um)
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
1
3
5
7
9
11
13
15
17
19
Days
AA
HA
HH

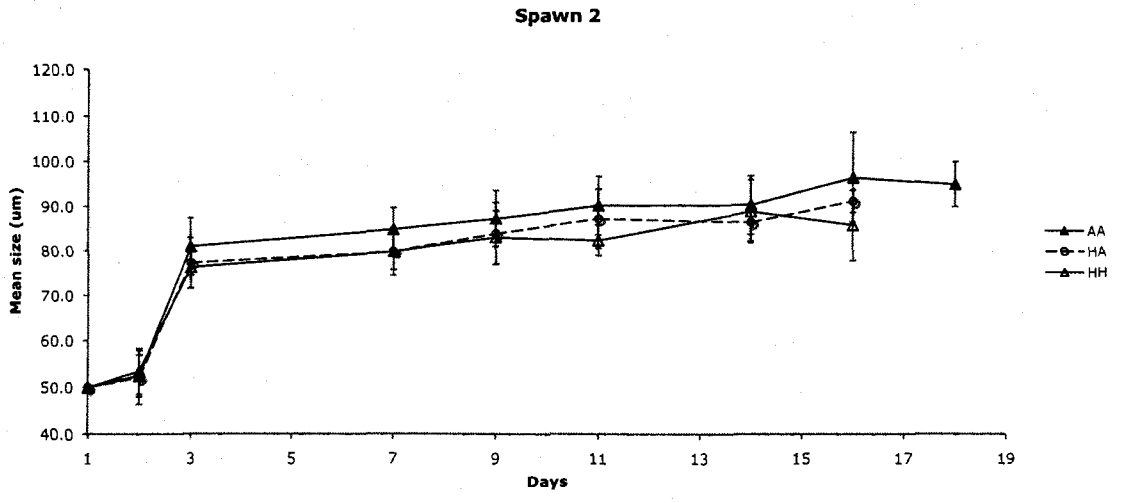

Spawn 2
Mean size (um)
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
1
3
5
7
9
11
13
15
17
19
Days
AA
HA
HH

Figure 2.5 RFLP patterns of the rDNA ITS-1 region digested with *Sau96* I to confirm the species designations of larvae from the HA hybrid cross (Lanes 3-7) and the pure HH (Lanes 8-12) and AA (Lanes 13-17) crosses. Lanes 1-2 and 18-19 are *C. hongkongensis* female, male and *C. ariakensis* female, male, respectively; Lane 20 is a $1kb^{+}$ size standard with molecular weight indicated at the right side. The arrow points to the extra band found in the hybrid which was not present in parental oysters or pure AA and HH crosses (see text).

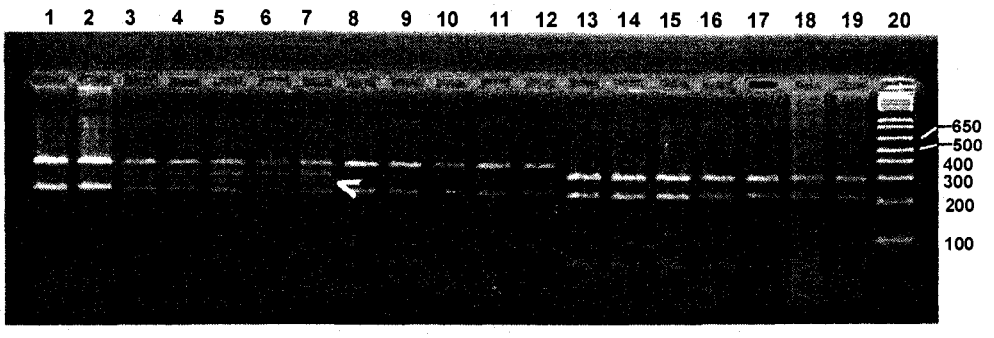

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
650
500
400
300
200
100

Figure 2.6 Factorial correspondence analysis (FCA) of genetic distances among wild populations of *C. ariaknesis* and *C. hongkongensis* based on three microsatellite markers. Three axes explain 45.2%, 9.1% and 6.7% of the total variance, respectively.

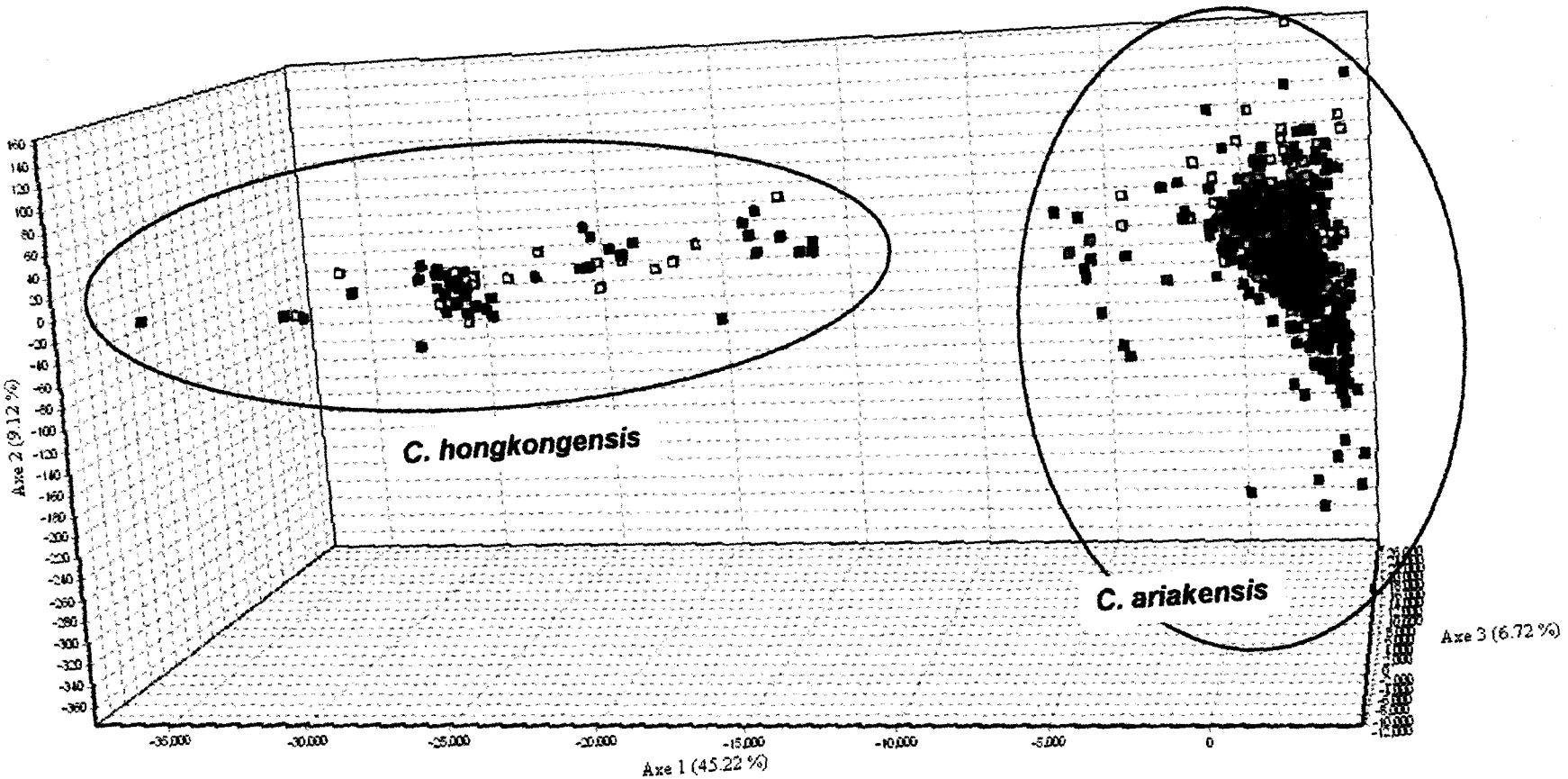
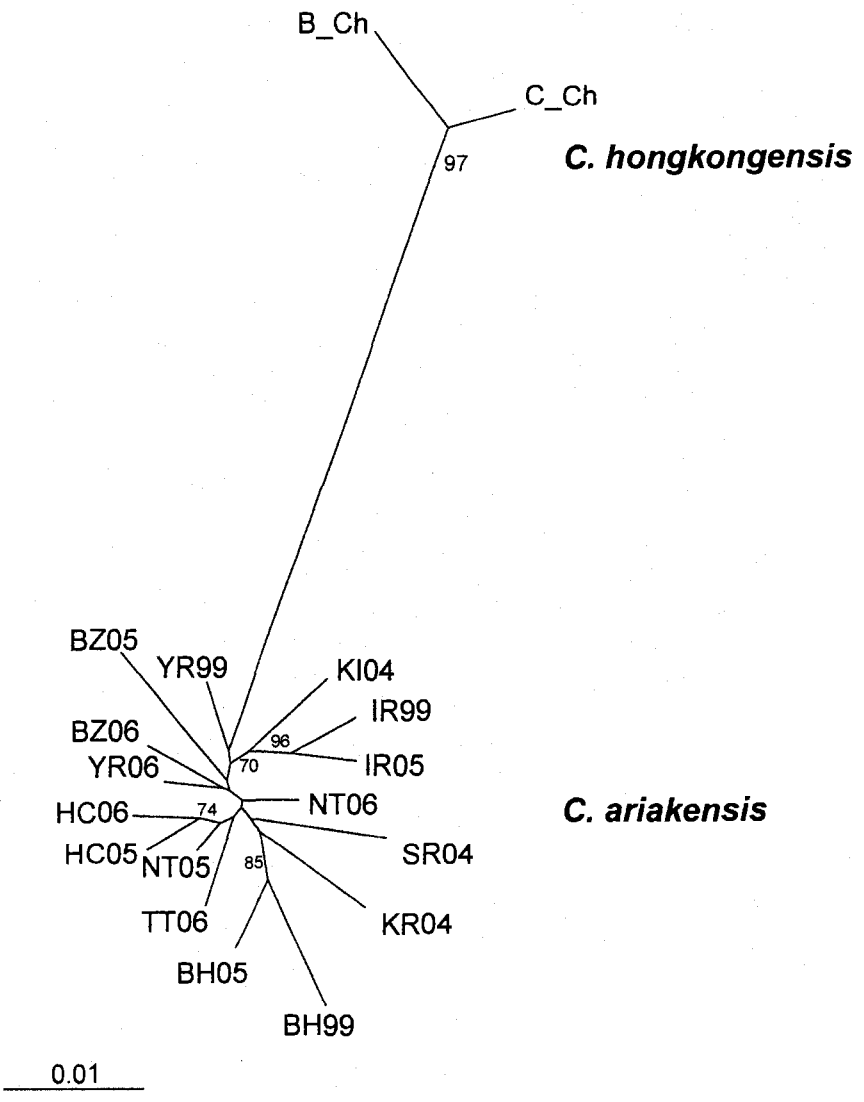

C. hongkongensis
C. ariakensis
Axe 1 (45.22 %)
Axe 2 (9.12 %)
Axe 3 (6.72 %)

Figure 2.7 Unrooted Neighbor-joining (NJ) tree based on a Cavalla-Sfourza and Edwards (1968) genetic distance matrix showing divergence between two *C. hongkongensis* (B_Ch and C_Ch) and 16 *C. ariakensis* wild samples. Length of each branch is proportional to the genetic distance. Numbers on the branches are percentages of bootstrapping support (> 50%) after 10,000 iterations. Sample abbreviations are as indicated in Table 3.1 of Chapter 3 and MATERIALS AND METHODS in this chapter. Inferred species clades are labeled in bold.


B_Ch
C_Ch
97
C. hongkongensis
BZ05
YR99
KI04
IR99
96
BZ06
YR06
70
IR05
HC06
74
NT06
C. ariakensis
HC05
NT05
85
SR04
TT06
KR04
BH05
BH99
0.01

CHAPTER 3

GENETIC POPULATION STRUCTURE OF *C. ARIAKENSIS* IN ASIA INFERRED BY MICROSATELLITE MARKER POLYMORPHISMS

INTRODUCTION

Asian oysters in the genus *Crassostrea* have been widely transplanted, and some species such as *C. gigas* are commercially important aquaculture species around the world (Ruesink et al. 2005). In contrast to the rapid growth of bivalve aquaculture, natural populations of *Crassostrea* oysters are believed to be declining in their native regions (Guo et al. 1999, 2006, Aranishi 2006) even as our knowledge of the taxonomy, abundance, and distribution of wild Asian *Crassostrea* oysters remains limited (Hedgecock et al. 1999, Boudry et al. 2003, Wang et al. 2004, Reece et al. 2008, Wang et al. 2008a). Even less is known about the genetic population structure of these oysters, important information needed for developing appropriate policies for stock management and conservation biology. With the rapid growth of oyster aquaculture and large breeding projects, molecular markers for studying the genetic variation among wild populations could help elucidate the genetic differences between wild populations, assess genetic variation within cultured stocks, determine genetic impacts of aquaculture on wild populations, and thereby promote sustainable aquaculture.

As addressed in previous chapters, *C. ariakensis* was recently proposed for introduction into the Chesapeake Bay on the east coast of the U.S. to revitalize the local oyster industry. Though some research has been conducted in the U.S. using diploid and triploid hatchery stocks of *C. ariakensis*, little is known about this species in its native region including its distribution and population genetic structure. According to the Code of Practice on Introductions and Transfers of Marine Organisms from the International Council for the Exploration of the Seas (ICES), prior to introduction of a non-native,

intensive research is required regarding the ecology, disease, and genetics of the species in its native region (ICES 2005).

Though sedentary as adults, oysters have high dispersal capability during their 2 – 3 week planktonic larval stage (Thorson 1950). However, unlike marine invertebrates found in the open ocean that often have little genetic differentiation over large geographic scales due to a lack of geographic barriers (Palumbi et al. 1997, DeWoody and Avise 2000), coastal invertebrate species are more influenced by historical or present-day biogeographic and oceanographic characters (Avise 1994, Kelly et al. 2006). According to ongoing distribution studies, *C. ariakensis* has been confirmed to occur along the coast of southern Japan, South Korea, and northern and southern China, probably with a patchy pattern of distribution (Wang et al. 2004, Zhang et al. 2005, Guo et al. 2006, Wang et al. 2006, Reece et al. 2008, Wang et al. 2008a, Yoon et al. 2008). Wang et al. (2004) suggested that two ecotypes of *C. ariakensis* might exist, one in northern and the other in southern China, with a geographic barrier proposed to exist in the vicinity of the Yangzi River. Zhang et al. (2005) could not resolve significant population structure within this species (i.e. the "N-type" as designated in Zhang et al. 2005) based on PCR/RFLP analysis of mtDNA and nuclear markers, as well as three microsatellite loci. This may have been due to low polymorphisms of the RFLP markers and null allele problems with the microsatellite loci, since primers were designed to target *C. gigas* sequences. For the current study, eight microsatellite markers specifically developed for *C. arakensis* (Chapter 1 and Xiao et al. 2008) were selected and wild *C. ariakensis* populations identified and confirmed throughout its currently recognized distribution were sampled in order to examine the genetic population structure of *C. ariakensis* in its native region.

