CHAPTER IV

DISCUSSION

I. Taxonomic difficulties of oysters genera *Crassostrea*, *Saccostrea* and *Striostrea*

The basic knowledge of genetic variation of oysters in Thailand are important for construction of appropriate breeding programs and management scheme in these species. However, relatively little is know about inter- and intraspecific genetic variability of oysters in Thailand. Taxonomy of these taxa are presently unclear. Oysters are variable in forms and can display ecomorphology (Littlewood, 1994). The external characteristics (e.g. shell morphology) are influenced by a variety of habitats and environmental conditions (xenomorphic variation) (Tack et al., 1992).

Moreover, it has been reported that interspecific hybridization among different oyster species (e.g. between *C. virginica and C. rhizophorae*) could generate viable and fertile progeny exhibiting variation of shell morphology and scar pigmentation (Hedgecock and Okazaki, 1984). This further complicated taxonomic difficulties of oysters based primarily on morphology alone.

Taxonomy of oysters (genera *Crassostrea* and *Saccostrea*) in Thailand are rather limited and not well studied. Almost all of the researches emphasized morphological characterization of these taxa. All *Saccostrea* oysters in Thailand was previously assumed to be *S. cucullata* (Amonjaruchit, 1988). Conversely, Brohmanonda et al. (1988) and Tookwinas (1991) classified small oysters collected from many provinces in Thailand as *S. commercialis*. Nevertheless, it has been subsequently proved that *S. commercialis* is not available in Thailand.

Yoosukh (2000) classified oysters (superfaminly Ostreoidea) using shell morphology to 2 families; Ostreidea and Gryphaeidea. The former could be further divided to three subfamilies; Ostreinae (Tribe Ostreini composing of *Planostrea pestrigris* and *Ostrea* sp.), Crassostreinae (*C. belcheri* and *C. lugubris*, *S. cucullata*,

S. mordax, *S. echinata* and *Saccostrea* spp.), and Lophinae (*Lopha cristagalii*, *Dendostrea folium* and *Dendostrea* sp.). Three biologically distinct species could not be identifies and were recognized as *Saccostrea* spp..

Although, all oysters investigated by this study belong to the Crassostreinae family, scientific names of Thai oysters are confused. For instance, the previously described *C. lugubris* in Thailand are currently regarded as *C. iredalei* while *S. mordax* is a synonym of *S. cucullata*. Moreover, *S. echinata* is presently recognized as *Stroiostrea* (*Parastroiostrea*) *mytiloides*. This indicated taxonomic difficulties of Thai oysters based primarily on morphology.

More recently, Visootiviseth et al. (1998) collected *Saccostrea* oysters from Sri Racha (Chonburi), Sam Saeb (Chumphon), Koh Talu (Chumphon), Koh Prab (Surat), Koh Chang (Trat) located in the Gulf of Thailand and Tubtieng (Trang) and Koh Khaoyai (Satun) located in the Andaman Sea. These specimens were morphometrically and electrophoretically analyzed. The results differentiated these oysters to three different groups, A (Sri Racha, Tubtieng, Koh Prab, Koh Khaoyai and Koh Chang species 1, B (Sam Saeb and Koh Chang species 2) and C (Koh Talu). These oysters were not named scientifically but recognized under S. *cucullata*.

Differentiation between *Crassostrea* and *Saccostrea* oysters can be carried out by the absence of chromata in the former but existence in the latter, respectively. Within the genus *Crassostrea*, oysters having black adductor muscle scars are taxonomically regarded to *C. iredalei* whereas those having white scars are *C. belcheri* (Visootiviseth et al., 1998). Within the genus *Saccostrea*, it is extremely difficult to differentiate *S. forskalli* and *S. mytiloides* from each other. In addition, four geographic samples (CsKB, S1SR, S2RN and S3SS) used in this study could not be unambiguously identified using morphological characters. Accordingly, molecular genetic approaches were applied to assist this important gap in these taxa.

