MARINE BIOTECHNOLOGY

Molecular Taxonomy of Cupped Oysters (*Crassostrea*, *Saccostrea*, and *Striostrea*) in Thailand Based on COI, 16S, and 18S rDNA Polymorphism

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Received: 22 September 2003 / Accepted: 20 July 2004 / Online publication: 14 July 2005

Abstract

Genetic diversity of oysters *Crassostrea belcheri* (Sowerby, 1871), *C. iredalei* (Faustino, 1932), *Saccostrea cucullata* (Born, 1778), *S. forskali* (Gmelin, 1791), and *Striostrea* (*Parastriostrea*) *mytiloides* (Lamarck, 1819) (Ostreoida, Mollusca) was analyzed by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) of 16S ribosomal DNA with *Acs*I, *Alu*I, *Dde*I, *Dra*I, *Rsa*I, and *Taq*I, 18S ribosomal DNA with *Hin*fI, and cytochrome oxidase subunit I with *Acs*I, *Dde*I and *Mbo*I. A total of 54 composite haplotypes were observed. Species-diagnostic markers were specifically found in *C. belcheri*, *C. iredalei*, and *S. cucullata*, but not in *S. forskali* and *Striostrea mytiloides*, which shared common composite haplotypes. Neighbor-joining trees constructed from genetic distances between pairs of composite haplotypes and species indicated large genetic differences between *Crassostrea* and *Saccostrea* (including *Striostrea mytiloides*), but closer relationships were observed within each genus. Four groups of unidentified oysters (*Crassostrea* sp. and *Saccostrea* sp. groups 1, 2, and 3) were also genetically analyzed. Fixed RFLP markers were found in *Crassostrea* sp. and *Saccostrea* sp. group 2, but not in *Saccostrea* sp. groups 1 and 3. Phylogenetic and genetic heterogeneity analyses indicated that *Crassostrea* sp. and *Saccostrea* sp. group 2 should be considered as newly unidentified oyster species in Thailand.

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Key words: PCR-RFLP — genetic markers — oysters — species-specific markers

Introduction

Oysters are benthic marine species inhabiting nearshore areas, shallow waters, bays, and estuaries widely distributed throughout tropical and subtropical regions (Hedgecock, 1995). Nine species of oysters belonging to the superfamily Ostreoidea were found in Thai waters (Yoosukh and Duangdee, 1999): *Hyotissa hyotis*, *Parahyotissa* (*Parahyotissa*) *imbricata*, *Lopha cristagalli*, *Dendostrea folium*, *Crassostrea belcheri*, *C. iredalei*, *Saccostrea cucullata*, *S. forskali*, and *Striostrea* (*Parastriostrea*) *mytiloides*. Nevertheless, only *Crassostrea*, *Saccostrea*, and *Striostrea* oysters are commercially important.

Oyster culture has been carried out in Thailand for several decades (Department of Fisheries, 1993). The annual oyster production since 1994 was estimated to be approximately 20,000 tons, accounting for 35% of the total production (Department of Fisheries, 1999). The main production of cultured oysters has been from Suratthani in the east of peninsular Thailand (10,782 tons, mainly *C. belcheri*) and Chonburi in the Gulf of Thailand (7,744 tons, mainly *Saccostrea* sp.) (Figure 1).

Effective breeding and fisheries management programs of oysters in Thailand require basic knowledge of levels of genetic diversity and differentiation for each species. Nevertheless, ecomorphological variation of external characteristics is

 DOI: 10.1007/s10126-004-0036-x • Volume 7, 306–317 (2005) •

Fig. 1. Map of peninsular Thailand indicating sample collection sites of oysters in this study: *C. belcheri, C. iredalei, S. cucullata, Striostrea mytiloides, S. forskali, Crassostrea* sp., *Saccostrea* sp. group 1; *Saccostrea* sp. group 2; and *Saccostrea* sp. group 3. Detailed information is given in Table 1.

commonly observed in oysters, particularly in members of the genus *Saccostrea* (Yoosukh and Duangdee, 1999; Yoosukh, 2000). This has prevented the development of closed life-cycle culture of these taxa. Species-specific markers of commercially cultured oysters are required for quality control of a particular seed species and for examination of larval distribution patterns of those oysters in Thailand (Klinbunga et al., 2003).