MATERIALS AND METHODS

Sampling

A total of 605 individual oysters were analyzed in this study. Tissue samples consisted of gill and/or mantle snips collected in the field and preserved in 95% ethanol for DNA extraction. Sixteen samples were collected from ten locations along the coastal waters of the northwest Pacific (Figure 3.1). Names of these samples were coded with two initials indicating the sampling location and two digits to indicate the year collected. Oysters from the Itoki River, Ariake Sea, Saga Prefecture, southern Japan (IR99, IR05) were collected from a traditional oyster farming area where local wild seed were used as broodstock. Korean samples KR04, SR04, and KI04 were from the Kahwa River, Sumjin River, and Kanghwa Island, Incheon, respectively. Four samples (BZ05, BZ06 and YR99, YR06) were collected from two proximal locations (Binzhou and Weifang, respectively) in the Yellow River Basin of Bohai Bay in northern China. Three samples (NT05, NT06, TT06) were also collected from an estuary of the Yangzi River with one (TT06) at the mouth and the other two (NT05, NT06) from Nantong, about 50 km upriver. Individuals from Haicheng in the Fujian Province of China were collected from a wild oyster reef in which *C. ariakensis* was the dominant oyster species. The samples BH99 and BH05 were taken from the Dafeng River near Beihai in the Guangxi Province of southern China, where *C. ariakensis* is cultured using local wild seed (Wang et al. 2004). This sampling range covered most sites where to date, substantial *C. ariakensis* populations have been confirmed (Wang et al. 2004, Zhang et al. 2005, Guo et al. 2006, Wang et al. 2006, Reece et al. 2008). To test for temporal stability, multiple samples were

collected from six locations, three of which were sampled 6-7 years apart, while another three were sampled in successive years (Table 3.1). Due to plasticity in shell morphology and the large number of *Crassostrea* oyster species that coexist in Asia (Boudry et al. 2003, Wang et al. 2004, Guo et al. 2006, Wang et al. 2006, Reece et al. 2008, Wang et al. 2008a), samples collected for this study were identified as *C. ariakensis* using the molecular genetic key of Cordes et al. (2008). Samples collected by our lab for a previous study were also positively identified using genetic markers (Zhang et al. 2005), as were samples collected by colleagues from the Haskin Shellfish Research Laboratory at Rutgers University (Wang and Guo 2006, Wang and Guo 2008).

DNA extraction and microsatellite amplification

Whole genomic DNA extraction and amplification of eight microsatellite loci (*CarG110*, *CarG4-60*, *Car130-08*, *Car119-6a*, *CarG122*, *Car11-70*, *CarG1-0b*) for all *C. ariakensis* samples followed the protocols described in Chapter 1 and Xiao et al. (2008). Fluorescence-labeled products were separated on an ABI-Prism 3130 Genetic Analyzer (Applied Biosystems) with a GenScan™ 500LIZ™ size standard (Applied Biosystems) for each sample.

Image processing and data analysis

Alleles were scored using the software package GeneMarker (SoftGenetics) and coded by their sizes in nucleotides. A panel including all the alleles detected in 605 individuals was created for each locus.

The software package Micro-Checker 2.2.1 (van Oosterhout et al. 2004) was used to check for possible null alleles and genotyping errors caused by stuttering and large allele dropout. Observed (H_O) and expected (H_E) heterozygosities, and Nei's (1978) gene diversity (H_S) were then calculated in the program GENETIX (Belkhir et al. 1996-2004). Weir and Cockerham's (1984) unbiased F-statistics (F_{IS} and θ_{ST}) were calculated using the web version of GENEPOP 3.4 (Raymond and Rousset 1995, http://genepop.curtin.edu.au/). Unbiased estimates of *P*-values for F_{IS} and θ_{ST} were performed using the Markov chain method with parameters set at 1,000 dememorizations, 1,000 batches, and 10,000 iterations per batch. To compare allelic richness (A) among populations with different sample sizes, the rarefaction method (Petit et al. 1998) implemented in FSTAT 2.9.3 (Goudet 2001) was used to estimate the expected number of alleles per locus in a sample size of 14 diploid individuals (the smallest sample size for this study). The statistical comparisons of A, H_O, H_E and F_{IS} among populations were done in MINITAB® (Minitab Inc., State College, PA).

The allele frequency table generated by GENETIX was imported into the program GENDIST in the PHYLIP 3.67 software package (Felsenstein 1989) to calculate Cavalli-Sforza & Edwards' genetic distances (D_{CE}, Cavalli-Sforza and Edwards 1967) among all samples. Neighbor-joining (NJ) trees were constructed using the D_{CE} matrix in the program NEIGHBOR. The robustness of each node was evaluated by bootstrapping with 10,000 iterations using SEQBOOT. The resulting consensus tree was calculated using CONSENSE and visualized in TREEVIEW 1.6.6 (Page 1996).

A post hoc AMOVA analysis was performed to determine appropriate temporal and geographic pooling of samples in the program ARLEQUIN 3.11 (Excoffier et al.

2005). Molecular variances within populations, among populations within groups, and among groups were assessed with exact tests based on 10,000 permutations.

In order to test the association between genetic and geographic distances, we performed Mantel tests (Mantel 1967) on the regressions between D_{CE} and the geographic distance matrix among samples. Tests were done using ISOLDE in GENPOP 3.4 with 10,000 permutations to assess the significance of each regression. Pearson's coefficiency (r) calculated by MINITAB was annotated to illustrate the fitness of regressions. Geographic distances were measured according to the shortest route over water, and distances between temporal collections were set to 0. To analyze the effect of single loci on this correlation, we retested the regressions with datasets generated by jackknifing one locus each time through the program GENETIX.

RESULTS

Allele frequency and genetic diversity within populations

Amplification of 605 individuals from 16 wild samples generated a total of 294 different alleles over the eight loci with an average of 16.6 alleles per locus per population. Seventy-two regionally specific alleles were detected, all of which had low frequencies (less than 5.3%) and with a majority (79.2%) present only once. Micro-Checker analysis indicated possible null alleles in 6 of 16 samples at locus *Car130-08* and in 12 samples at locus *Car115-a0*. Scoring errors resulting from stuttering were indicated in 2 cases at locus *CarG122* based on heterozygote deficiencies.

In order to evaluate the performances of these markers, allelic richness (A), observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficients (F_{IS}), and gene diversity (H_S) were calculated at each locus for every sample (Table 3.2). Single locus HWE tests for each sample revealed 18 significant values out of 128 tests; 11 were at locus *Car115-a0*, 6 were at locus *Car130-08*, and another at locus *CarG122*. The significant deviations from HWE at these loci were probably caused by the presence of null alleles or stutters as indicated by the Micro-Checker analysis. Estimations using all eight loci indicated that 7 of the 16 samples were out of HWE after sequential Bonferroni correction ($\alpha = 0.05$, K = 16), all of which showed lower observed heterozygosity than expected. The number of significant deviations from HWE was reduced to one ($\alpha = 0.01$ level) and F_{IS} decreased significantly for all samples when the locus *Car115-a0* was excluded from the analysis, indicating that most deviations were due to this locus.

Multi-locus gene diversities, observed, and expected heterozygosities were comparable across all populations, with mean values of 0.818 (± 0.017), 0.766 (± 0.030), and 0.805 (± 0.017), respectively. However, mean *A* across all loci was significantly larger (11%, P = 0.001) for four southern samples (BH99, 05 and HC05, 06) as compared to the other samples. Further analysis found that this was primarily caused by one locus, *Car115-a0*. When this locus was excluded from the analysis, the difference in mean *A* between the four southern samples and the other 12 samples decreased to 6%, and P was increased to 0.032.

Variation in *A* values at the locus *Car115-a0* (Table 3.2) was significantly higher (P < 0.001) for the four southern samples (18.9 – 20.8) compared with the 12 samples collected from the northern sites (11.3 – 16.7). At the same time, F_{IS} values at this locus were marginally higher (P = 0.027) for the southern samples compared to the rest of the samples. Since a large number of deviations from HWE were observed at the locus *Car115-a0* (see above), analyses of population structure were performed both with and without this locus to detect artifacts from a single locus.

Inter-population genetic differentiations and structure

Multi-locus global θ_{ST} among populations was small (0.018) but highly significant (P < 0.001), indicating genetic heterogeneity among the natural populations. Single locus effect on global θ_{ST} was tested through a jackknife procedure (Table 3.4). The variance among eight tests only accounted for 10% of the total mean, indicating this genetic heterogeneity was not simply an effect from a single locus. Pairwise θ_{ST} values among all sixteen samples (Table 3.3) ranged from -0.002 (BZ05 vs. BZ06) to 0.041 (NT06 vs.

BH99). Ninty-nine (82.5%) of the 120 comparisons were significant after Bonferroni corrections. The other 21 non-significant results consisted of the six comparisons between temporal samples, six comparisons between geographically contiguous groups less than 50 km apart, 8 comparisons involving samples SR04, KI04 and BZ05 (all with low sample sizes ≤ 20), and one comparison between HC05 and BH99. Nine of 15 comparisons involving sample BZ05 were not significant, indicating that the low sample size (14) may have reduced the power of our tests to differentiate this population with others.

The overall pattern of genetic differentiation was visualized by a NJ tree based on the D_{CE} matrix of the 16 samples (Figure 3.2). In general, the tree topology was consistent with the results from the population pairwise θ_{ST} matrix and the geographic locations of the samples. Temporal samples generally clustered together in the tree, however, the four samples from within the Yellow River basin (YR99, YR06, BZ05, BZ06) formed a clade (< 50% support) with the two 2006 samples from the two sites forming a moderately supported (64%) sub-clade. Similarly, the two Haicheng samples (HC05 and HC06) did not form a monophyletic clade, although they fell into a clade with the neighboring Beihai samples with high boostrap support (95%). In addition, the geographically contiguous samples NT and TT from the Yangzi River estuary, collected from sites that are less than 50 km apart were also closely grouped. In fact, three geographic groups, including a Japanese group (IR99 and IR05), a southern China group (BH99, BH05, HC05 and HC06) and the Yangzi River estuary group comprised of NT05, NT06, and TT06, were distinctly separated from each other and formed individual clades with high bootstrap support (> 90%). Each of the three Korean samples (KR04, SR04 and KI04)

was separated from the clades described above, and formed a separate clade, though with relatively low bootstrap support (< 60%).

The AMOVA analysis first tested the temporal genetic variation among samples by grouping the temporal samples from a given location and found that the variance among temporal samples at six locations only accounted for 0.14% of the total variance and was not significant (P = 0.220 ± 0.004) (Table 3.5). In contrast, 1.87% of variance was from among the ten geographic samples and was significant, which indicated a lack of temporal genetic variation and confirmed the presence of genetic structuring among wild geographic populations of *C. ariakensis* in its native region. Temporal samples at each of the six locations (IR, BZ, YR, NT, HC and BH) were subsequently pooled based on their genetic homogeneity. Since close genetic relationships among geographically contiguous samples <50 km apart (BZ and YR from the Yellow River basin, TT and NT from the Yangzi River estuary; Fig. 3.1) were implied by the results from both the pairwise θ_{ST} values and the NJ (see above for details), adjacent samples from the two river systems were further grouped to test for fine-scale population subdivision in our samples. Little and non-significant variance (0.08%, P = 0.292) among geographically contiguous samples suggested that oysters from each of these two regions (Yangzi River estuary and Yellow River basin) came from genetically homogenous populations. Ultimately, eight genetically differentiated populations (Figure 3.2) were detected among these 16 samples, and the variance among populations accounted for 2.03% of the total variance.

Mantel test

A significant linear correlation ($D_{CE} = 0.017 + 4.09\times10^{-6}$ distance, $r^2 = 0.428$, $P < 0.01$) between genetic distances (D_{CE}) and geographic distances was detected, indicating a pattern of isolation by distance with a general increase in D_{CE} of 0.00409 per 1000 km distance (Figure 3.3). Jackknife testing on this regression showed that both the slopes and Pearson's coefficients (r) varied little when individual loci were sequentially excluded from the analysis (Table 3.4), suggesting that the linear regression was not driven by artifacts from any single locus.

DISCUSSION

Based on the polymorphisms at eight microsatellite markers, the magnitude and pattern of genetic structure was assessed among sixteen samples of natural Asian *C. ariakensis* collected from ten sites where large populations have been identified (Zhang et la. 2005, Guo et al. 2006, Wang et al. 2006, Reece et al. 2008, Wang et al. 2008a). Small, but highly significant, genetic heterogeneity was found among these populations, and a pattern of isolation by distance was observed indicating a correlation between genetic distances (D_{CE}) and geographic distances. Hereafter, the robustness of this structure and the possible biological and geological factors that would account for this genetic variability will be discussed.

Robustness of the genetic structure analysis in wild populations

Levels of genetic differentiation in terms of pairwise F_{ST} values among these *C. ariakensis* samples were small (ranging from -0.002 to 0.041), but often highly significant. A recurrent problem associated with such small F_{ST} values is the ability to discriminate real population structure from background noise due to sampling errors (Waples 1998). A 'sweepstakes' effect, caused by large variances in individual reproductive success, has been previously observed in the highly fecund marine organism *C. gigas* (Hedgecock 1994), which resulted in large inter-annual differences in genetic heterogeneity. Temporal genetic differentiation in the current study, however, either between samples with 6 - 7 year intervals (corresponding to 2 – 3 generations; IR, YR and BH) or those from consecutive years (BZ, NT, HC), only accounted for 0.14% of the

total variation and were all non-significant, indicating little temporal genetic variation in natural *C. ariakensis* populations and eliminating the role of temporal variation as a confounding factor in the results of the spatial genetic structure analysis. Small sample sizes of some populations such as BZ05 (14) and KI04 (20) may have limited the power of the analysis to detect differences among some populations, as indicated by a number of non-significant pairwise F_{ST} values (Table 3.3) in which these samples were involved. The overall genetic structure nonetheless did not change significantly after these samples (KI04 and BZ05) were dropped from the analysis (data not show). Therefore, problems associated with small sample sizes of a few samples in this study did not influence the overall genetic structure, though they might have affected determination of the genetic structure at a local scale where these samples were involved. The consistency among temporal samples also points to the relatively small influence of sample sizes at some locations (BZ05 and BZ06). Moreover, the significant linear pattern of isolation by distance also increases the confidence that the small genetic differentiation among populations is not due to sampling errors (Palumbi 2003).

Non-amplifying (null) alleles are often the result of high mutation rates in primer binding regions and are extremely common in marine bivalves (Hedgecock et al. 2004, Reece et al. 2004), raising concerns when applying microsatellite markers to population genetic studies in molluscs (Hedgecock et al. 2004). From a recent simulation study, microsatellite null alleles were found to cause a small but significant overestimation of F_{ST} values (Carlsson 2008). Here, the eight polymorphic microsatellite markers used in this study had minimized the null allele presence through initial primer development and extensive optimization (Xiao et al. 2008). A high number (68.8%) of samples, however,

exhibited deviations from HWE at a single locus *Car115-a0*. Most of these samples were in HWE at the other 7 loci, indicating that population effects, such as the Wahlund effect or inbreeding, could be excluded as causing these deviations. Presence of null alleles at the *Car115-a0* locus was indicated by Micro-Checker and may still be a problem for this locus, even though the primers and amplification parameters were optimized and initial testing did not detect any null alleles in eight family crosses and a wild population (Xiao et al. 2008). It is interesting that locus *Car115-a0* showed a significantly higher allelic diversity (P = 0.000) along with marginally higher positive F_{IS} values (P = 0.027) in four southern Chinese samples (HC05, HC06, BH99, BH05) compared to the northern locations. It would be difficult to explain these two concurrent observations based solely on the presence of null alleles. Further investigation is needed to determine whether other problems such as homoplasy, along with null alleles, are causing the significant differences in allelic diversity and F_{IS} values found in the four southern Chinese samples. Another plausible explanation is selection. Selection on certain types of markers (such as allozymes) has been commonly used to explain inconsistent observations of genetic structure in some marine species (Karl and Avise 1992, Pogson et al. 1995, Arnaud-Haond et al. 2003). It was also applied as a reason to discard any individual locus at which strong structure was observed while not the other loci, since most structure analyses make the assumption of marker neutrality. Selection against deleterious alleles was recently hypothesized to explain the distorted marker segregation in oysters in the laboratory (McGoldrick and Hedgecock 1997, Bierne et al. 1998, Launey and Hedgecock 2001). For the current study, in order to eliminate any undue influence of the *Car115-a0* locus on the results, analyses of genetic differentiation and structure for the natural *C.*

ariakensis populations were conducted with and without this locus. Though exclusion of the locus *Car115-a0* did reduce multi-locus F_{IS} values and the number of populations out of HWE, this marker did not affect the global F_{ST} value or the overall pattern of IBD. The effects of the other seven loci were also tested by removing them one at a time from the analysis. No significant changes were found, indicating overall measures of genetic structure were not affected by any single locus and further confirming the existence of overall genetic structure among wild *C. ariakensis* populations.