II. RAPD analysis: a potential tool for population genetic and systematic studies of *Crassostrea*, *Saccostrea* and *Striostrea* oysters in Thailand

RAPD analysis has been successfully used for several applications in population genetic studies of molluscs, for example, examination of genetic diversity in the eastern oyster, *C. virginica* (Hirschfeld et al., 1999), identification of polymorphic DNA markers in genetic studies of the sea scallop, *Placopecten magellanicus* (Patwary et al., 1994), and determination of systematic relationships and genetic diversity in intertidal limpets, *Siphowaria spp*. (Chambers et al., 1998). Nevertheless, there have been no extensive studies on genetic diversity and species-diagnostic markers of all commercial oysters in Thailand which are necessary to elevate culture and management efficiency of these taxa (Klinbunga et. al., 2000a).

The RAPD analysis was chosen to study genetic diversity and to identify species-specific markers of oysters in Thailand. The number of monomorphic bands of each species (observed in at least 95% of overall individuals in a particular species) varied greatly among different oyster species. Using this simple approach, several unique RAPD fragments exhibiting fixed frequencies (species-specific markers) were observed in wild individuals of commercially cultured oyster species (*C. belcheri*, *C. iredalei* and *S. cucullata*).

Although a number of monomorphic RAPD fragments in *S. mytiloides* and *S. forskali* were found, these bands were also found in other species obviated the possibility to obtain diagnostic markers in these morphological problematic species. At the intraspecific level, population-specific markers were not observed in any oyster species. Therefore, intraspecific population structure of interested species should be further carried out to investigate whether the gene pool of each oyster species in Thai waters is homogeneous or not.

Although the sample size of each species used in this study was relatively small, wild specimens were collected from different geographic locations situated in different coastal sides of peninsular Thailand. Accordingly, these specific markers did not originated from artefacts or accidents due to experimental or sampling errors.

All RAPD primers used in this study also generated fragments with fixed frequencies in both *S. commercialis* and *P. viridis* indicating the possibility to use these primers to identify useful markers in these two species. These markers were, however, not regarded to be species-specific because representative specimens of these species were originated from only one location (Brisbane in Australia and Chonburi in Thailand, respectively) and should not represent overall genetic constituents of each species.

All RAPD bands generated by each primer were polymorphic (observed in less than 95% of investigated individuals) for overall samples. This was similar to results from studies of genetic diversity of three sympatric mud crabs of the genus *Scylla* in eastern Thailand (Klinbunga et al., 2000b). Data from the present study suggested the potential of RAPD analysis for determination of genetic differences of oysters at both inter- and intraspecific levels.

A large number of genotypes of *Crassostrea* and *Saccostrea* oysters in Thailand was generated from each RAPD primer indicating extremely high levels of genetic diversity of these taxa. To eliminate effects influenced by different sample sizes, a ratio between the number of genotypes per primer and the number of investigated specimens in a particular species, which is more reliable than the number of genotypes alone, was introduced. The lowest level of this parameter was observed in *C. belcheri* (0.58) whereas other species showed higher levels of this ratio (0.90 in *C. iredalei*, 0.98 in *S. cucullata* and 1.0 in *S. mytiloides* and *S. forskali*). This suggested that inbreeding is presently not a major concern for natural oyster populations in Thailand.

Hirschfeld et al. (1999) used the RAPD technique to determine genetic diversity of the eastern oyster (*C. virginica*) from four wild-naturalized stocks (Wellfleet, Wareham River, East Wardham/Onset and Barnstable Harbor) and one cultured stock (Cotuit) in Massachusetts, USA. Ten oligonucleotide primers were selected and screened to amplify DNA from 79 samples originating from five sampling sites. The highest level of genetic polymorphism were found in specimens

from Barnstable Harbor (74%) followed by those from Wellfleet (71%), Wareham River (70%), East Wareham/Onset (62%), and Cotuit (54%), respectively.

Significant differences in RAPD polymorphism between three wild (Barnstable Harbor, Wellfleet, and Wareham River) and the cultured samples were found ($P < 0.05$). Nonetheless, the East Wareham/Onset sample did not show significant genetic difference with the cultured Cotuit population ($P > 0.05$). These results suggested that the founder population of Cotuit may have originated from the East Wareham/Onset. The information on genetic variation of cultured and wild stocks of *C. virginica* in Massachusetts may be useful for proper management of resources in this taxon.