Visootiviseth et al. (1998) examined taxonomically problematic *Saccostrea* spp. collected over their geographic distribution in Thai waters using morphometric and allozyme (*Pgi, Lap, Pgm, Mpi-2, Ap, Est-2, Aat-2, Mdh-2 and Idh-1*) analyses. All oysters could be split into A, B, or C groupings depending on morphology and their multilocus genotypes at *Pgi, Pgm, Mpi-2, Lap*, and *Idh-1 loci*. Although these oysters were differentiated into 3 groups, they were only recognized under *S. cucullata*.

More recently, Day et al. (2000) used allozymes and shell morphology to distinguish sympatric *Saccostrea* oysters collected from 12 sample sites throughout Thailand (Ko Chang, Trat; Ban Si Racha and Bang Saen, Chonburi; Ko Samet, Ban Sam Saeb, Ban Pak Nam, Ko Jorakae, and Ko Talu, Chumporn; Ko Prab, Suratthani; Ko Patra, Satun, and Ban Kantang, Trang) and electrophoretically determined at 8 enzymatic loci. Four polymorphic loci (*Lap, Mpi, Pgm*, and *Pgi*) were observed. The principal component analysis (PCA) of these loci allocated all individuals into 3 discrete clusters corresponding to interspecific differences. They were then identified as *S. commercialis* (note that *S. commercialis* is currently recognized as *S. glomerata*, and hereafter the new scientific name is used throughout this report; Anderson and Adlard, 1994) and *S. manilai*, which were sympatrically found in coastal and estuarine sites throughout the Gulf of Thailand, and *S. cucullata*, which was restricted to offshore isles.

Report of low genetic diversity of *C. virginica* from the Atlantic coast and the Gulf of Mexico were based on analysis of 16S ribosomal DNA by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP). The most common haplotype (AAAAAAAAAA) was found in 95% of overall specimens (N = 410). Most of the remaining mitotypes were represented by either one or two individuals. The average haplotype and nucleotide diversity within populations were low in all geographic samples (0.1079 and 0.1309%, respectively). No genetic differentiation was found between different geographic samples either within or between regions ($P > 0.05$) of this oyster (Small and Chapman, 1997).

The taxonomic status of the Portuguese oyster (*C. angulata*) and the Pacific oyster (*C. gigas*) has been questionable because no morphological or genetic information distinguishing these species has been reported. Boudry et al. (1998) used mitochondrial DNA–RFLP analysis to investigate genetic differentiation between populations of these taxa. Restriction analysis of COI of *C. gigas* (N = 203) and *C. angulata* (N = 50) with *Taq*I, *Sau*3AI, *Hha*I, and *Mse*I illustrated 6 mitotypes composed of A (ccab), B (cdab), C (dcad), D (dcab), E (dcbd), and J (acab). The C and A mitotypes were found in 76% and 88% of *C. gigas* and *C. angulata*, respectively. These mtDNA markers offered partial differentiation between *C. angulata* and *C. gigas*. Notably, the power of discrimination reported in their study was underesti-

Table 1. Sample Collection Sites and Sample Sizes of Local Oysters and Ingroup (*S. glomerata*) and Outgroup (*P. viridis*) References Used in This Study

Sample[a]	*Abbreviation*[b]	*Sample size (N)*
Local oyster, *Crassostrea belcheri*		
East of PT		11
Suratthani	CbSRE	8
Songkhla	CbSKE	3
West of PT		6
Ranong	CbRNW	3
Krabi	CbKBW	3
Local oyster, *Crassostrea iredalei*		
East of PT		16
Chonburi	CiCBE	6
Prachuapkririkhan	CiPJE	4
Songkhla	CiSKE	6
West of PT		5
Phangnga	CiPNW	4
Ranong	CiRNW	1
Local oyster, *Saccostrea cucullata*		
East of PT		11
Trat	ScTDE	5
Chantraburi	ScCTE	6
West of PT		12
Ranong	ScRNW	6
Phuket	ScPKW	6
Local oyster, *Striostrea mytiloides*		
East of PT		8
Chantraburi	SmCTE	2
Samut Sakhon	SmSSE	6
West of PT		9
Phuket	SmPKW	3
Ranong	SmRNW	6
Local oyster, *Saccostrea forskali*		
East of PT		37
Chantraburi	SfCTE	12
Chonburi, Angsila	SfCBAE	4
Chonburi, Sichang Island	SfCBSE	10
Prachuapkririkhan	SfPJE	3
Suratthani	SfSRE	3
Songkhla	SfSKE	5
West of PT		9
Ranong	SfRNW	5
Satun	SfSTW	4
Unidentified local species *Crassostrea* sp.		
Krabi (west of PT)	CsKBW	9
Saccostrea group 1		
Suratthani (east of PT)	S1SRE	8
Saccostrea group 2		
Ranong (west of PT)	S2RNW	9
Saccostrea group 3		
Samut Sakhon (east of PT)	S3SSE	5
Ingroup reference, *Saccostrea glomerata*		
Brisbane, Australia	Sglo	6
Outgroup reference, *Perna viridis*		
Chonburi, Thailand	Pevi	5