Population genetic structure

Global genetic differentiation among these natural populations of *C. ariakensis* was small (0.018) but highly significant, indicating population heterogeneity in this region. The genetic differentiations among these populations followed a pattern of isolation by distance (IBD), with no significant genetic differentiations among populations within small geographic scales (< 50 km), but strong structure among populations hundreds to thousands of kilometers apart. Eight genetically distinguished populations were identified, based on the AMOVA, pairwise F_{ST}, and/or NJ analyses, composed of a Japanese population (IR), three populations along the coast of South Korea (KR, SR, and KI) and four populations along the coast of China. The four Chinese populations included a Yellow River basin population (YR) located within Bohai Bay, northern China, a population from the Yangzi River estuary (YzR), a central coast population (HC) and a southern coast population (BH). Whether this pattern of differentiation is due to real genetic discontinuities or patchy sampling along a continuous gradient (isolation by distance) is addressed later in the discussion.

Geographic distances among South Korean samples were intermediate to the distances between those samples showing no structure and strong structure. These three populations also showed significant genetic differentiations among them. The small sample sizes of these populations (20 – 33), as well as one of the Yellow River basin samples (BZ05, n = 14), might influence estimation of their genetic differentiations due to random drift resulting from the high probability of sampling errors (Walpes 1998).

Although the two temporal samples from Haicheng (HC05, HC06) did not form a monophyletic clade in the NJ tree, they did form a clade together with the two temporal samples from neighboring Beihai (BH99, BH06). Relatively small θ_{ST} values (0.003 – 0.008) including one non-significant comparison (θ_{ST} = 0.003, P = 0.031) between the two Haicheng samples and two Beihai samples might indicate some gene flow among these two populations, though oyster transplantation by local farmers between these two locations could not be excluded. Records for anthropogenic transportation of *C. ariakensis* along the region surveyed by this study are hard to interpret due to notorious nomenclature problems (Zhou and Allen 2003, Wang et al. 2004, Reece et al. 2008). There were some reports of *C. gigas* being introduced from Japan to northern and southern China (Guo et al. 1999, Li et al. 2006) and from the U.S. west coast to Japan and South Korea (Ruesink et al. 2005 and references herein), though there was no known transportation of *C. ariakensis* between northern and southern China (Wang et al. 2004). Furthermore, oyster aquaculture is very popular and intensive within southern China including Fujian, Guangdong and Guangxi provinces (Guo et al. 1999, Zhou and Allen 2003). In spite of some confusion regarding which species is being cultured (Guo et al. 1999, Reece et al. 2008, Cordes et al. in press), *C. ariakensis* has been identified in both

oyster farming areas and in the wild throughout this region (K.S. Reece, unpublished data). It is not clear, however, if there have been un-documented transportation of *C. ariakensis* in southern China.

Small but significant levels of genetic differentiation have also been observed in other marine bivalves across similar geographic scales, including *Ostrea edulis*, *C. angulata*, *C. gigas*, and *Pinctada maxima* (Huvet et al. 2000, Launey et al. 2002, Sellos et al. 2003, and Lind et al. 2007, respectively), and are comparable to observations for many marine fishes (Ward et la. 1994, Walpes 1998). Lack of geographic barriers in the ocean, high dispersal capability during their pelagic larval stage, and high effective population size normally contribute to the genetic homogeneity in marine fish (DeWoody and Avise 2000). IBD patterns similar to those seen in this study of *C. ariakensis* were discovered in other coastal bivalve species (Murray-Jones and Ayre 1997, Launey et al. 2002, Mariani et al. 2002, Ríos et al. 2002, Rose et al. 2006, Lind et al. 2007), though the regression slopes were quite variable among taxa. This could be due to differences in biological traits such as duration of the planktonic larval stage, egg size, and drifting of adults (Bonhomme and Planes 2000, Kinlan and Gaines 2003, Lester and Ruttenberg 2005, Bradbury and Bentzen 2007), as well as other factors including the range of different genetic marker types (with varying levels of polymorphism) used in the different studies (Diaz-Almela et al. 2004).

In species sedentary as adults but having a long pelagic larval stage, dispersal of larvae is considered to be one of the major factors influencing patterns of geographic distribution and population structure in marine systems (DeWoody and Avise 2000, Kinlan and Gaines 2003). A patchy pattern of *C. ariakensis* population distribution was

indicated after several surveys in China (Guo et al. 2006, Wang et al. 2006). This patchy pattern could suggest a non-continuous and fragmented natural habitat for *C. ariakensis* along the coast of China, which might limit larval dispersal and preclude geographically continuous settlement along this region.

Some physical and geological barriers to genetic continuity in other regions, such as along the coast southern Georgia and Florida of southeast U.S., along the coast of Spain, France and Mediterranean Sea, were also suggested by other research in marine fish and invertebrates (Avise 1994, McMillan and Palumbi 1995, Ríos et al. 2002, Nikula and Väinölä 2003). These barriers to gene flow were often associated with a strong shift of gene frequencies over a short geographic range (Reeb and Avise 1990, Karl and Avise 1992, Hare and Avise 1998), or significant changes in genetic patterns between populations across the barriers compared with those among populations on either side (McMillan and Palumbi 1995, Barber et al. 2002, Planes and Fauvelot 2002, Ríos et al. 2002, Nikula and Väinölä 2003). Wang et al. (2004) observed morphological differences and distinct 16S and COI mitochondrial haplotypes between *C. ariakensis* in southern China (Beihai, Guangxi Province and various locations in Guangdong Province) and northern China (Weifang, Yellow River basin) after surveying 18 *C. ariakensis* individuals, and suggested long-term isolation and subsequent population subdivision between these geographic populations. A biogeographical barrier was suggested to exist along the coast in this region close to the Yangzi River estuary (Wang et al. 2004), and was supported by distributions of other marine invertebrate species as described by Xu (1997). The Yangzi River estuary was also suggested as a barrier causing sharp genetic differentiations among populations of *C. plicatula*, *C. gigas*, and *Coelomactra antiquata*

(the xishishe clam) in China (Kong et al. 2007, Yu et al. 2008), but this conclusion was weakened by the fact that they did not explicitly exclude other species or hatchery animals from their samples, therefore they did not verify whether the genetic variation due to inter- and intra-species differences or between wild and hatchery populations (Kong et al. 2007, Wang et al. 2008a, Yu et al. 2008). Data from the current study did show significant genetic differentiation between the Yellow River basin population (YR, corresponding to northern samples in Wang et al. 2004) and the Beihai population (BH, where some southern samples were collected by Wang et al. 2004), though no significant change in the genetic pattern (such as larger F_{ST} values) among populations across this proposed barrier was observed compared with the overall structure, and results of the Mantel test were consistent with a pattern of IBD (Figure 3.3). It is unclear, however, whether the significant genetic differentiation between YR and BH populations at the extremes of the studied geographic range is a result of a fairly gradual accumulation of small non-significant genetic shifts that became significant over a large scale (IBD), or because of one or more sharp genetic discontinuities related to specific barriers. The fact that *C. ariakensis* populations are scattered in distribution over a wide geographic scale (Guo et al. 2006, Wang et al. 2006) suggests that the strong genetic differentiations between populations so far apart (> 1500 km) could be a consequence of patchy distribution, long geographic distances, and the associated factors influencing larval dispersal as described above. Unfortunately, our sampling density was not high enough to discriminate between these two scenarios (continuous genetic differentiation or sharp genetic discontinuity) due to limitations on finding and sampling natural populations of *C. ariakensis* in its native region. Without more intensive sampling along this region, it is

hard to determine whether or not there is a geographic barrier causing a sudden genetic shift in *C. ariakensis* populations along the coast of China.

Wang et al. (2004) found that northern haplotypes in both COI and 16S genes were genetically closer to those found in Japan and South Korea (ÓFoighil et al. 1995, Kim et al. 2000), while southern haplotypes were closer to those found in Hong Kong (Lam and Morton 2003) indicating close genetic relationships among oysters within these two areas (northern area including northern China, Korea and Japan vs. southern area centered in southern China). However, haplotypes from neither the north nor the south were reciprocally monophyletic in either COI and 16S mitochondrial gene trees (Wang et al. 2004). Likewise, results from the current study showed significant genetic differentiation among populations within both the northern and southern sampling regions, such as the Yellow River basin population vs. the Yangzi River population vs. Japanese population, and the Haicheng population vs. the Beihai population. Indeed, the levels of genetic differentiation within the northern samples were not significantly lower than those observed between the north and the south, For example, over similar geographic distances (about 600 km), F_{ST} values between theYellow River basin and Yangzi River populations in the north (0.011 – 0.026) were similar to those observed between the Yangzi River and the Haicheng populations further south (0.017 – 0.030) (P = 0.322, t-test). Overall, these data do not support the notion of a clear North-South genetic discontinuity.

Wang et al. (2004) did not explain how the Yangzi River estuary might function as a barrier to gene flow between northern and southern *C. ariakensis* populations. Xu (1997) argued that due to the different temperature and salinity characters of water

masses between northern areas (including Bohai Bay and the Yellow Sea north to the Yangzi River estuary) and the East China Sea (south to the Yangzi River estuary), some marine bivalve species adapted to low temperature and low salinity water in northern areas may be prevented from moving further south to the East China Sea, which is affected by a current with more saline and warmer water from south, and *vice versa.* He also mentioned that the large freshet from the Yangzi River in the summer might obstruct reciprocal invasions of some bivalve species adapted to high salinity waters, while not affecting those coastal species able to inhabit a broad spectrum of salinity and temperature ranges. Apparently, *C. ariakensis* can live across such a broad spectrum, as shown by its confirmed distribution to date (Wang et al. 2004, 2008, Zhang et al. 2005, Reece et al. 2008, Yoon et al. 2008). Estuarine habitats with seasonal freshets like the Yangzi River also seem suitable for *C. ariakensis* and its larvae, since samples for this study were collected in that (NT05, NT06, TT06) as well as other estuaries (BZ05, BZ06, YR99 and YR06 were from Yellow River estuary; BH99 and BH05 were collected from Dafeng River estuary; see MATERIALS AND METHODS for details). It has been reported that *C. ariakensis* was often found to occur near rivers (Wang et al. 2006, Guo et al. 2006), and local fishermen in China often collect oyster seeds from upper-river areas (Guo et al. 1999). Langdon and Robinson (1996) speculated that this species is "dependent on estuaries for natural larval growth and recruitment" after some laboratory spawning trials. Therefore, it is questionable if a large amount of fresh water discharge from the Yangzi River in summer would be a barrier for larval dispersal of this species. Stable prevailing currents moving uni-directionally might also be a barrier to the gene flow among populations across the currents, whereas assisting gene flow along the

currents, like the case of pearl oyster populations distributed along the coast of the Central Pacific islands (Arnaud-Haond et al. 2003). The water currents in the native region of *C. ariakensis*, however, are quite variable among seasons (Figure 3.1). Though a major northeastward current joined with the Yangzi River freshet moves from the south to the Sea of Japan through the strait between the Korean peninsula and Japan during summer, there is not much evidence showing that there is more gene flow among the populations along this route relative to that among populations across it. For example, genetic differentiations between Yangzi River population (NT and TT) and the Korean population (KR, SR) or the Japanese population (IR) were significant (along the current), but not substantially lower than those between KR and IR, which are located across the major current. Therefore, the hypothesis of a genetic barrier formed by the Yangzi River estuary was not supported by the data from this research. But again, samples from more locations north and south of the Yangzi River estuary could produce a finer resolution of genetic population structure in this species and help to confirm or refute this hypothesis.

Discrepant results, however, can be obtained when different types of markers are used to study phylogeographic changes in marine bivalves. For example, anonymous nuclear genes and mtDNA revealed a strong genetic break with little gene flow between Atlantic and Gulf populations of the eastern oyster (*C. virginica*), while allozyme markers showed genetic homogeneity, possibly due to balancing selection on the protein-coding loci (Karl and Avise 1992). Diaz-Almela et al. (2004) found much higher genetic differentiation and differences in gene diversity variance among European flat oyster (*Ostrea edulis*) populations at a mitochondrial gene than was detected using several microsatellite loci, and speculated a sex-biased gene flow in this species. Wang et al.

(2004) used two mitochondrial genes (16S, COI), to examine genetic population structure in *C. ariakensis*, while the current research used eight nuclear microsatellite markers. Whether a similar phenomenon occurred in this Asian oyster species requires further investigation, especially in light of the small sample sizes (a total of 18 *C. ariakensis* individuals) used by Wang et al. (2004).

Summary

In conclusion, genetic heterogeneity does exist among *C. ariakensis* populations across its known native range. A small global genetic differentiation and a genetic pattern of isolation by distance were discovered, which is not surprising since it is also commonly observed in other marine invertebrates. There are, however, few reports on the genetic structure of marine coastal species in the northwest Pacific. A few studies assessing the genetic variation of coastal marine resources have focused either on local stocks (Shen et al. 2003, Chen et al. 2006, Yu and Chu 2006a, Yu and Chu 2006b, Kong et al. 2007) or cultured populations (Aranishi 2006, Li et al. 2006, Yu et al. 2008). No consistent pattern of genetic differentiation was revealed by these studies. Therefore, this research represents a novel observation on wild population genetic structuring in a coastal bivalve species along the coast of the northwest Pacific.

As addressed above, the observed pattern of isolation by distance seen in this study was not sufficient to reject the hypothesis of a distinct geographic barrier existing along the coast of China. Higher sampling density in the distribution region, especially around the hypothesized barrier would be necessary to confirm or refute this hypothesis. Furthermore, the partial genetic structuring seen in this study across small geographic

scales (e.g. among the three South Korean samples) could be an artifact of sample size, so increasing *n* for those populations could resolve some inconsistencies at some branches of the species' phylogenetic tree.

Finally, *C. ariakensis* from some native populations (particular the Japanese, Yellow River basin, and Beihai populations) have been transported into U.S. hatcheries, and their progeny were used in a variety of research projects (Breese and Malouf 1977, Langon and Robinson 1996, NRC 2004). It was unclear how much genetic variation existed within these hatchery stocks of *C. ariakensis* and what the genetic relationship is with their natural source populations. Therefore, in Chapter 4, these investigations were further extended to include the hatchery stocks of *C. ariakensis*, in order to get a more complete picture of the genetic variations within and among all wild populations and hatchery stocks of *C. ariakensis.*

Table 3.1 Source, sample code, collection date (month/year), type, and sample sizes of all populations used in this study. [a]

Samples from an oyster farming area where local natural seeds were collected for culturing.

Source	Sample code	Collection date	Sample type	Sample size
Ariake Sea, Saga Prefecture, Kyushu, Japan	IR99, IR05	5/99, 10/05	wild, [a]cultured	49, 50
Kahwa River, South Korea	KR04	04	wild	33
Sumjin River, South Korea	SR04	04	wild	20
Kanghwa Island, Incheon, South Korea	KI04	04	wild	20
Binzhou, Shandong Province, China	BZ05, BZ06	8/05, 4/06	wild	15, 35
Yellow River, Weifang, Shandong Province, China	YR99, YR06	6/99, 4/06	wild	32, 50
Nantong, Jiangsu Province, China	NT05, NT06	5/05, 4/06	wild	50, 50
Tangtou, Shanghai, China	TT06	8/06	wild	37
Haicheng, Fujian Province, China	HC05, HC06	4/05, 4/06	wild	50, 45
Dafeng River, Beihai, Guangxi Zhuang, China	BH99, BH05	5/99, 3/05	wild, [a]cultured	26, 43

Table 3.2 Estimated allelic richness (A) standardized with rarefaction method (Petit et al. 1998), observed and expected heterozygosities (H_O, H_E), inbreeding coefficient (F_{IS}), and gene diversity (H_S) for each sample at each locus, across all loci ([a]), and across all loci excluding *Car115-a0* ([b]). **Bold** fonts denote significant P values after Bonferroni correction (K=16) at $\alpha = 0.01$ ([**]) and 0.05 ([*]) levels.