Disregarding *Crassostrea sp.*, *Saccostrea sp.* group 1, *Saccostrea sp.* group 2 and *Saccostrea sp.* group 3, the average number of bands per primer and the percentage of polymorphic bands of *Crassostrea* oysters was much lower than those of the genus *Saccostrea* indicating higher genetic diversity levels of the latter group. The level of polymorphic RAPD bands in *Crassostrea* was comparable to that of five geographic populations of the giant tiger shrimp, *Penaeus monodon* in Thailand (Tassanakajon et al., 1998), specific pathogen free and wild stocks of *P. vannamei* originating from Sinaloa and Oaxaca in Mexico (Garcia, 1994) and three species of mud crabs of the genus *Scylla* in eastern Thailand (Klinbunga et al., 2000b). Conversely, the percentage of polymorphic bands in each *Saccostrea* species (97.64% and 99.30% in *S. cucullata* and *S. forskali*, respectively) was higher than those taxa.

Genetic differences within each *Crassostrea* species were much lower than those within *S. cucullata*, *S. forskali* and *S. mytiloides*. The results were in accord with those based on morphological studies (Brock, 1990) and allozyme electrophoresis (Buroker et al., 1979; Hedgecock, 1995; Visootiviseth et al., 1998).

At the intraspecific level, oysters from different geographic coastal regions had greater genetic divergence than those located within the same coastal areas implying the possible intraspecific genetic differentiation of each oyster species in Thailand. High genetic diversity within each oysters may have reflected correlation between genetic adaptation to climatic or environmental variation (Harry, 1985).

Considering unidentified oyster species, all of these groups exhibited high genetic variability within each geographic sample. The percentage of polymorphic bands were 85.92%, 90.11%, 77.63% and 89.55% in unidentified *Crassostrea* sp. and *Saccostrea* groups 1, 2 and 3, respectively. Several RAPD fragments with fixed frequencies were observed in these oysters. Some of which in *Crassostra sp.*, *Saccostrea* group 1 and 2 samples were not found in others and could represent specificity of each group (Appendix C).

Using allozyme (*Lap*, *Idh-1*, *Aat-2*, *Pgi*, *Pgm*, *Est-2*, *Mpi-2*, *Mdh-1* and *Ap*) analysis, Visootiviseth et al. (1998) classified, presumably, *S. cucullata* to be groups A, B and C using multilocus genotypes and phylogenetic relationships of these loci. An *Idh-1* allele was fixed in *Saccostrea* group C whereas other two alleles were found in specimens of group A and B (sampling locations of their experiments were described previously) and could be used for diagnostic purpose. Several unique alleles (found in only one species with relatively high frequencies) of a single locus were found in C but no alleles clearly differentiated those *Saccostrea* oysters. Results from morphometric analysis illustrated comparable conclusions with an exception that Trat 1 (A) and Trat2 (B) were sister taxa when analyzed by this approach.

III. Do oysters in Thailand exhibited biological species complexes?

A neighbor-joining tree constructed from genetic distance between pairs of geographic samples illustrated a clear separation between *Crassostrea* and *Saccostrea* oysters. Within the former group, clear phylogenetic differences between oysters from different coastal areas (between the east and west of Peninsular Thailand) were consistent observed in *C. belcheri* but not in *C. iredalei*.

Within the latter genus, *S. cucullata* was well allocated to a separate group. All three *S. mytiloides* samples were also clustered together. Interestingly, *S. forskali* samples could be differentiated to 2 different groups; one that exhibited phylogeographic relationships between samples collected from the east coast (SfCT, Chantraburi; SfCBA, Angsila Chonburi; SfPJ, Prachuapkririkhan and SfSK, Songkhla) and the west coast (SfRN, Ranong and SfST, Satun) and the other that showed phylogenetically closed relationships with *S. mytiloides* (SfCTS and SfSR

collected from Koh Sichang in Chonburi and Koh Prab in Surattani, respectively). The phylogenetic results suggested that *S. forskali* from Koh Sichang, (Chonburi) and Koh Prab (Suratthani) were genetically different from that originating from other locations.