[a]PT indicates peninsular Thailand.
[b]Species names: Cb indicates *C. belcheri*; Ci, *C. iredalei*; Sc, *S. cucullata*; Sf, *S. forskali*; Sm, *Striostrea* (*Parastriostrea*) *mytiloides*; Cs, *Crassostrea* sp., Sglo, *S. glomerata*, Pevi, *P. viridis*. Names are followed by sample location, CB, CT, PK, PJ, PN, RN, SK, SR, SS, ST, and TD, and coastal region (E indicates east and W indicates west).

mated because some of the studied populations were initially misidentified.

The objectives of this study were to determine the genetic diversity of oysters in Thailand and to identify species-specific RFLP markers using restriction analysis of mitochondrial genes (16S rDNA and COI) and nuclear genes (18S rDNA). The knowledge obtained can be applied to the construction of appropriate fisheries management programs, identification of seed and broodstock species of oysters, and clarification of the possible existence of newly unidentified oysters in Thailand.

Materials and Methods

Sampling. Indigenous oysters in Thai waters, including *C. belcheri* ($N = 17$), *C. iredalei* ($N = 21$), *S. cucullata* ($N = 23$), *S. forskali* ($N = 46$), and *Striostrea mytiloides* ($N = 17$), as well as those having taxonomic difficulties, *Crassostrea* sp. ($N = 9$), unidentified *Saccostrea* sp. group 1 ($N = 8$), group 2 ($N = 9$), and group 3 ($N = 5$), were collected (Figure 1 and Table 1). Taxonomic identification was carried out according to Vaught (1989) and Yoosukh and Duangdee (1999). The Australian rock oyster, *S. glomerata* ($N = 6$), and the mussel *P. viridis* ($N = 5$), collected from Brisbane (Australia) and Chonburi (eastern Thailand), were included as ingroup and outgroup references, respectively.

DNA Extraction and PCR Amplification. Genomic DNA of each oyster was individually extracted from the adductor muscle using a phenol–chloroform–sodium dodecylsulfate (SDS) method (Klinbunga et al., 2001). The concentration of extracted DNA was estimated spectrophotometrically. DNA was stored at 4°C until required.

Three gene segments of each oyster including cytochrome oxidase subunit I (COI) (LCO1490, 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′; and HCO2198, 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-

3′; Folmer et al., 1994), 16S ribosomal DNA (F, 5′-CGC CTG TTT AAC AAA AAC AT-3′; and R, 5′-GGT CTG AAC TCA GAT CAG ATC ACG T-3′; Small and Chapman, 1997), and 18S rDNA (F, 5′-TGG ATC CGG GCA AGT CTG GTG CC-3′; and R, 5′-TGA AGT CAA GGG CAT CAC AGA CC-3′; Klinbunga et al., 2003) were amplified by PCR.

The amplification reaction was carried out in a 50-μl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 100 μM of each dNTP, 2.5 mM (COI) or 2.0 mM (16S and 18S) $MgCl_2$, 0.2 μM (COI) or 0.5 μM (16S and 18S) of each primer, 1 U of *AmpliTaq* DNA polymerase (PerkinElmer/Cetus), and 50 ng of DNA template. PCR was carried out using conditions described by Khamnamtong (2000). Five microliters of the PCR product were electrophoresed through a 1% agarose gel to determine whether the reaction was successful. Specimens showing expected product sizes were subjected to restriction analysis.

Restriction Enzyme Digestion and Electrophoresis. In a 15-μl reaction volume, the amplified 16S rDNA of each oyster was digested with *Acs*I (A/GAATTT/C), *Alu*I (AGCT), *Dde*I (CTNAG), *Dra*I (TTTAAA), *Rsa*I (GTAC), and *Taq*I (TCGA); 18S rDNA with *Hin*fI (GANTC); and gel-purified COI with *Acs*I, *Dde*I and *Mbo*I (GATC). The digests were size-fractionated through 2.5% to 3.5% Metaphor agarose gels (BMA) prepared in 1× TBE, stained with ethidium bromide, and visualized under a UV transilluminator (Sambook et al., 1989).