	CarG110	*CarG4-60*	*Car119-6a*	*Car11-70*	*Car130-08*	*CarG122*	*CarG1-0b*	*Car115-a0*	**Multi loci[a]**	**Multi loci[b]**
IR99										
A	6.8	16.8	9.3	14.0	13.7	2.7	6.5	16.5	10.8	10.0
H_O	0.776	1.000	0.837	0.878	0.604	0.306	0.652	0.750	0.725	0.722
H_E	0.793	0.940	0.850	0.932	0.915	0.351	0.707	0.936	0.803	0.784
P	0.130	0.072	0.147	0.041	**0.000****	0.103	0.077	**0.000****	**0.000****	**0.000****
F_{IS}	0.032	-0.054	0.026	0.068	0.349	0.138	0.088	0.209	0.107	0.090
H_S	0.801	0.950	0.859	0.941	0.925	0.355	0.715	0.946	0.811	0.792
IR05										
A	7.6	16.3	9.6	13.1	13.0	2.0	6.5	14.7	10.4	9.7
H_O	0.878	0.920	0.816	0.860	0.592	0.440	0.638	0.780	0.741	0.735
H_E	0.835	0.936	0.867	0.906	0.918	0.343	0.663	0.921	0.799	0.781
P	0.141	0.158	0.070	0.071	**0.000****	0.052	0.131	**0.000****	**0.000****	**0.001***
F_{IS}	-0.041	0.027	0.069	0.061	0.364	-0.273	0.048	0.163	0.083	0.070
H_S	0.843	0.946	0.876	0.915	0.928	0.347	0.670	0.931	0.807	0.789
KR04										
A	10.1	18.2	10.6	13.0	14.5	2.0	7.3	17.3	11.6	10.8
H_O	0.879	0.879	0.909	0.909	0.844	0.121	0.636	0.677	0.732	0.740
H_E	0.874	0.949	0.896	0.913	0.922	0.165	0.694	0.934	0.793	0.773
P	0.215	0.028	0.240	0.221	0.031	0.206	0.105	**0.000****	**0.000****	0.005
F_{IS}	0.010	0.089	0.001	0.019	0.100	0.281	0.098	0.290	0.093	0.059
H_S	0.887	0.963	0.910	0.927	0.937	0.168	0.704	0.949	0.806	0.785
SR04										
A	6.1	19.5	13.7	12.9	14.4	2.0	7.3	16.7	11.6	10.8
H_O	0.850	1.000	0.900	0.900	0.800	0.250	0.750	0.850	0.788	0.779
H_E	0.764	0.943	0.900	0.904	0.914	0.219	0.736	0.930	0.789	0.768
P	0.184	0.500	0.283	0.262	0.025	0.752	0.251	0.050	0.121	0.284
F_{IS}	-0.088	-0.035	0.026	0.030	0.150	-0.118	0.007	0.111	0.027	0.012
H_S	0.783	0.967	0.923	0.927	0.937	0.224	0.755	0.954	0.809	0.788
KI04										
A	8.3	19.6	7.7	12.7	12.0	2.0	12.3	14.4	11.1	10.6
H_O	0.950	1.000	0.900	0.750	0.800	0.400	0.850	0.790	0.805	0.807
H_E	0.849	0.949	0.840	0.911	0.901	0.420	0.881	0.904	0.832	0.822
P	0.189	0.572	0.257	0.006	0.043	0.387	0.190	0.029	0.019	0.064
F_{IS}	-0.094	-0.028	-0.046	0.202	0.138	0.073	0.061	0.154	0.058	0.043
H_S	0.871	0.973	0.862	0.935	0.924	0.431	0.904	0.929	0.853	0.843
YR99										
A	7.7	17.2	10.0	12.3	13.7	2.0	9.6	13.0	10.7	10.4
H_O	0.875	0.938	0.875	1.000	0.906	0.313	0.688	0.875	0.809	0.799

H_E	0.803	0.943	0.865	0.906	0.920	0.305	0.694	0.875	0.789	0.777
P	0.141	0.243	0.223	0.066	0.200	0.443	0.221	0.232	0.312	0.647
F_{IS}	-0.074	0.022	0.005	-0.088	0.031	-0.010	0.025	0.015	-0.009	-0.013
H_S	0.816	0.958	0.879	0.920	0.935	0.310	0.705	0.888	0.801	0.789
YR06										
A	7.7	19.1	10.6	12.4	13.5	3.1	7.9	11.3	10.7	10.6
H_O	0.760	0.940	0.880	0.860	0.920	0.200	0.720	0.714	0.749	0.754
H_E	0.802	0.959	0.883	0.910	0.918	0.236	0.719	0.884	0.789	0.775
P	0.094	0.137	0.172	0.059	0.202	0.085	0.162	**0.000****	**0.001***	0.023
F_{IS}	0.062	0.030	0.014	0.065	0.008	0.161	0.009	0.202	0.060	0.037
H_S	0.810	0.969	0.892	0.919	0.928	0.238	0.727	0.893	0.797	0.783
BZ05										
A	6.9	19.1	10.7	9.9	10.7	2.9	7.8	15.0	10.4	9.7
H_O	0.800	1.000	0.867	0.933	0.867	0.133	0.733	0.714	0.756	0.762
H_E	0.813	0.938	0.867	0.864	0.876	0.331	0.756	0.916	0.795	0.778
P	0.233	0.642	0.287	0.337	0.256	0.020	0.231	0.003	0.006	0.057
F_{IS}	0.051	-0.032	0.034	-0.045	0.045	0.619	0.064	0.255	0.084	0.055
H_S	0.841	0.970	0.897	0.894	0.906	0.343	0.782	0.950	0.823	0.805
BZ06										
A	7.6	20.0	10.4	12.4	15.7	2.0	7.3	14.1	11.2	10.8
H_O	0.771	1.000	0.857	0.943	0.943	0.229	0.686	0.743	0.771	0.776
H_E	0.817	0.960	0.883	0.905	0.932	0.202	0.691	0.906	0.787	0.770
P	0.108	0.388	0.148	0.250	0.286	0.637	0.187	**0.001***	0.039	0.320
F_{IS}	0.070	-0.027	0.044	-0.027	0.003	-0.115	0.023	0.194	0.034	0.008
H_S	0.829	0.974	0.896	0.918	0.945	0.205	0.701	0.919	0.799	0.781
NT05										
A	8.7	18.9	7.0	12.9	15.5	3.2	11.6	14.0	11.5	11.1
H_O	0.820	0.940	0.760	0.959	0.760	0.400	0.860	0.840	0.792	0.786
H_E	0.847	0.958	0.755	0.917	0.930	0.349	0.871	0.929	0.820	0.804
P	0.118	0.148	0.150	0.173	**0.000****	0.099	0.158	0.008	0.009	0.050
F_{IS}	0.042	0.029	0.004	-0.035	0.192	-0.136	0.023	0.105	0.043	0.033
H_S	0.856	0.968	0.763	0.927	0.939	0.353	0.880	0.938	0.828	0.812
NT06										
A	7.3	19.2	6.9	12.0	16.5	2.2	10.1	15.7	11.3	10.6
H_O	0.860	1.000	0.880	0.900	0.900	0.100	0.780	0.816	0.780	0.774
H_E	0.819	0.959	0.792	0.907	0.942	0.198	0.843	0.933	0.799	0.780
P	0.139	0.191	0.052	0.174	0.061	**0.003***	0.055	**0.000****	0.021	0.159
F_{IS}	-0.040	-0.033	-0.101	0.018	0.054	0.502	0.085	0.136	0.035	0.017
H_S	0.827	0.968	0.800	0.916	0.951	0.200	0.852	0.943	0.807	0.788
TT06										
A	8.6	17.0	7.5	11.9	14.8	3.1	11.4	14.7	11.1	10.6
H_O	0.919	0.892	0.750	0.892	0.806	0.460	0.914	0.800	0.804	0.805

H_E	0.851	0.947	0.811	0.901	0.926	0.370	0.892	0.927	0.828	0.814
P	0.125	0.040	0.080	0.194	**0.003***	0.094	0.237	**0.002***	0.017	0.115
F_{IS}	-0.066	0.072	0.090	0.023	0.144	-0.228	-0.010	0.152	0.043	0.026
H_S	0.863	0.960	0.823	0.913	0.939	0.375	0.905	0.941	0.840	0.826
HC05										
A	9.3	18.0	10.8	13.1	16.5	2.8	9.3	20.8	12.6	11.4
H_O	0.840	0.980	0.816	0.960	0.837	0.380	0.820	0.625	0.782	0.805
H_E	0.862	0.953	0.880	0.922	0.941	0.318	0.778	0.967	0.827	0.808
P	0.131	0.288	0.042	0.188	**0.002***	0.118	0.137	**0.000****	**0.000****	0.204
F_{IS}	0.035	-0.018	0.082	-0.031	0.121	-0.186	-0.044	0.363	0.065	0.014
H_S	0.870	0.963	0.889	0.931	0.950	0.321	0.786	0.977	0.836	0.816
HC06										
A	8.6	17.1	10.3	14.2	16.3	3.7	9.4	19.3	12.4	11.4
H_O	0.778	0.889	0.796	0.933	0.864	0.489	0.628	0.558	0.742	0.768
H_E	0.829	0.946	0.862	0.930	0.943	0.412	0.760	0.957	0.830	0.812
P	0.080	0.030	0.050	0.232	0.009	0.061	0.005	**0.000****	**0.000****	**0.001***
F_{IS}	0.073	0.072	0.088	0.008	0.096	-0.175	0.185	0.426	0.118	0.065
H_S	0.838	0.957	0.872	0.941	0.954	0.417	0.769	0.968	0.839	0.821
BH99										
A	8.7	15.2	10.6	14.2	16.8	3.1	8.8	18.9	12.0	11.0
H_O	0.962	0.885	0.808	0.885	0.846	0.269	0.654	0.769	0.760	0.758
H_E	0.812	0.919	0.859	0.925	0.928	0.270	0.675	0.948	0.792	0.770
P	0.035	0.147	0.116	0.125	0.036	0.183	0.209	**0.000****	0.003	0.076
F_{IS}	-0.165	0.057	0.079	0.063	0.107	0.022	0.051	0.207	0.060	0.034
H_S	0.828	0.937	0.876	0.943	0.946	0.275	0.689	0.966	0.807	0.785
BH05										
A	9.2	16.4	9.7	13.2	13.0	3.4	9.4	20.3	11.8	10.6
H_O	0.884	0.905	0.829	0.861	0.767	0.279	0.643	0.575	0.718	0.738
H_E	0.865	0.934	0.842	0.912	0.899	0.338	0.717	0.960	0.808	0.787
P	0.192	0.116	0.157	0.066	**0.003***	0.062	0.047	**0.000****	**0.000****	**0.001***
F_{IS}	-0.010	0.044	0.027	0.068	0.157	0.184	0.115	0.412	0.124	0.073
H_S	0.875	0.946	0.852	0.923	0.909	0.341	0.726	0.972	0.818	0.796

Table 3.3 Pairwise θ_{ST} (above diagonal) and P values (below diagonal) among sixteen *C. ariakensis* samples. **Bold** fonts denote cases with non-significant genetic differentiation after Bonferroni correction (K = 15, $\alpha = 0.05$).

	IR99	IR05	KR04	SR04	KI04	YR99	YR06	BZ05	BZ06	NT05	NT06	TT06	HC05	HC06	BH99	BH05
IR99	-	0.000	0.019	0.025	0.024	0.020	0.023	0.017	0.015	0.031	0.031	0.031	0.015	0.015	0.022	0.024
IR05	**0.037**	-	0.018	0.022	0.025	0.018	0.022	0.021	0.017	0.033	0.035	0.033	0.016	0.017	0.024	0.021
KR04	0.000	0.000	-	0.007	0.018	0.016	0.013	0.007	0.007	0.021	0.022	0.025	0.005	0.008	0.011	0.009
SR04	0.000	0.000	0.002	-	0.015	0.012	0.009	0.004	0.007	0.023	0.018	0.023	0.019	0.021	0.032	0.026
KI04	0.000	0.000	0.000	0.000	-	0.010	0.016	0.002	0.014	0.004	0.008	0.0041	0.012	0.016	0.030	0.028
YR99	0.000	0.000	0.000	0.000	**0.007**	-	0.003	0.004	0.000	0.019	0.023	0.026	0.015	0.017	0.026	0.027
YR06	0.000	0.000	0.000	0.000	0.000	**0.010**	-	0.002	-0.002	0.025	0.025	0.026	0.018	0.019	0.027	0.028
BZ05	0.000	0.000	0.000	**0.054**	**0.159**	**0.448**	**0.144**	-	-0.002	0.011	0.013	0.012	0.004	0.005	0.016	0.018
BZ06	0.000	0.000	0.000	0.000	0.001	**0.415**	**0.261**	**0.775**	-	0.021	0.019	0.024	0.012	0.012	0.019	0.022
NT05	0.000	0.000	0.000	0.000	**0.031**	0.000	0.000	**0.009**	0.000	-	0.000	0.001	0.017	0.021	0.031	0.033
NT06	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	**0.521**	-	0.002	0.023	0.030	0.041	0.038
TT06	0.000	0.000	0.000	0.000	0.003	0.000	0.000	**0.005**	0.000	**0.079**	**0.039**	-	0.018	0.025	0.036	0.033
HC05	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.124**	0.000	0.000	0.000	0.000	-	-0.001	0.003	0.003
HC06	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.006**	0.000	0.000	0.000	0.000	**0.182**	-	0.005	0.008
BH99	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.031**	0.000	-	0.003
BH05	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	**0.080**	-

Table 3.4 Estimates of Weir and Cockerham's (1984) global F_{ST} (θ_{ST}), and regressions of Cavalli-Sfourza and Edwards' (1968) genetic distance measure (D_{CE}) versus geographic distances among 16 natural samples through a jackknife procedure which reiterates the analysis while excluding one of the eight loci each time. S= slope for linear regression, r= Pearson correlation for regressions.

Locus excluded	Regression		θ_{ST}
	S ($\times 10^{-6}$)	r	
CarG110	3.42	0.601	0.017
CarG4-60	4.35	0.656	0.020
Car119-6a	4.34	0.679	0.016
Car11-70	4.62	0.684	0.021
Car130-08	4.24	0.628	0.020
CarG122	4.11	0.649	0.019
CarG1-0b	4.42	0.689	0.017
Car115-a0	3.18	0.537	0.018
Mean	4.09	0.641	0.018
Standard error	0.51	0.051	0.002

Table 3.5 Results of an AMOVA analysis for 16 *C. ariakensis* samples. *Denotes a significant result ($P < 0.05$). [a] Genetic relationships were inferred from pairwise θ_{ST} analysis and NJ tree (see text for details).

Assumption	No. of groups	No. of populations	Source of variation	Percentage of variation	*P*
Temporal stability	10	16	within populations	97.99	0.000 ± 0.000*
			within groups	0.14	0.220 ± 0.004
			among groups	1.87	0.000 ± 0.000*
Genetically closely related among geographically-contiguous samples[a]	8	10	within populations	97.95	0.000 ± 0.000*
			within groups	0.08	0.292 ± 0.005
			among groups	1.96	0.002 ± 0.000*
Eight populations	1	8	within populations	97.97	
			within groups	2.03	0.000 ± 0.000*
			among groups		

Figure 3.1 Map of sample locations and water circulations in the northwest Pacific Ocean (redrawn after Sündermann and Feng 2004). ●= locations of *C. ariakensis* populations (see Table 1 for detailed descriptions of site abbreviations). (a)= water circulation in winter. (b)= water circulation in summer.