Two oyster individuals are morphologically classified to be *S. forskali* (Oy030) and *C. iredalei* (Oy039), respectively. These specimens possessed *C. iredalei*-specific markers (1250 bp) when analyzed with the primer OPB08. The Oy030 also possessed a 745 bp fragment from the UBC210. The remaining *C. iredalei*-specific markers were not observed. The loss of species-specific markers should have been resulted from introgressive hybridization between *C. iredalei* and an another oyster species, presumably *S. forskali*.

The species status of Oy095 and Oy104 could not be clearly concluded morphologically. The former was classified as a hybrid between *C. belcheri* and *C. iredalei* whereas the latter was regarded as C. *iredalei*-like oysters. Based on RAPD analysis, both individuals possessed all species specific fragments in *C. iredalei*. No species-specific fragments of *C. belcheri* were detected in the Oy095. Accordingly, these specimens are actually *C. iredalei* rather than those of hybrid origins.

Oy121 and Oy122 can only be classified to *Saccostrea*. Using all references diagnostic RAPD markers and morphological characters, these specimens still could not be differentiated unambiguously. Accordingly, the parallel study of this thesis on PCR-RFLP of 18S and 16S rDNA and the intergenic COI-COII of the same sample set may provide a clearer conclusion.

Specimens from four geographic locations, originating from Krabi (Klong Bo Tho), Suratthana (Koh Prab), Ranong (Koh Nui) and Samut Sakhon, could not assigned their correct scientific names. They was then called as *Crassostra* sp., *Saccostrea* sp. group 1, *Saccostrea* sp. group 2, and *Saccostrea* sp. group 3, respectively.

The *Crassostrea sp.* oysters showed the absence of chromata indicating that they are belonging to the genus *Crassostrea*. No species-specific marker for *C. belcheri* and *C. iredalei* were found in all investigated individuals of *Crassostrea sp.*. Additionally, the phylogenetic tree also dissociate this sample set from that of *C. belcheri* and *C. iredalei*. Accordingly, these specimens should be regarded as a new species of *Crassostrea* oysters in Thailand. Previously, Dilokrattanatrakul C. (1998) found unidentified *Crassostrea* oysters at Klon Thom (Krabi). Therefore, more than two *Crassostrea* species are existed in Thai waters.

The unknown *Saccostrea sp.* group 1 from Koh Prab (Suratthani) was phylogenetically clustered with *S. forskali* from the same location. A lack of diagnostic RAPD markers in *S. forskali* and *S. mytiloides* resulted in the failure to clarify the genetic status of these individuals.

The unknown *Saccostrea sp.* group 2 from Koh Nui (Ranong) was recognized as a new species. Specimens from this geographic location displayed several fixed fragments found in all individuals of this group using primer OPA09, OPB08, UBC210 and UBC220. The average similarity index within this group was slightly lower than *Crassostrea* oyster but greater than any other *Saccostrea* oysters. A lack of 750 bp (OPB08) and 1800 bp (UBC220) indicated that they are not *S. cucullata*. Several bands which were found in all *Saccostrea sp.* group 2 individuals are not a genetic character of *S. mytiloides* and *S. forskali*. Therfore, we considered this oyster group as a new unidentified species. The phylogenetic tree allocated *Saccostrea sp.* group 2 to a new branch supporting the earlier conclusion.

The *Saccostrea sp.* group 3 was collected from Samut Sakhon and preliminary identified morphologically as *S. mytiloides*. No species-specific marker found in this study were existed in this group of oysters but results of phylogenetic tree showed its closely relationship with *S. mytiloides* from same location than did *S. mytiloides* from Ranong and Phuket. Therefore, it was regarded as a conspecific population of *S. mytiloides*.

The *Saccostrea* oysters could display ecomorphological variation (Tack et al., 1992). Accordingly, two sympatric species may be morphologically similar and

misidentified to be a single species. On the other hand, allopatric populations inhabiting different habitats may show ecomorphological variation but the species status is questionable. Phylogenetic relationships of oysters in Thailand indicated the existence of new species and species complexes in *Saccostrea* oysters in Thailand.

All *Saccosstea* oysters in Thailand was previously assumed to be only *S. cucullata* (Amonjaruchit, 1988) or *S. commercialis* (Brohmanonda et al., 1988 and Tookwinas, 1991). Results from our study indicated that at least four *Saccostrea* species were locally distributed in Thailand. This is in agreement with those based on allozyme analysis (Visootiviseth et al., 1998).