Data Analysis. Sizes of RFLP bands were estimated by comparing their relative electrophoretic mobility with that of a 100-bp incremental DNA marker (Promega). Restriction patterns generated from each restriction endonuclease were given letter designations alphabetically according to their appearance. Composite haplotypes were generated from combination of restriction patterns of 16S rDNA, 18S rDNA, and COI, respectively. The presence (1) and absence (0) of restricted fragments of each oyster were recorded in a binary matrix. Genetic distance between pairs of composite haplotypes (d), haplotype (h), and nucleotide diversity (π) within species and nucleotide divergence between species (d_A) was estimated (Nei and Li, 1979; Nei and Tajima, 1981; Nei, 1987) using REAP 4.0 (McElroy et al., 1991).

Neighbor-joining trees (Saitou and Nei, 1987) based on genetic distance (d) between composite haplotypes and interspecific divergence (d_A) were constructed using Neighbor implemented in PHYLIP (Felsenstein, 1993). Specimens of each oyster were divided into two hierarchical groups, the east (Gulf of Thailand) and the west (Andaman Sea) coasts of peninsular Thailand. Geographic heterogeneity among different coastal regions of oysters was analyzed using a Markov chain method (Guo and Thompson, 1992) routine in GENEPOP (Raymond and Rousset, 1995).

Results

In total, 90 restriction patterns were observed from analysis of 161 oyster individuals and 5 mussels (*P. viridis*). Ten different restriction endonucleases were used for RFLP analysis (Figure 2, A and B). Digestion of 16S rDNA (560 bp) with *Acs*I, *Alu*I, *Dde*I, *Dra*I, *Rsa*I, and *Taq*I generated 12, 12, 9, 6, 4, and 3 patterns, respectively; 18S rDNA (900 bp) with *Hin*fI, 3 patterns; and COI (710 bp) with *Acs*I, *Dde*I, and *Mbo*I, 6, 13, and 22 patterns, respectively (Table 2).

A molecular taxonomic key for identification of Thai oysters based on randomly amplified polymorphic DNA (RAPD) (Amparyup, 1999; Klinbunga et al., 2001) and RFLP (Klinbunga et al., 2003, and this study) were collectively constructed (Table 3). The *Hin*fI–digested 18S rDNA could differentiate *Crassostrea* (pattern A), *Saccostrea* and *Striostrea* (pattern B), and the mussel *P. viridis* (pattern C) unambiguously. At least one restriction enzyme could differentiated *C. belcheri*, *C. iredalei*, *S. cucullata*, *Crassostrea* sp., and *Saccostrea* sp. group 2 (Table 3).

Fifty-four composite haplotypes were generated (Table 4). Disregarding effects influenced by different sample sizes, the number of composite haplotypes found in *C. belcheri*, *C. iredalei*, *S. cucullata*, *S. forskali*, *Striostrea mytiloides*, *S. glomerata*, and *P. viridis* was 3, 3, 5, 22, 10, 2, and 1, respectively. The unidentified *Crassostrea* sp. and *Saccostrea* sp. groups 1, 2, and 3 exhibited 2, 6, 3, and 2 composite haplotypes, respectively. Species-specific (or fixed) RFLP patterns and composite haplotypes were observed in *C. belcheri*, *C. iredalei*, *S. cucullata*, *Crassostrea* sp., and *Saccostrea* sp. group 2, but not in *S. forskali*, *Striostrea mytiloides*, and *Saccostrea* sp. groups 1 and 3 (Tables 3 and 4).