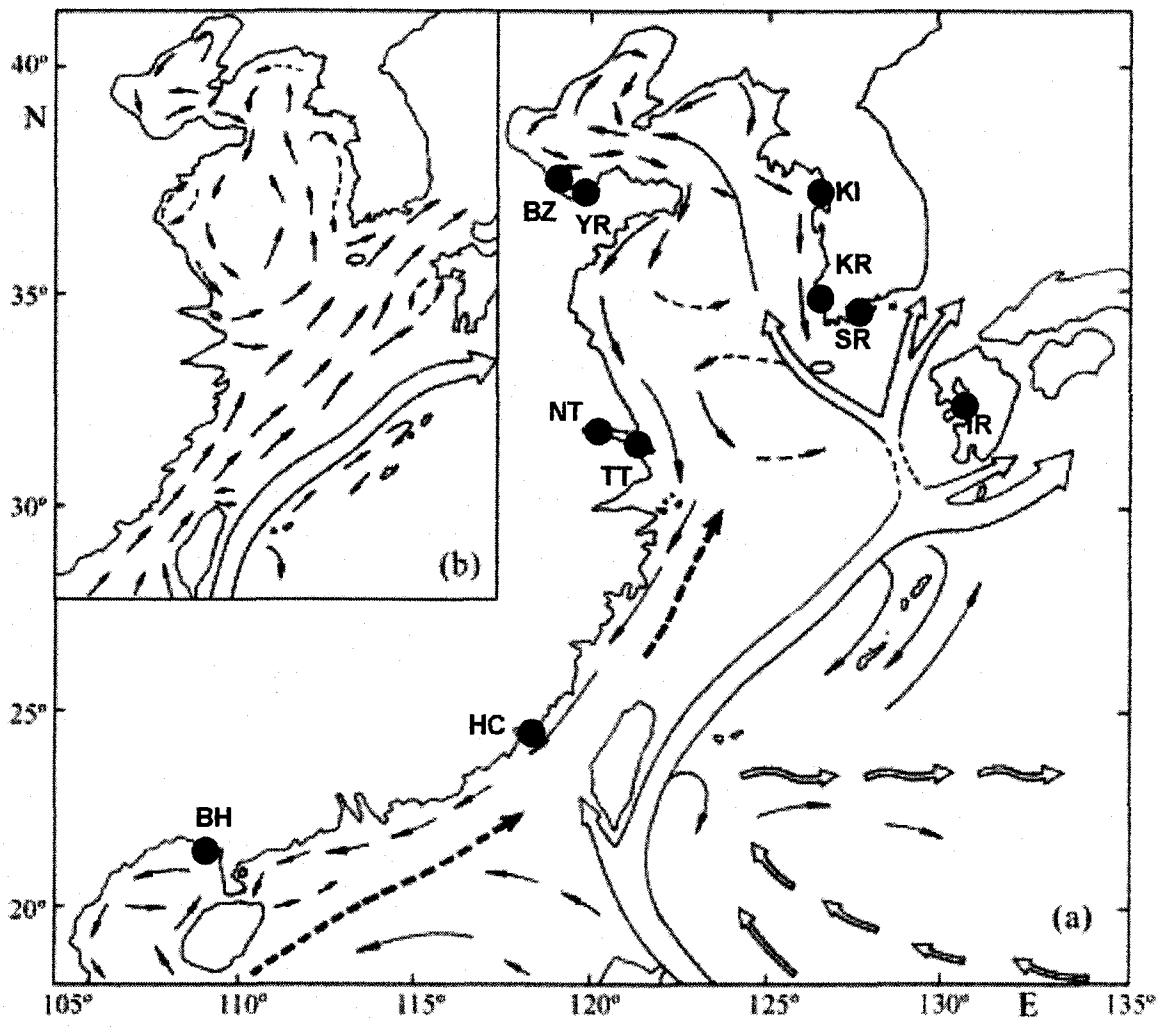

40°
N
35°
30°
25°
20°
105°
110°
115°
120°
125°
130°
E
135°
BZ
YR
KI
KR
SR
NT
TT
IR
HC
BH
(a)
(b)

Figure 3.2 Neighbor-Joining (NJ) phenogram of sixteen *C. ariakensis* samples based on Cavalli-Sforza and Edwards' genetic distances (D_{CE}). Numbers on the internal branches are bootstrap support values higher than 50% after 10,000 permutations over alleles. Sample abbreviations were listed in Table 3.1. Names of eight inferred genetically distinguished populations were labeled.

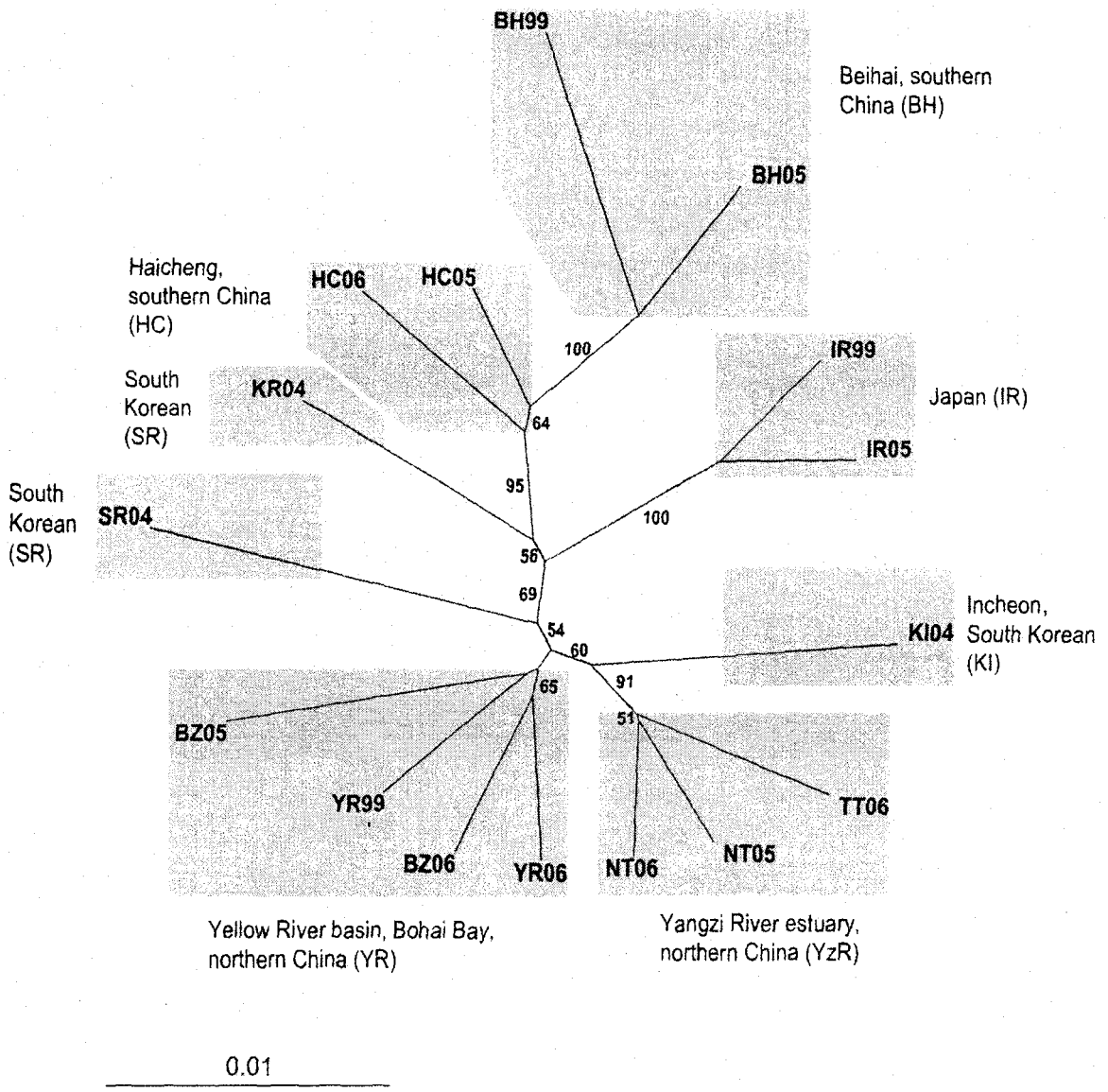
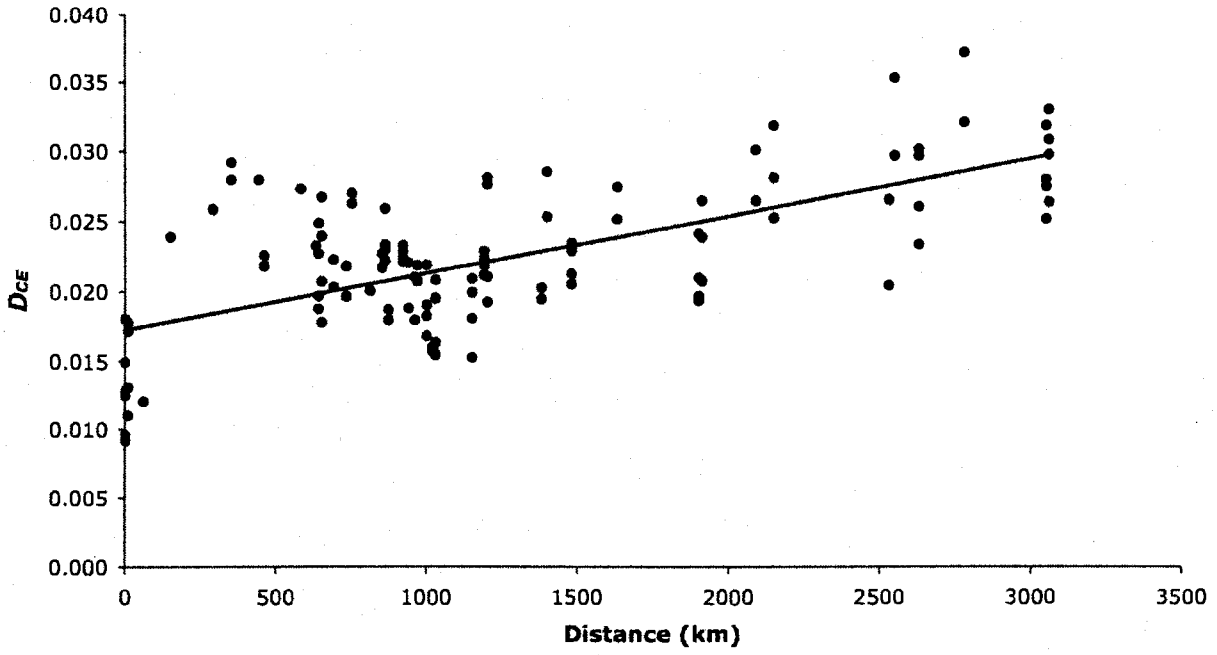

BH99
Beihai, southern China (BH)
BH05
Haicheng, southern China (HC)
HC06
HC05
100
IR99
South Korean (SR)
KR04
64
Japan (IR)
IR05
95
South Korean (SR)
SR04
100
56
69
54
60
Incheon, South Korean (KI)
KI04
91
65
51
BZ05
YR99
TT06
BZ06
YR06
NT06
NT05
Yellow River basin, Bohai Bay, northern China (YR)
Yangzi River estuary, northern China (YzR)
0.01

Figure 3.3 A significant linear regression between D_{CE} based on eight microsatellite loci and geographic distances (km) among the natural samples ($D_{CE} = 0.017 + 4.09\times10^{-6}$ distance, $r^2 = 0.428$, $P < 0.01$).


0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
D_{CE}
0
500
1000
1500
2000
2500
3000
3500
Distance (km)

CHAPTER 4

GENETIC VARIABILITY IN U.S. HATCHERY STOCKS OF *C. ARIAKENSIS*: COMPARISONS WITH NATURAL POPULATIONS IN ASIA AND IMPLICATIONS FOR INTRODUCTION

INTRODUCTION

Introduced stocks of *C. ariakensis* have been hatchery reared along the U.S. west coast in Oregon and Washington since the 1970s, but no natural populations have been established in the region, possibly due to the low water temperatures in the Pacific northwest (Breese and Malouf 1977, Perdue and Erickson 1984, Langdon and Robinson 1996). Recently, proposals to introduce a non-native oyster species to the Chesapeake Bay region of the U.S. east coast have led to research and testing of nonnative oyster species including *C. gigas* and *C. ariakensis*. Since the 1990s, *C. ariakensis* broodstocks have been imported from the US west coast to Rutgers University and to VIMS from the U.S. west coast, northern China, and southern China (NRC 2004, Zhang et al. 2005). Though no reproductively capable *C. ariakensis* have been approved for in-water testing along the U.S. east coast, sterile triploid *C. ariakensis* derived from these hatchery stocks (Allen et al. 2003) have been used for various laboratory and comparative field studies on taste rating (Grabowski et al. 2003), biology and ecology of this species (Calvo et al. 2001, Grabowski et al. 2004, Bishop and Hooper 2005, Hudson et al. 2005, Alexander et al. 2008, Kingsley-Smith and Luckenbach 2008, McGhee et al. 2008, Paynter et al. 2008, Tamburri et al. 2008), non-indigenous pathogens (Burreson et al. 2004, Bishop et al. 2006, Graczyk et al. 2006, Moss et al. 2007, Alavi et al. 2008, Schott et al. 2008), and disease tolerance (Calvo et al. 2001, Grabowski et al. 2004, Moss et al. 2006). Little is known, however, about the genetic makeup of these stocks, the variation among these stocks, and the genetic differentiation from wild populations, which might be associated with differences in biology, behavior, and performance under various environmental conditions.

Studies on other marine bivalves show that allelic reduction is quite common in hatchery lines, and it is often associated with deviations in allelic or genotypic frequencies compared with the natural source populations (Hedgecock and Sly 1990, Gaffney et al. 1996, Yu and Guo 2005, Carlsson et al. 2006). This drift is thought to arise due to bottleneck effects from the small effective number of parents typically contributing to spawns in hatcheries and nonrandom selection that often occurs during breeding and larval recruitment in the hatchery (Hedgecock and Sly 1990, Gaffney et al. 1996, Boudry et al. 2002, Yu and Guo 2005). Consequently, inbreeding and a concomitant decrease in various performance measures can occur in hatchery stocks (Hedgecock et al. 1995, Bierne et al. 1998, Ernande et al. 2003). Currently, levels of genetic variation among *C. ariakensis* hatchery stocks have not been thoroughly assessed, which could provide useful information for appropriate broodstock management. An initial study (Zhang et al. 2005) showed reduced genetic diversity in five VIMS hatchery stocks compared to wild populations. Unfortunately, this study did not provide detailed information on the genetic structuring within and among *C. ariakensis* hatchery and wild populations.

The social, economical, and ecological risks of introducing the non-native *C. ariakensis* to the Chesapeake Bay ecosystem is a concern (Ruesink et al. 2005, Simberloff 2005, Bishop et al. 2006), and no introduction (or even field testing) of fertile non-native oysters has been approved to date (though currently under consideration), and an environmental impact statement (EIS) is being drafted (http://www.nao.usace.army.mil/oysterEIS/homepage.asp). However, accidental release or intentional, illegal introduction of reproductively capable *C. ariakensis* is still possible

through various means (Simberloff 2005). The highly polymorphic microsatelllite markers used here have proven adept at differentiating among natural populations of *C. ariakensis* (Chapter 3). The present study further tested the utility of these markers to differentiate among hatchery stocks, to assess the relationship between these stocks and their natural source populations, and to assign *C. ariakensis* of unknown origin back to their source domestic stocks or wild populations in order to monitor *C. ariakensis* that might be introduced into Chesapeake Bay.

MATERIALS AND METHODS

Samples

A total of 245 individuals from five hatchery samples used in this study and confirmed to be *C. ariakensis* by Zhang et al. (2005) are listed in Table 4.1. They were sampled in 2002, and four of them (NCA, WCA, SCA99 and SCA00) were from stocks maintained by the ABC at VIMS; the other one (TUI) was from Taylor Shellfish Farms Inc., located on the U.S. west coast. TUI and WCA were derived from the 'Oregon Strain', which was inadvertently introduced into the U.S. in the 1970s (Breese and Malouf 1977). In comparison to TUI, which has been hatchery-reared for several generations, WCA was directly collected in 1999 from a *C. ariakensis* stock being held in the waters along the coast of Washington State that does not spawn naturally. NCA was derived directly from wild broodstock brought to VIMS from the Yellow River Basin, Shandong Province, northern China, and spawned in 1999 at the ABC; similarly, SCA99 and SCA00 were derived from a wild broodstock collected in Beihai, Guangxi Province, southern China. SCA99 was spawned in 1999 and another group from the same broodstock was spawned in 2000. For the purpose of this study, TUI and WCA were considered to be long-established stocks due to the long separation time (around 30 years) from their natural source populations in Japan (Breese and Malouf 1977). NCA, SCA99, and SCA00 were considered recent stocks, since their parental broodstocks were transported into the U.S. quite recently and each stock had undergone only one generation of hatchery propagation at the time of sampling.