Typically, the *Saccostrea* oysters originating from Angsila (Chonburi) had been recognized as *S. commercialis* (Department of Fisheries, 1993). Specimens from this locales (SfCNA) was included in this study. Based on our RAPD results, they are not *S. commercialis* due to a lack of species-diagnostic markers typically found in that species. Phylogenetic inference of SfCNA oysters indicated that they should be referred to *S. forskali in lieu*.

Tsuchiya and Liardwitayapasit (1986) identified small rock oysters collected from the rocky shores of Koh Sichang (Chonburi) as *S. mordax*. Oyster specimens from this location were also included in the analysis and regarded to be *S. forskali*. Although it has been reported that *S. mordax* is a homospecific of *S. cucullata*, species-specific markers of *S. cucullata* were not found in these oysters.

Recently, Charoensit (1994) examined interspecific and intergeneric hybridization between *C. belcheri*, *C. iredalei* (synonym of *C. lugubris*) and *S. cucullata* in laboratory conditions. Hybrid progeny between two *Crassostrea* species survived to the spat stage whereas intergeneric hybridization between female *C. iredalei* and male *S. cucullata* were successful. Nevertheless, *S. cucullata* used in that studies may not be a true *S. cucullata* due to taxonomic difficulties of small oysters in Thailand. All previous aquacultural studies in Thailand that included *S. cucullata* in the experiments should be carefully followed by interested researchers because a member of species complexes rather than a single species may be used in

those experiments under the scientific name of *S. cucullata*. Two species-specific markers in this species are useful to eliminate this taxonomic problems.

Further population and systematic studies of *Saccostrea* oysters, particularly, *S. mytiloides* and *S. forskali* should be carried out using other molecular genetic approaches for example, analyses of RFLP of conserved genes or allozyme polymorphism of these taxa. Cross-hybridization between suspected taxa and complexity of gene pools of each oyster species should be carried out in details (Bank et al., 1993).

IV. Cloning and characterization of species-specific RAPD fragments in *C. belcheri*

C. belcheri is the most commercially important oyster species in Thailand (Charoensit, 1995). Selection of correct seed and broodstock of this species is one of the main important factors for successful culture of *C. belcheri* in Thailand. Although species-specific RAPD markers of three commercially cultured oyster species allow direct examination of this purpose, RAPD-PCR is sensitive to reaction conditions and even slight changes of those may affect the reproducibility of amplification products (Hadry et al.,1992).

This technique also requires good quality DNA template for reliable and consistent results which mat not be possible for field specimens. This may significantly cause false negative results from investigated samples which actually are *C. belcheri*. To eliminate this problem, *C. belcheri*-specific RAPD fragments were converted to sequence-characterized amplified region (SCAR) markers (Weising et al., 1995).

A total of ten species-specific fragments from four primers (OPA09, OPB01, OPB08 and UBC220) was observed in *C. belcheri*. Development of SCAR markers were not attempted from any of six *C. belcheri*-amplified fragments exhibiting sizes greater than 1000 bp (1650 bp and 1550 bp fragments from OPB08, 2100 bp, 1400 bp and 1250 bp fragments from OPB01 and a 1050 bp fragment from UBC220) and from that of 250 bp generated by OPA09 due to difficulties arising from sequencing of

large RAPD fragments and less possibility to design suitable primers from a 250 bp fragment.

Jones et al. (1997) developed species-specific primers discriminating schistosome-intermidiate hosts of *Bulinus* taxa (Gastropoda : Planorbidae) by converting RAPD markers into SCAR markers. Diagnostic RAPD markers for either *B. senegalensis* of *B. forskalii* from West Africa were cloned, sequenced. Species-specific primers of these organisms were designed. The PCR primers specifically amplify a 553 bp species-specific product in *B. forskalii* and a 564 bp and a 370 bp species-specific products in *B. senegalensis.* Specificity of primers was confirmed by tested against widespread samples throughout each species range. This result demonstrated the use of RAPD and SCAR markers for discrimination of species complexes in *Bulinus.*

Using the same approach, three *C. belcheri*-specific RAPD fragments (650 bp band from OPB01 and 835 bp and 600 bp bands from OPB08) were cloned and sequenced. The actual molecular length of pPACB1, pPACB2 and pPACB3 was 637 bp, 811 bp and 563 bp, respectively(Fig. 3.16). Comparisons of these sequences with those previously deposited in GenBank did not reveal significant similarity with any sequence. specific primers were designed from nucleotides of each DNA fragments and tested for primer sensitivity.