Genetic distances between pairs of composite haplotypes within *Crassostrea* oysters (0.0039–0.0485) were generally lower than those within *Saccostrea* and *Striostrea* oysters (0.0029–0.0867). Haplotype and nucleotide diversity within species were low in *Crassostrea* oysters (0.1857–0.3889 and 0.0912%–0.3538%), but high diversity was observed in *Saccostrea* and *Striostrea* oysters (0.4000–0.9044 and 0.1447%–3.2388%; Table 5). Large nucleotide divergence (5.410%–11.272%) was observed between *Crassostrea* and small oysters (*Saccostrea* and *Stri-*

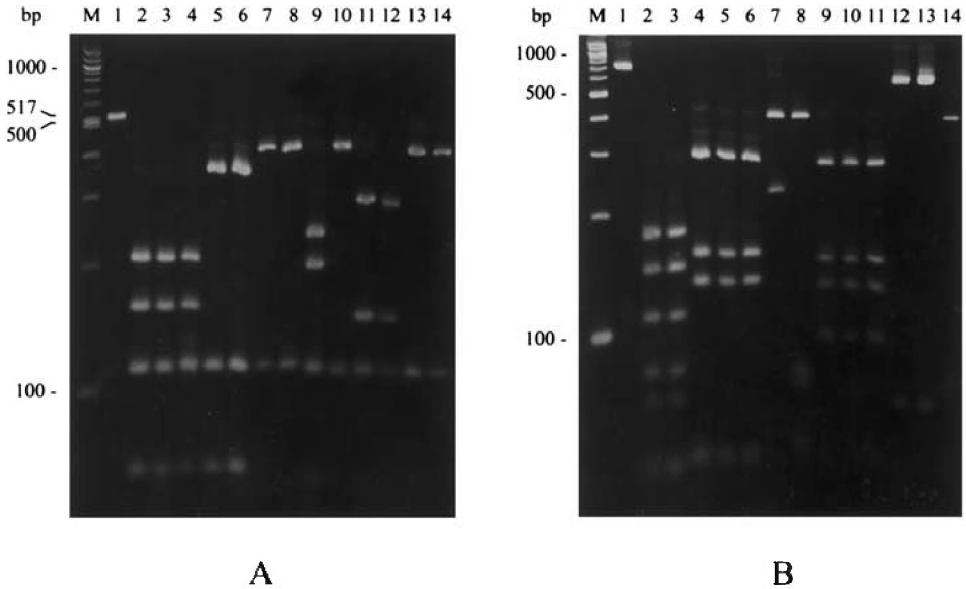

Fig. 2. An example of restriction patterns of amplified 16S rDNA of *C. belcheri* (pattern A, lanes 2–4), *C. iredalei* (B, lanes 5, 6), *Crassostrea* sp. (D, lanes 7, 8), *S. cucullata* (C, lane 9), *S. forskali* (D, lane 10), *Saccostrea* sp. group 2 (J, lanes 11, 12), and *Striostrea mytiloides* (D, lanes 13, 14) digested with *Acs*I (**A**) and COI of *C. belcheri* (pattern A, lanes 2, 3), *C. iredalei* (C, lanes 4–6), *Crassostrea* sp. (K, lane 7), *S. cucullata* (D, lane 8), *S. forskali* (F, lanes 9–11), *Saccostrea* sp. group 2 (M, lanes 12, 13), and *Saccostrea* sp. group 1 (G, lane 14) digested with *Mbo*I (**B**). Lanes M and 1 are a 100-bp DNA marker and undigested 16S rDNA (A) and COI (B), respectively.

ostrea), but lesser divergence was found within each group (5.004%–7.836% and 0.432%–6.819% for *Crassostrea* and *Saccostrea* oysters, respectively; Table 6).

Neighbor-joining trees constructed from genetic distances between pairs of composite haplotypes and nucleotide divergence between species indicated well separated allocation of *C. belcheri*, *C. iredalei*, and *Crassostrea* sp., but close relationships between *Saccostrea* and *Striostrea* oysters (Figures 3 and 4). Geographic heterogeneity analysis revealed a lack of population differentiation in *C. belcheri*, *C. iredalei*, and *S. cucullata* ($P > 0.0083$ following a sequential Bonferroni procedure; Rice, 1989), but significant heterogeneity was observed in *S. forskali* ($P < 0.0001$) and *Striostrea mytiloides* ($P < 0.0001$). All investigated oysters exhibited significant genetic differences interspecifically ($P < 0.0001$) except between *Striostrea mytiloides* and *S. forskali* ($P = 0.1148$) and *Saccostrea* sp. group 3 ($P = 0.5475$).

Discussion

Oysters are highly variable in form and can display ecomorphological variations (Littlewood, 1994). Their external characteristics (e.g., shell morphology) are influenced by a variety of habitats and environmental conditions (Tack et al., 1992). Accordingly, classification of oysters based on morphological characters alone is often insufficient for taxonomic identification.