Samples from eight genetically heterogeneous natural populations (as inferred by the population genetic structure analysis in Chapter 3) were also included in the analyses (Table 4.1). They were used for comparisons of genetic variability and diversity between hatchery stocks and wild populations and as reference populations for assignment tests. Two additional wild samples, which were not included in the wild population genetic structure analysis, were genotyped and used as test samples for the assignment analyses conducted here. These test samples were composed of 50 individuals collected from the Itoki River in Japan (IR05a) and 36 individuals from the Yellow River in China (YR05a) in 2005. These additional samples were confirmed to be *C. ariakensis* using the RFLP/PCR molecular identification key of Cordes et al. (2008).

DNA extraction, microsatellite amplification and data analysis

Genomic DNA extraction, PCR amplifications of eight microsatellite markers (*CarG110, CarG4-60, Car119-6a, CarG122, Car130-08, CarG1-0b, Car11-70, Car115-a0*), and separation of PCR products on a PRISM® ABI 3130 genetic analyzer followed the protocols described in Chapter 1. Allele sizes in base pairs (bp) were called based on comparisons to the panels generated by scoring 605 wild individuals of *C. ariakensis* from Japan, South Korea, and China (Chapter 3), using the software package GENEMARKER.

Based on multi-locus genotype data the inter- and intra-population variation as measured by various parameters (A, H_O, H_E, F_{IS}, F_{ST}) were analyzed using the same procedures as described in Chapter 3. In order to compare the allelic richness (A) of hatchery stocks and natural populations, A was adjusted to a sample size of 14 (the

smallest sample size in natural populations) by a rarefaction method (Petit et al. 1998). Significant levels of per sample F_{IS} and F_{ST} values were assessed by bootstrapping with 10,000 iterations at each locus and over all loci in GENETIX. The genetic relationship between hatchery and natural populations was visualized through construction of a Neighbor-Joining (NJ) tree using the software package PHYLIP 3.67 (Felsenstein 1989) based on Cavalli-Sforza & Edwards' (1967) genetic distances.

In the assignment analyses, eight wild populations (IR, KR, SR, KI, YR, YzR, HC and BH; listed in Table 4.1) were used as the reference genotypic database for wild sources; the five hatchery samples served unaltered as the reference for hatchery sources since significant genetic differentiation was observed among all pairwise comparisons (see RESULTS for details). To assess the ability of the markers to correctly assign individuals back to their source populations, we first conducted self-assignment tests (where the population of origin for a sampled individual is considered the source) for the eight natural populations and five hatchery populations by a 'leave-one-out' procedure that excludes the individual to be assigned from the population during computation (Piry et al. 2004). We then assigned 136 putatively 'unknown' individuals to either wild or hatchery sources. To do this, ten individuals from each of the five hatchery populations (named TUIa, WCAa, NCAa, SCA99a and SCA00a) were randomly drawn from the hatchery populations and removed from the 'reference' database. These 50 hatchery individuals and 86 test individuals from two additional natural samples (IR05a and YR05a) were treated as putatively 'unknown' samples and assigned to the natural or hatchery sources based on their multi-locus genotype profiles. A Bayesian method (Rannala and Mountain 1997) implemented in the program GENECLASS 2 (Piry et al.

2004) was used to compute the probability of an individual being classified to each 'reference' population. The 'reference' population with the highest assignment probability was chosen as the assigned source for this individual, and compared with the known information from sampling.

RESULTS

Genetic diversity within stocks and comparisons with natural populations

A total of 152 alleles were amplified at eight microsatellite loci in 246 individuals from five hatchery stocks. Only 52.6% of the alleles that amplified in wild samples (Chapter 3) were observed in the hatchery stocks. In addition, three alleles (from loci *CarG4-60*, *Car130-08* and *Car115-a0*) were not observed in the wild samples, while they were amplified in hatchery stocks. One allele was only found in the Japanese derived hatchery stocks TUI and WCA; the other two were found only in stocks derived from the southern Chinese populations SCA99 and SCA00.

Adjusted allelic richness (A) across all loci ranged from an average of 3.3 (TUI) to 9.1 (SCA99) alleles per locus per population (Table 4.2). There was a significant difference in A values among these five hatchery stocks, and the long-established stocks (TUI and WCA) had much lower values for A compared to the recently derived stocks ($P = 0.000$, two tailed Mann-Whitney test). The overall mean A for these five hatchery populations was 6.8 alleles per locus/per population.

Multi-locus analysis of the hatchery stocks indicated that only the TUI stock showed significant negative F_{IS} (-0.226). However, individual HWE tests for each stock at every locus (Table 4.2) revealed that 16 of 40 tests were significantly out of HWE after Bonferroni correction ($K = 8$, $\alpha = 0.05$), and 11 (69%) of them were due to an excess of heterozygotes. TUI had the highest number (5 out of 8) of deviations from HWE, all due to $H_O > H_E$.

When comparing genetic diversity in terms of allelic richness (A) and the observed (H_O) and expected (H_E) heterozygosities between the hatchery stocks and wild populations (data from Chapter 3), significant reductions of A and H_E (P = 0.001, 0.012, respectively; two tailed Mann-Whitney test), but not H_O (P = 0.615) were found in the hatchery populations. We further analyzed the variation of these three parameters in 5 hatchery and 16 natural samples and found that fluctuations of H_O and H_E across all samples were correlated with A ($R^2 = 0.431$, $P = 0.001$ and $R^2 = 0.862$, $P = 0.000$, respectively) (Figure 4.1). The values for all three parameters (A, H_O, H_E) were decreased significantly (all P values < 0.05) in the two mature stocks TUI and WCA; while the three recent stocks (NCA, SCA99 and SCA00) had reduced values of allelic richness (A, P = 0.009) but not heterozygosity (H_O and H_E, P = 0.615 and 0.105, respectively). In addition, the magnitude of reduction in the hatchery samples was higher in A compared with H_O and H_E. In general, average A and H_E values for all hatchery populations were decreased by 40.0% and 13.7%, respectively, compared with the mean values over all wild populations. These hatchery stocks, however, did not contribute equally to the reductions. The average A, H_O, and H_E values of the two long-established stocks (TUI and WCA) were decreased by 60.4%, 10.9%, and 26.4%, respectively, compared with their wild source population (IR). In comparison, the average reduction in A in the three recent hatchery stocks was much lower, with 17.7% for NCA compared with its source population YR, and 29.5% for SCA99 and SCA00 compared to the wild BH population, while H_E and H_O values did not decrease significantly.

Genetic differentiation among hatchery stocks and wild populations

Population pairwise θ_{ST} values among hatchery samples (Table 4.3) ranged from 0.054 (SCA99 vs. SCA00) to 0.238 (TUI vs. SCA00) (global $\theta_{ST} = 0.1352$), and were clearly larger than those previously observed among natural populations (Chapter 3). All pairwise θ_{ST} values among hatchery populations were highly significant ($P < 0.001$), and those comparisons that involved the long-established stocks (TUI and WCA) all had θ_{ST} values > 0.100, and were larger than those observed among recent stocks (< 0.065). Genetic relationships among these hatchery populations and the eight wild populations from Chapter 3 were visualized by an unrooted NJ tree (Figure 4.2). Hatchery populations derived from the same natural sources, such as TUI and WCA (Japan), and SCA99 and SCA00 (southern China), were grouped together with high bootstrapping support (> 90%). TUI and WCA were genetically closer to their wild source population (IR) with a relatively high bootstrap support value (75%). SCA99 and SCA00 were also grouped with their wild source population (BH), but with low bootstrap support (< 50%). Similarly, the NCA stock (derived from broodstock collected from the Yellow River region in China) was genetically closest to the northern Chinese (including YR and YzR populations) and Korean populations (KR04, SR04 and KI04) without strong bootstrap support values (< 50%). Long branch lengths indicated significant drift of the hatchery stocks away from their parental sources.

Assignment tests

The results of all assignment tests are shown in Table 4.4, including two groups of self-assignments and assignments of some additional and/or random samples to natural

and/or hatchery origins. The self-assignment tests correctly assigned 84.0% - 97.9% of hatchery individuals back to their hatchery stocks, while variable percentages (20.0% - 72.7%) of wild individuals were assigned back to their specific natural populations. The three Korean populations, which had small sample sizes (20 – 33), had quite low incidences of correct assignments (20.0% - 30.0%). Nevertheless, a comprehensive pairwise assignment table (Table 4.4) indicated that large proportions of wild individuals were assigned to geographically proximal populations. For example, 15.0% - 40.0% of individual oysters from the three Korean populations (KR, SR and KI) were assigned to the Yellow River basin population (YR), and an additional 20.0% and 35.0% of individuals from the SR and KI populations, respectively, were assigned to the Yangzi River population (YzR). Similarly, a substantial proportion of oysters (14.7%) from Haicheng (HC) were classified to the other southern Chinese population (BH), and *vice versa.*

Tests using both hatchery stocks and natural populations as references for seven putatively 'unknown' samples resulted in overall 96.3% of individuals being correctly classified generally as wild or hatchery oysters. The exceptions were that one individual from IR05a was assigned to a hatchery stock (SCA99), and likewise, 3 from NCAa and one from SCA99a were assigned to wild populations instead of their source hatchery stocks. And similar with self-assignment tests, results from these 136 putatively 'unknown' samples indicated that a high percentage (70.0% - 90.0%) of hatchery oysters were precisely assigned to their specific hatchery stock of origins and a moderate percentage (63.9% - 68.0%) of the wild individuals (YR05a and IR05a) were assigned to their specific natural geographic origins.

DISCUSSION

Genetic makeup of the hatchery stocks

Results of this study led to three main interrelated observations regarding the genetic variability within and among hatchery stocks of *C. ariakensis*: 1) there was significant reduction of genetic variability in terms of allelic richness and observed and expected heterozygosities in the hatchery stocks, 2) the greatest levels of genetic differentiation were found among hatchery stocks and between the hatchery stocks and wild populations, and 3) deviations from HWE were largely due to excess observed heterozygosity compared to expected, especially in the highly inbred population TUI.

The three measures of genetic diversity commonly used as indicators of genetic bottleneck effects in natural and cultivated marine organisms are the number of polymorphic loci, allelic diversity, and heterozygosity, though the first two were suggested as more useful measures of bottlenecks than heterozygosity in allozyme studies (Leberg 1992). Although the number of polymorphic loci was commonly used in allozyme studies as a measure of bottlenecking, the overall high levels of polymorphism found in microsatellite loci make this measure less meaningful for this class of markers. In a hatchery setting, reductions in allelic diversity are believed to be caused by small effective population sizes (N_e) resulting from a small number of animals being used as broodstock and non-equal gametic viability and/or spawning condition of the potential parents (Hedgecock and Sly 1990, Hedgecock 1994). Reductions in heterozygosity, however, do not always respond immediately to these conditions, depending on the number and severity of the bottlenecks, the original N_e, and the evenness of allelic

frequencies after the bottleneck (Hedgecock and Sly 1990, Leberg 1992). Reductions in both types of diversity are commonly observed in cultivated fishes (Allendorf and Phelps 1980, Ryman and Ståhl 1980), while hatchery propagated marine bivalves have been typically observed to lose only allelic diversity (Hedgecock and Sly 1990, Yu and Guo 2005, Carlsson et al. 2006). In the present case, reductions in genetic diversity were greatest in the long-established hatchery stocks (TUI and WCA) that had been isolated from their source populations for the longest times. This is not surprising since these stocks have been domesticated for over 30 years and have undergone multiple generations of hatchery spawning. In contrast, the more recently domesticated stocks showed reductions in allelic diversity but not in heterozygosity, consistent with previous reports that allelic diversity is more sensitive to bottleneck events and that a time lag exists before measurable decreases in heterozygosity are observed (Nei et al. 1975, Hedgecock and Sly 1990, Leberg 1992, Petit et al. 1998). In the current study, variations in H_O and H_E were correlated with fluctuations of A (Figure 4.1). A relation curve based on studies of 78 animal species (DeWoody and Avise 2000) showed that heterozygosity changed little once the effective number of alleles reached 10. However, if inbreeding is continued over several generations so that allelic diversity continues to decrease, heterozygosity will eventually decrease correspondingly.

Unlike in natural populations where deviations from HWE were primarily due to heterozygote deficiencies (Chapter 3), overall a high proportion (68.8%) of the significant F_{IS} values in these hatchery stocks were due to heterozygote excess. In fact, 83.3% of the deviations observed in the TUI stock were due to an excess of observed heterozygotes compared to what was expected based on HWE, and a closer look at the

genotype frequencies found that a few genotypes were highly prevalent in this hatchery population (data not show), which is not an uncommon observation in hatchery propagated inbred oyster families (Saavedra and Guerra 1996, McGoldrick and Hedgecock 1997, Bierne et al. 1998, Marsic-Lucic and David 2003). Uneven parentage contribution and selection against deleterious homozygotes due to identical-by-descent markers might contribute to the distorted genotypic frequencies in those hatchery populations, particularly in the short term (McGoldrick and Hedgecock 1997, Bierne et al. 1998, Launey and Hedgecock 2001). Furthermore, the distorted genotypic frequencies observed here indicate non-equal contributions to the progeny genotypes from the parents, and there were probably only a few successful breeders (N_e) at each spawning. This "founder effect" can cause shifts in allele frequencies at individual loci by random chance (i.e. drift). Loss of rare alleles and a high prevalence of a few common alleles can result in very different allelic distributions. Relatively rapid divergence from source populations and from the other stocks derived from the same source is not uncommon with hatchery stocks and this phenomenon is often observed in highly fecund species like oysters (Hedgecock 1994, Saavedra and Guerra 1996, Vercaemer et al. 2006). This likely explains the large genetic distance among hatchery stocks and between the hatchery stocks and wild populations in this study.

Implications for *C. ariakensis* introduction

Based on the results described above, three possible effects of genetic bottlenecks on these hatchery stocks are discussed below. First, loss of genetic diversity over the long term may cause inbreeding depression. Though the cause of inbreeding depression and its

converse heterosis (hybrid vigor) remains poorly understood in marine bivalves, positive correlations between multi-locus heterozygosity and fitness-related traits were commonly observed in highly inbred families and populations of bivalves (Gaffney et al. 1990, Hedgecock et al. 1995, McGoldrick and Hedgecock 1997, Bierne et al. 1998, David et al. 1998, Naciri-Graven et al. 2000, Launey and Hedgecock 2001, Hedgecock et al. 2007). High genetic load of deleterious recessive mutations in some bivalve species such as *C. gigas* has also been suggested and provides some basis for inbreeding depression (Launey and Hedgecock 2001). Reduction in heterozygosity, though undetectable in the first several generations of inbreeding (Hedgecock and Sly 1990), could become significant after continued use of a small numbers of individuals for spawning, which are drawn from stocks that are already showing reduced allelic diversity. Stock erosion may occur due to detrimental changes in fitness parameters such as growth and early recruitment, which has been shown to be correlated with reduced heterozygosity (Hedgecock et al. 1995, David 1998, Bierne et al. 1998). Therefore, successive introduction of broodstock from wild populations is recommended in order to enrich and maintain healthy genetic pools in existing hatchery stocks, especially for those stocks isolated from their source for the longest times (TUI and WCA).