A pair of primers containing each insert was designed and used to amplify *C. belcheri* total DNA (pPACB1-F/R, pPACB2-F/R, pPACB3-F/R). The sizes of PCR products were identical to those expected from their DNA sequences (536 bp, 600 bp and 506 bp, respectively). Heterozygotes were not observed in investigated *C. belcheri* individuals when analyzed with these primer sets implying the retention of dominant segregated fashion of the original RAPD species-specific markers.

Primer sensitivity was tested by PCR amplification using homologous DNA template between 16.25 pg to 25 ng. The sensitivity of detection roughly reached 30 pg of DNA template for each pair of primers. The sensitivity level allows the possibility to use these SCAR markers to study the dispersal process of *C. belcheri* planktonic larvae over vast geographic areas in Thailand.

Only, a pPACB1 primer pair was chosen for the specificity test in all local oyster species, the Australian oyster, *S. commercialis*, and the mussel, *P. viridis*. A positive 536 bp band was found in all *C. belcheri* individuals but was not observed in other species. The results eliminated the possibility that a 650 bp fragment in *C. belcheri* and a 700 bp fragment in *C. iredalei* were homologous. This indicated a successful development of a species-specific SCAR marker of *C. belcheri* in Thailand. This and other (pPACB2, pPACB3 and RAPD) markers would offer accurate identification and selection of seed and broodstock of *C. belcheri* to elevate culture efficiency of this economically important species in Thailand.

V. Applications of knowledge on genetic diversity levels and species-specific markers of three oyster species in Thailand

Identification of species-specific markers in *C. belcheri*, *C. iredalei* and *S. cucullata* could assist selection of appropriate larval and broodstock species for aquaculture production. These markers can be utilized for studies of larval distribution patterns and recruitment of commercially cultured Thai oysters. However, more accurate results for detection of *C. belcheri* at different developmental larval stages would be obtained using primers for pPACB1. This information will enhance aquaculture output without adversely affecting native populations leading to sustainable farming activity of these taxa (Hedgecock, 1995).

In this study, *C. iredalei*-specific RAPD fragments were used to confirm the existence of hybridization origins of specimens which are morphologically identified to be *S. forskaii*-like or *C. iredalei*-like oysters. This indicated that additional SCAR marker may be further developed in this species and used for taxonomic identification and determining degrees of interspecific hybridization of commercially culture oysters in Thailand.

In December 1996, a massive die-off (approximately 90%) of *C. belcheri* broodstock in Suratthani (the most important cultivated area of this species in Thailand) occurred suggesting that stock enhancement programs should be carried out. Data on the present study indicated high genetic heterogeneous of Thai oysters

genera *Crassostrea*, *Saccostrea* and *Striostrea*. At present, data on population structure of each species has not been reported. Therefore, selection of appropriate broodstock for aquacultural and restocking purposes should be from that locally residing in the same area in which those activity is occurred. This prevents anthropological disturbances of the gene pool of each oysters.

RAPD-PCR analysis has been successfully used to determine population differentiation in the black tiger prawn, *P. monodon* (Tassanakajon et al., 1998), to identify species-specific markers in mud crabs (*S. serrata*, *S. oceanica* and *S. tranquebarica*) in eastern Thailand and to demonstrated a lack of interspecific hybridization between these taxa (Klinbunga et al., 2000b). The present study illustrate the use of this approach to identify large genetic differences between Thai oysters and several species-specific RAPD fragments in *C. belcheri*, *C. iredalei* and *S. cucullata*. These genetic markers can be further used for aquacultural (selection of a particular broodstock species and determination of seed species) and biological (patterns of distribution and recruitment) applications in these taxa (Linstrom, 1998).