Second, given the high genetic differentiation among the hatchery stocks derived from different source populations, there might be phenotypic divergence among stocks. As the conceptual basis of selective breeding, phenotypic differences are often intentionally selected to produce broodstock with specific traits: however, this process can be unintentionally associated with a decrease in genetic diversity, and particularly in allelic diversity (Yu and Guo 2005, Carlsson et al. 2006). Likewise, involuntary artificial

selection pressure resulting from time and energy constrains under laboratory or hatchery conditions, has been shown to cause change and reduce variance in growth and development of domesticated oysters. For example, Taris et al. (2006) reported that by culling *C. gigas* larvae in early stages, the stocks resulted in individuals that were an average of 10% larger and had a 12% shorter time to settlement than control groups, and the variance in the parameter means was reduced by 30-40% and 55%, respectively. It is still unclear whether or not the genetic shifts in the *C. ariakensis* hatchery stocks compared to their wild source populations is linked to any changes in growth performance, early recruitment or development, as results of side-by-side performance trials comparing within and among hatchery stocks and wild populations have not been reported, and there is a lack of information on the basic biology of *C. ariakensis* in its native region. Differences in larval settlement and swimming, however, were observed among different stains of *C. ariakensis* (Luckenbach 2004, Tamburri et al. 2008). Numerous comparative studies between *C. ariakensis* and *C. virginica* on biological traits such as growth and early development have been conducted using a single strain of *C. ariakensis* for each study. The most commonly used stocks were derived from the so-called "Oregon Strain" from the west coast of the U.S. (Langdon and Robinson 1996, Calvo et al. 2001, Hudson et al. 2005, Paynter et al. 2008, Kingsley-Smith and Luckenbach 2008), which corresponds to the TUI and WCA stocks used in this study. These stocks showed the greatest genetic bottleneck effects and genetic drift from their natural source populations and the other hatchery stocks. Therefore, interpretations of comparisons among studies conducted in the U.S. over the last decade might be

compromised due to the use of different stocks which might differ not only genetically, but also in performance and fitness parameters.

Moreover, the specific strain used and its genetic character has to be taken into account when we evaluate the performance of *C. ariakensis* transplants, given the various environmental conditions in locations from which stocks were derived, including different disease challenges. Moss et al. (2007 and 2008) did a disease survey of *C. ariakensis* and other related oysters in Asia, and found that a herpes-like virus (OsHV-1) and *Perkinsus olseni* was prevalent throughout the sampling area used in the present study, from Japan to South Korea and northern and southern China, while a newly described *Perkinsus* species (*P. beihaiensis*) was also found only in southern China. Whether these disease challenges could result in different disease tolerance or resistance of oysters from different locations is still under investigation. There are, however, many reports of varying disease resistance in different geographic strains of bivalves. For example, Bushek and Allen (1996) found that different broodstocks of *C. virginica* exhibited distinct levels of *P. marinus* resistance; Brown et al. (2006) reported higher Dermo disease resistance in a North Carolina strain of *C. virginica* than in Chesapeake Bay conspecifics; and natural resistance to QPX was discovered among different geographic strains of the hard clam *Mercenaria mercenaria* (Ragone Calvo et al. 2007). Therefore, the stock or strain of *C. ariakensis* used in field or laboratory studies should be considered when evaluating the performance of this species in the U.S.

Genetic tracking

Assignment tests based on an individual's genotypic profile have been widely used to detect immigrants, identify hybrids, trace the origins of animals and plants, and detect dispersal patterns (Paetkau et al. 1997, Maudet et al. 2002, Castric and Bernatchez 2003, Manel et al. 2005, Vercaemer et al. 2006). An introduction of fertile *C. ariakensis* oysters to Chesapeake Bay has not been approved but is currently under consideration, and an environmental impact statement (EIS) evaluating various alternative strategies for oyster restoration in the region is being drafted. Accidental release or intentional, illegal introduction of reproductively capable *C. ariakensis*, however, has been a concern (Simberloff 2005), and the ability to trace such introductions of *C. ariakensis* could be important for controlling these non-native oysters.

Multiple tests conducted in this study first indicated that a high percentage (overall 96.3%) of putatively unknown oysters could be correctly classified generally as wild or hatchery oysters. Secondly, the domesticated oysters could be further tracked back to their specific hatchery origins with high accuracy (> 80%). The ability to accurately assign wild oysters back to their specific source population, however, was low to moderate (20.0% - 72.7%), probably due to reduced genetic differentiation among these natural populations compared to the relatively high differentiation among the hatchery stocks. It has been reported that a 100% correct assignment could be achieved by scoring 10 microsatellite loci on 30 – 50 individuals from each of 10 populations with F_{ST} values around 0.1 (Cornuet et al. 1999), which is in the range of what was observed among the hatchery populations in the present study (F_{ST} = 0.132). However, for those populations with F_{ST} values approximately 10 fold lower, (i.e. around 0.01, comparable to

the F_{ST} value of 0.020 that we observed among the eight natural populations), the percentage of correct assignments seldom reach 50%, even using 20 loci and 90 individuals per population (Cornuet et al. 1999). Additionally, low sample sizes of some wild populations in this research (i.e. KR, SR and KI) probably also affected the probability of correct assignments (Cornuet et al. 1999, Manel et al. 2004).

A closer look at the pairwise assignments found that a substantial proportion of wild individuals were assigned to their geographically proximal populations (see RESULTS). This is not surprising given a genetic pattern of isolation by distance observed in the wild populations (Chapter 3). Since the probability of correct assignments largely depends on population genetic differentiations (Cornuet et al. 1999, Manel et al. 2004), relatively smaller genetic differentiation and higher gene flow among populations with shorter geographic distances, compared with populations farther apart, probably resulted in a relatively high number of individuals being misassigned to a population geographically proximate to their sampled populations.

Based on the testing results discussed above, an assignment strategy is proposed: 1. an unknown sample could be first classified generally as hatchery or wild oysters with an average accuracy of 95%; 2. hatchery oysters could be further assigned to the specific hatchery stocks with a high probability (average 92%); 3. if the unknown sample was assigned as originating from a wild Asian population, the geographically proximate populations are also likely to be the origin population, due to the low genetic differentiation among them; therefore, they could be traced back to a wild geographic group which is either a Japanese group, a northern Chinese group (including KI, SR, IN, YR and YzR populations), or a southern Chinese group (including HC and BH). In

reality, it is more likely that hatchery *C. ariakensis* would be released into the environment, since only hatchery stocks of diploid and triploid *C. ariakensis* have been used in the U.S. for research, at least to date (Grabowski et al. 2003, Hudson et al. 2005, Calvo et al. 2001, Grabowski et al. 2004, Burreson et al. 2004, Graczyk et al. 2006, Calvo et al. 2001, Moss et al. 2006).

Table 4.1 Sample information for five *C. ariakensis* hatchery stocks and eight natural populations from Asia used for comparisons of genetic variability in Chapter 4. Based on results of the population genetic study presented in Chapter 3, 16 wild samples[2] were combined into 8 populations[1] (also see Figure 3.2). Two test samples comprised of additional individuals from two wild samples (IR05a, YR05a) used for validating the assignment tests.

Sample code[1]	Sample code[2]	Collecting date	Sample type	Sample size	Source
TUI		3/02	hatchery	48	Taylor Shellfish Farms, Inc., derived from 'Oregon Strain' in west coast of U.S.
WCA		12/02, 1/03	hatchery	50	ABC, derived from 'Oregon Strain' in west coast of U.S.
NCA		7/02, 12/02	hatchery	50	ABC, F_1 generation of Yellow River stock (YR) in northern China, spawned in 1999.
SCA99		7/02, 12/02	hatchery	50	ABC, F_1 generation of Beihai stock (BH) in southern China, spawned in 1999.
SCA00		7/02, 12/02	hatchery	47	ABC, F_1 generation of Beihai stock (BH) in southern China, spawned in 2000.
IR	IR99, IR05	5/99, 10/05	wild	49, 50	Ariake Sea, Saga Prefecture, Kyushu, Japan
KR	KR04	04	wild	33	Kahwa River, South Korea
SR	SR04	04	wild	20	Sumjun river, South Korea
KI	KI04	04	wild	20	Kanghwa Island, Incheon, South Korea
YR	YR99, YR06, BZ05, BZ06	6/99, 4/06, 8/05, 4/06	wild	32, 50, 15, 35	Yellow River basin, Shandong Province, China
YzR	NT05, NT06, TT06	5/05, 4/06, 8/06	wild	50, 50, 37	Yangzi River estuary, China
HC	HC05, HC06	4/05, 4/06	wild	50, 45	Haicheng, Fujian Province, China
BH	BH99, BH05	5/99, 3/05	wild	26, 43	Dafeng River, Beihai, Guangxi Zhuang, China
IR05a		10/05	wild	50	Ariake Sea, Saga Prefecture, Kyushu, Japan.
YR05a		5/05	wild	36	Yellow River, Weifang, Shandong Province, China.

Table 4.2 Microsatellite diversity in five hatchery populations of *C. ariakensis*. *A* is allelic richness adjusted to a sample size of N = 14 by the rarefaction method (Petit 1998); H_O and H_E are observed and expected heterozygosity, respectively; F_{IS} is Weir & Cockerham's (1984) inbreeding coefficiency estimate; *P* is the probability that F_{IS} is null. Numbers in **boldface** are significantly different from zero after Bonferroni correction (K = 8, $\alpha = 0.05$).

	CarG110	*CarG4-60*	*Car119-6a*	*Car11-70*	*Car130-08*	*CarG122*	*CarG1-0b*	*Car115-a0*	**multi-locus**
TUI									
A	2.9	5.2	3.3	3.8	3.2	2.0	2.0	4.1	3.3
H_O	0.750	0.771	1.000	0.854	0.302	0.521	0.229	0.787	0.652
H_E	0.508	0.697	0.615	0.658	0.462	0.385	0.203	0.690	0.527
P	**0.000**	0.089	**0.000**	**0.001**	**0.002**	**0.000**	**0.000**	0.052	**0.000**
F_{IS}	-0.469	-0.096	-0.620	-0.289	0.356	-0.343	-0.119	-0.131	-0.226
WCA									
A	3.5	8.7	5.4	6.5	5.1	2.3	2.0	7.1	5.1
H_O	0.840	0.900	0.841	0.680	0.378	0.300	0.435	0.860	0.654
H_E	0.655	0.848	0.721	0.809	0.690	0.285	0.386	0.820	0.652
P	**0.001**	0.121	0.017	**0.005**	**0.000**	0.240	0.112	0.207	0.405
F_{IS}	-0.273	-0.052	-0.155	0.169	0.462	-0.041	-0.117	-0.039	0.007
NCA									
A	5.5	13.5	7.1	9.8	11.8	2.0	6.6	14.1	8.8
H_O	0.640	0.900	0.840	0.960	0.860	0.340	0.820	0.940	0.788
H_E	0.621	0.911	0.810	0.848	0.895	0.282	0.759	0.925	0.756
P	0.327	0.192	0.271	**0.003**	0.094	**0.000**	0.112	0.341	0.055
F_{IS}	-0.021	0.022	-0.027	-0.122	0.049	-0.195	-0.071	-0.007	-0.031
SCA99									
A	7.8	11.8	8.8	9.5	11.0	3.2	6.7	14.0	9.1
H_O	0.860	1.000	0.816	0.920	0.837	0.440	0.745	0.717	0.792
H_E	0.836	0.900	0.861	0.864	0.883	0.426	0.722	0.911	0.800
P	0.316	**0.000**	0.093	0.087	0.063	0.350	0.313	**0.000**	0.150
F_{IS}	-0.018	-0.102	0.062	-0.055	0.063	-0.023	-0.021	0.223	0.021
SCA00									
A	7.5	10.7	6.9	8.7	8.1	3.4	6.5	9.8	7.8
H_O	0.766	0.957	0.936	0.915	0.447	0.298	0.783	0.848	0.744
H_E	0.730	0.890	0.809	0.850	0.807	0.268	0.687	0.870	0.739
P	0.227	0.041	**0.003**	0.079	**0.000**	**0.000**	0.025	0.175	0.436
F_{IS}	-0.039	-0.065	-0.147	-0.065	0.455	-0.100	-0.129	0.037	0.004
Overall									
A	5.4	10.0	6.3	7.6	7.9	2.6	4.7	9.8	6.8
H_O	0.771	0.906	0.887	0.866	0.565	0.380	0.603	0.831	0.726
H_E	0.670	0.849	0.763	0.806	0.747	0.329	0.551	0.843	0.695

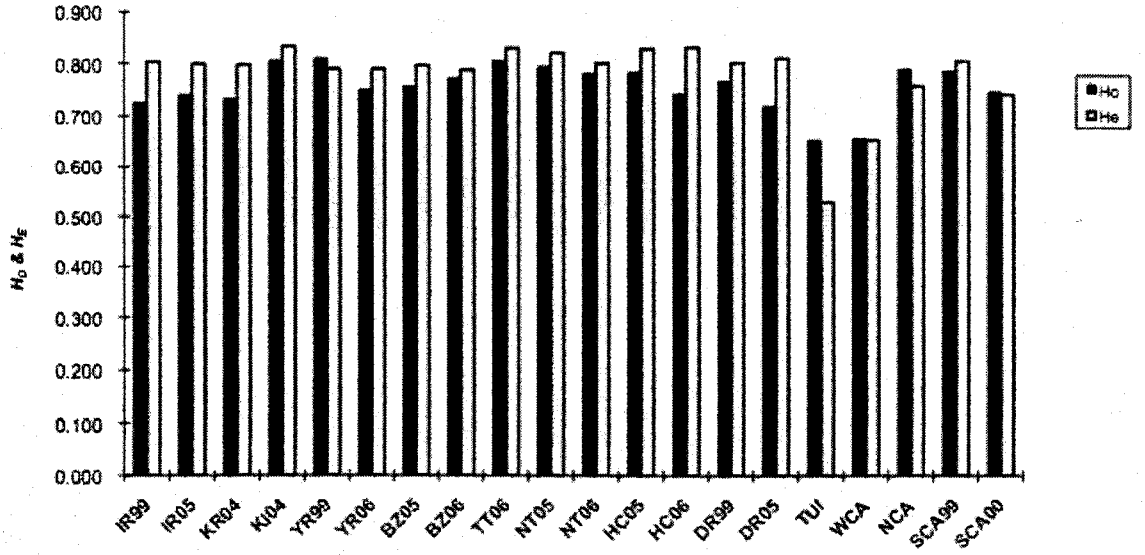
Table 4.3 Pairwise θ_{ST} (above diagonal) and *P* values (below diagonal) among five hatchery populations of *C. ariakensis*. All comparisons were highly significant after Bonferroni correction (K = 5, $\alpha = 0.01$).

	NCA	WCA	SCA99	SCA00
TUI	0.228	0.123	0.194	0.238
NCA	-	0.149	0.062	0.055
WCA	<0.001	-	0.101	0.150
SCA99	<0.001	<0.001	-	0.054
SCA00	<0.001	<0.001	<0.001	-

Table 4.4 Percentages (%) of individuals assigned to various hatchery and wild reference samples using Rannala & Mountain's (1997) Bayesian method: a) % of correct self- assignments for eight wild populations, b) % of correct self- assignments for five hatchery samples and c) % of assignments for seven putatively 'unknown' samples to each of the reference populations from a) and b) above. See Table 4.1 for sample abbreviations. Numbers in bold are proportions of oysters correctly classified to its sample of origin. *Self assignment tests (Piry et al. 2004, also see text) were performed for a) and b) (also see above), and consequently no tests were conducted to assign the eight wild populations to the five hatchery populations and *vice versa*, as indicated by the empty grey cells.

	Sample size	IR	KR	SR	KI	YR	YzR	HC	BH	TUI	WCA	NCA	SCA99	SCA00
a) *eight wild populations*[*]														
IR	99	**72 (72.7)**	5 (5.1)	2 (2.0)	3 (3.0)	4 (4.0)	6 (6.1)	4 (4.0)	3 (3.0)					
KR	33	2 (6.1)	**8 (24.2)**	3 (9.1)	1 (3.0)	8 (24.2)	3 (9.1)	5 (15.2)	3 (9.1)					
SR	20	2 (10.0)	2 (10.0)	**6 (30.0)**	1 (5.0)	3 (15.0)	4 (20.0)	2 (10.0)	0 (0.0)					
KI	20	0 (0.0)	1 (5.0)	0 (0.0)	**4 (20.0)**	8 (40.0)	7 (35.0)	0 (0.0)	0 (0.0)					
YR	132	4 (3.0)	7 (5.3)	2 (1.5)	12 (9.1)	**83 (62.9)**	15 (11.4)	9 (6.8)	0 (0.0)					
YzR	137	4 (2.9)	4 (2.9)	3 (2.2)	7 (5.1)	15 (10.9)	**87 (63.5)**	14 (10.2)	3 (2.2)					
HC	95	7 (7.4)	9 (9.5)	0 (0.0)	3 (3.2)	6 (6.3)	9 (9.5)	**47 (49.5)**	14 (14.7)					
BH	69	1 (1.4)	10 (14.5)	1 (1.4)	0 (0.0)	1 (1.4)	1 (1.4)	20 (29.0)	**35 (50.7)**					
b) *five hatchery populations*[*]														
TUI	48									**47 (97.9)**	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)
WCA	50									3 (6.0)	**46 (92.0)**	0 (0.0)	1 (2.0)	0 (0.0)
NCA	50									0 (0.0)	0 (0.0)	**42 (84.0)**	0 (0.0)	8 (16.0)
SCA99	50									0 (0.0)	0 (0.0)	1 (2.0)	**47 (94.0)**	2 (4.0)
SCA00	47									0 (0.0)	0 (0.0)	1 (2.1)	2 (4.3)	**44 (93.6)**
c) *seven putatively unknown samples*														
IR05a	50	**34 (68.0)**	3 (6.0)	1 (2.0)	0 (0.0)	3 (6.0)	4 (8.0)	0 (0.0)	4 (8.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)
YR05a	36	0 (0.0)	1 (2.8)	0 (0.0)	2 (5.6)	**23 (63.9)**	8 (22.2)	1 (2.8)	1 (2.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TUIa	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**9 (90.0)**	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)
WCAa	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**9 (90.0)**	0 (0.0)	1 (10.0)	0 (0.0)
NCAa	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (20.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**7 (7.0)**	0 (0.0)	0 (0.0)
SCA99a	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**9 (90.0)**	0 (0.0)
SCA00a	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)	**9 (90.0)**

Figure 4.1 Multilocus observed (H_O) and expected (H_E) heterozygosities and adjusted allelic richness (A) for all 21 samples including 16 wild (IR99, IR05, KR04, SR04, KI04, YR99, YR06, BZ05, BZ06, TT06, NT05, NT06, HC05, HC06, BH99, BH05) and five hatchery samples (TUI, WCA, NCA, SCA99 and SCA00). See Table 4.1 for sample abbreviations. Long-established hatchery stocks (TUI and WCA) have significantly lower values of A, H_O, and H_E, but recently derived hatchery stocks (NCA, SCA99, and SCA00) only have reduced A. Correlations of H_O and H_E with A are significant ($R^2 = 0.431$, $P = 0.001$ and $R^2 = 0.862$, $P < 0.001$, respectively).


0.900
0.800
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H_O & H_E
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He
IR99
IR05
KR04
KI04
YR99
YR06
BZ05
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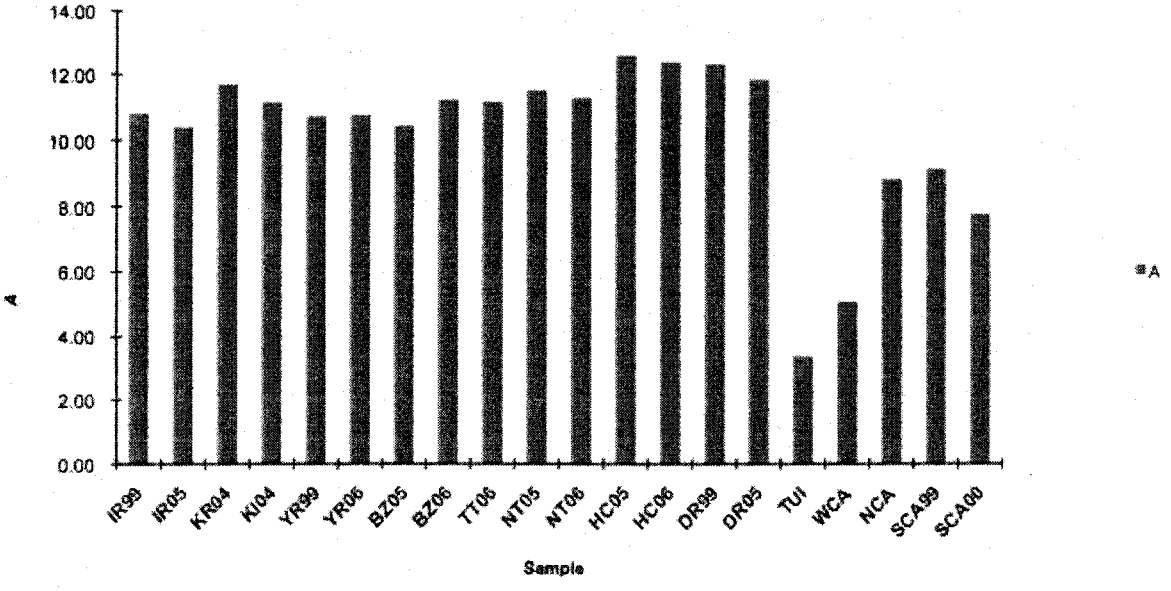
SCA00


14.00
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A
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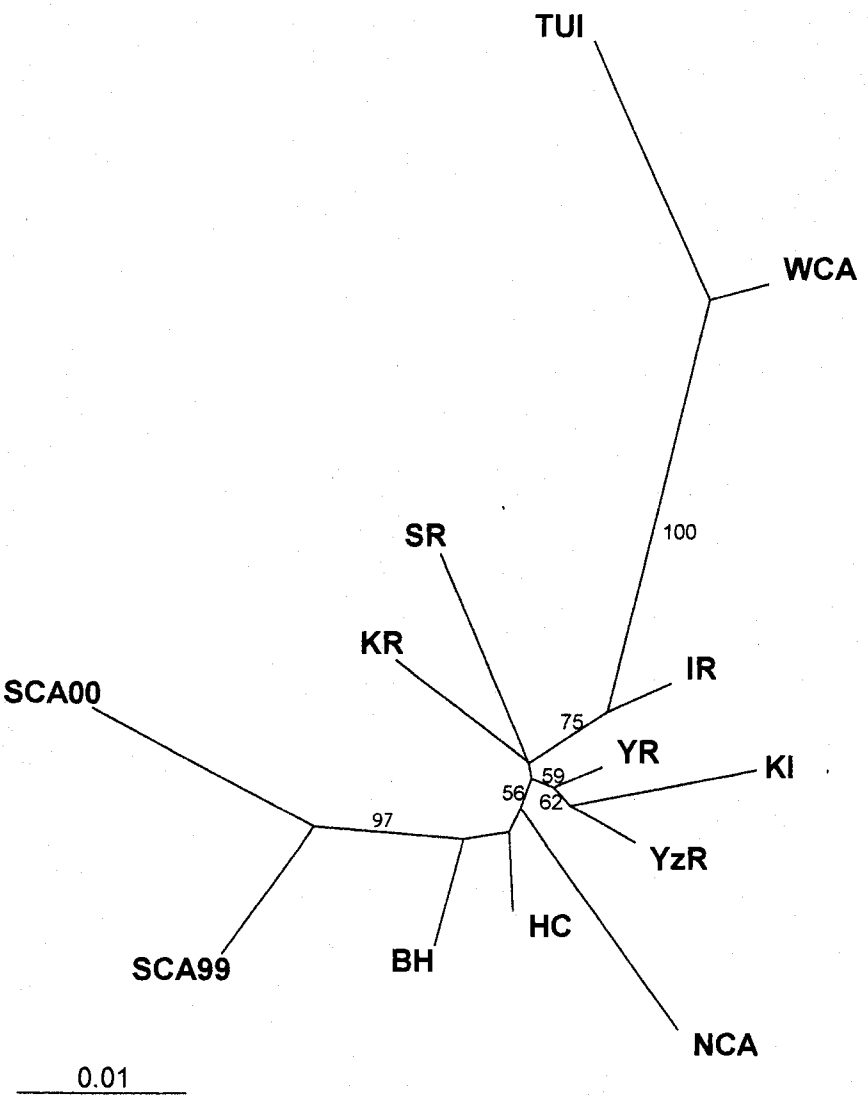
YR06
BZ05
BZ06
TT06
NT05
NT06
HC05
HC06
DR99
DR05
TUI
WCA
NCA
SCA99
SCA00
Sample

Figure 4.2 Unrooted Neighbor-Joining (NJ) tree based on Cavalli-Sforza & Edwards' (1967) genetic distances among five hatchery stocks (TUI, WCA, NCA, SCA99, and SCA00) and eight wild populations (IR, KR, SR, KI, YR, YzR, HC and BH) of *C. ariakensis*. Numbers on internal branches are percentages of bootstrap support > 50% after 10,000 iterations.


TUI
WCA
100
SR
KR
IR
SCA00
75
YR
KI
59
56
62
97
YzR
HC
SCA99
BH
NCA
0.01

SUMMARY:

MAJOR ACHIEVEMENTS AND FUTURE PROSPECTS

Through this doctoral research, additional evidence was obtained to support the two-species hypothesis regarding the taxonomic status of *C. ariakensis* and *C. hongkongensis* (Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008). In addition, a set of novel microsatellite markers was developed specifically for *C. ariakensis* (Xiao et al. 2008) and was used to assess genetic variability among and within natural populations of *C. ariakensis* in Asia and hatchery stocks in the United States. These markers also proved suitable for future tracking of *C. ariakensis* introductions in the Chesapeake Bay. Here the major achievements of this research and prospects for future study are summarized and discussed.

A semi-gametic incompatibility was observed between *C. ariakensis* and *C. hongkongesis* during a laboratory hybridization experiment, indicating partial reproductive isolation between these two probable species. Additional evidence for the distinct species status was gained from the molecular marker work, which indicated low transferability of primers targeting homologous microsatellite loci between these two taxa (i.e. primers designed based on *C. ariakensis* microsatellite flanking region sequences failed to successfully amplify these targeted loci in *C. hongkongensis* individuals). There was an order of magnitude larger genetic divergence between these two taxa (F_{ST} = 0.328) than that observed within each group, and no natural hybrids of these two species were found in multiple samples comprised of thousands of individual animals collected from their native region. Finally, there is some evidence from hatchery spawns for differences among these species in their reproductive cycles. These results are congruent with the molecular phylogenies based on several mitochondrial gene sequences (Boudry et al. 2003, Lam and Morton 2003, Wang et al. 2004, Reece et al. 2008) and one nuclear

region (Reece et al. 2008). More work, however, is obviously needed to further characterize these two species, since information on the physiology, biology and ecology of both is largely unknown, and earlier literature may have confused these two, as well as several other *Crassostrea* species, in their native region due to morphologically based misidentifications (Lam and Morton 2003, Wang et al. 2004, Guo et al. 2006). The fertilization ratio of a hybrid cross between *C. ariakensis* (male) and *C. hongkongensis* (female) was comparable to those observed in two pure *C. ariakensis* and *C. hongkongensis* control crosses. The hybrid larvae from this cross were inviable in this study; however, even larvae from pure crosses failed to survive through early metamorphosis. Therefore, it cannot be confidently determined that there is complete reproductive isolation by one-way gametic incompatibility and larvae inviability between *C. ariakensis* and *C. hongkongensis*. Future studies might focus on identifying any differences between normal and hybrid larvae in post-metamorphosis development.

Based on the results from this laboratory hybridization experiment, as well as current and previous genetic studies and some biological and ecological observations, *C. hongkongensis* samples were dropped from the remaining intra-species genetic studies of *C. ariakensis*. Analyses of 16 natural samples from Japan, South Korea and China using eight polymorphic microsatellite loci found slight but significant genetic differentiation among the samples ($F_{ST} = 0.018$, $P < 0.001$), which could be characterized by a pattern of isolation by distance. Eight genetically differentiated populations (global $F_{ST} = 0.020$) were further identified in the native region according to various analyses, including one population from southern Japan (IR), three populations along the coast of South Korea (KR, SR and KI) and four along the coast of China (YR: Yellow River basin; YzR:

Yangzi River estuary; HC: Haicheng, southern China; BH: Beihai, southern China). The relatively uniform genetic distances that constitute the overall genetic structure, however, could not be used as support for the hypothesis of a distinct biogeographic barrier (e.g. the Yangzi River estuary; Wang et al. 2004) to the genetic continuity of *C. ariakensis* populations in their native range. This could be due, however, to limited sampling along the coast of China. Therefore, adding samples from intermediate locations to those targeted in the current sample set might provide a finer-scale spatial genetic structure of *C. ariakensis*.

Genetic differentiation among five hatchery stocks in the U.S. ($F_{ST} = 0.132$) was five-fold larger than that observed among wild populations, indicating these hatchery stocks were genetically divergent. The NJ tree showed that each hatchery population was still relatively closer to its natural source population than to other wild populations, although substantial drift from these source populations was evident. Significant reduction in genetic diversity was observed in all hatchery stocks compared to source populations. There were decreases on both allelic diversity and heterozygosity by 60% and 11% - 26%, respectively, for the two mature stocks (TUI and WCA), which have been domesticated for about 30 yrs, compared to their source wild populations; whereas only decreased allelic diversity (18% - 30%) was found in the three new stocks (NCA, SCA99 and SCA00) separated from their sources in northern and southern China by only one generation at the time of sampling. In addition, an unexpected large number of H_O excess relative to H_E was observed in these hatchery stocks. Apparently, a genetic bottleneck due to a relatively small number of animals used for spawning, combined with a likely uneven contribution to the next generation from the parents, has affected the

genetic make-up of these hatchery populations. Further investigation is needed, nonetheless, to determine whether the genetic bottlenecks, genetic drift from natural populations, and large differentiations among these hatchery stocks, are associated with any possible phenotypic heterogeneity among these hatchery populations, as some differences in larval performance have already been reported (Luckenbach 2004, Tamburri et al. 2008). Specifically, it would be helpful to evaluate the performance of these hatchery stocks in terms of disease tolerance and growth under different environmental conditions. The source populations were collected from sites with wide variations in environmental parameters such as temperature, salinity, substrate, etc. In addition, as the hatchery stocks are quite divergent, different hatchery stocks might be better suited for planting and/or aquaculture development at particular sites.

Finally, the microsatellite markers developed here have proven useful for genetic tracking of *C. ariakensis* stocks, and assignment of hatchery individuals back to their source stocks could be done with a high degree of confidence. Though assignment of wild individuals back to their specific wild population of origin proved less reliable, a high percentage of individuals could be assigned to their source population or geographically proximal populations. A strategy for future tracking of unknown *C. ariakensis* in the Chesapeake Bay was proposed. The unknown sample will be first classified generally as originating from hatchery or wild populations, and subsequently be assigned to either a specific hatchery stock or a general geographic group of wild populations that would include the assigned population and those geographically proximate populations which formed a distinct genetic region based on both the F_{ST} and NJ analyses.

For future work, a comparative study on the post-metamorphic development of hybrid and pure crosses between *C. ariakensis* and *C. hongkongensis* should be conducted, as well as an ecological survey of these two species in order to determine which of the mechanisms indicated by the current research is most likely responsible for the reproductive isolation between these two taxa. Furthermore, adding more wild samples to the intra-specific genetic study could resolve a finer genetic structure for the wild *C. ariakensis* populations in the native region. Specifically, samples from intermediate locations along the coast of China could help test the proposed biogeographic barrier near the Yangzi River estuary (Wang et al. 2004). Finally, since high levels of genetic differentiation and genetic bottlenecking were observed among the hatchery stocks, side-by-side comparisons among the hatchery stocks would be informative to determine the possible phenotypic differences among these hatchery stocks with regards to disease tolerance and growth, associated with the genetic differentiation. In the future, the genetic make-up of stocks used for various research conducted in the U.S. should be taken into account when interpreting results, considering the heterogeneous environmental conditions of the locations in the native regions from which these stocks were derived.

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