Although several molecular genetic studies of oysters have been carried out (Hedgecock and Okazaki, 1984; Reeb and Avise, 1990; Karl and Avise, 1992, 1993; Banks et al., 1993; Anderson and Adlard, 1994; Littlewood, 1994, Foltz and Hu, 1996; Small and Chapman, 1997; Hare and Avise, 1998), knowledge about levels of genetic diversity and population differentiation of oysters in Thai waters is rather limited. Previously, genetic diversity and species-specific markers of local oysters in Thailand were examined by RAPD analysis (Klinbunga et al., 2000, 2001), which is less reliable than data from RFLP analysis of 18S rDNA and COI (Klinbunga et al., 2003). In the present study we extended the information by digestion of 16S rDNA gene segment with 6 restriction enzymes (*Acs*I, *Alu*I, *Dde*I, *Dra*I, *Rsa*I, and *Taq*I) to identify more species-specific markers in 3 commercially important species (*C. belcheri*, *C. iredalei*, and *S. cucullata*), clarified newly unidentified oyster species (*Crassostrea* sp. and *Saccostrea* sp. group 2), and revealed the existence of population differentiation between main fisheries regions (the Andaman Sea and Gulf of Thailand) of *S. forskali* and *Striostrea mytiloides*, for which no data are available at present.

Ó Foighil et al. (1995) sequenced 16S rDNA (433 bp) of *C. virginica*, *C. gigas*, and *C. ariakensis* and illustrated interspecific sequence divergence of 4.9% to 16.0%. Several restriction site differences

Table 2. Restriction Fragment Patterns Resulting from Digestion of 16S rDNAs of Oysters in Coastal Thai Waters with Restriction Endonucleases[a]

Gene segment and enzyme	*Pattern observed[b] (bp)*
16S rDNA–*Acs*I	A: 220, 160, 120, 60
	B: 380, 120, 60
	C: 240, 200, 120
	D: 430, 120
	E: 560
	F: 280, 150, 80 (50)
	G: 320, 150, 80
	H: 230, 150, 120 (50)
	I: 280, 150, 120
	J: 300, 150, 120
	K: 400, 140
	L: 260, 150, 120 (20)
16S rDNA–*Alu*I	A: 420, 80, 60
	B: 250, 170, 80, 60
	C: 250, 180, 70, 60
	D: 250, 180, 130
	E: 170, 165, 85, 80, 60
	F: 250, 170, 130
	G: 250, 170, 70 (60)
	H: 250, 90, 80, 70 (60)
	I: 210, 170, 100, 80
	J: 250, 180, 60, 50
	K: 250, 140, 130
	L: 250, 170, 115
16S rDNA–*Dde*I	A: 430, 105
	B: 230, 220, 105
	C: 500, 50
	D: 300, 135, 70 (50)
	E: 300, 190, 70
	F: 430, 70 (50)
	G: 430, 65 (50)
	H: 210, 105, 95, 80, 75
	I: 430, 60, 50
16S rDNA–*Dra*I	A: 560
	B: 330, 220
	C: 295, 250
	D: 250, 210, 80
	E: 250, 240
	F: 320, 205
16S rDNA–*Rsa*I	A: 560
	B: 500 (60)
	C: 350, 200
	D: 210, 160, 80
16S rDNA–*Taq*I	A: 360, 85, 70 (60)
	B: 430, 70 (60)
	C: 425, 70, 60

[a]For 18S rDNA and COI results, see Klinbunga et al., 2003 (Table 2).
[b]Numbers in parentheses indicate missing bands. When possible, a single band of their sum was inferred.

among these oysters were found, and PCR-RFLP distinguished these oysters unambiguously.

Several species-diagnostic RAPD markers of commercially important oysters *C. belcheri*, *C. iredalei*, and *S. cucullata* in coastal Thai waters had been previously identified (Klinbunga et al., 2001, 2003). In addition, Amparyup (1999) had examined genetic diversity and molecular diagnostic markers of 4 groups of taxonomically unclear oysters that could only be differentiated at the genus levels (*Crassostrea* sp., *Saccostrea* sp. groups 1, 2, and 3 in this study) using 5 RAPD primers (OPA01, OPA09, OPB08, UBC210, and UBC220). One and two diagnostic markers for *Saccostrea* sp. group 2 (2300-bp RAPD fragment from OPB08) and *Crassostrea* sp. (1400-bp and 2100-bp RAPD fragments from UBC210 and UBC220, respectively) were identified. Likewise, molecular diagnostic RFLP markers based on both single and composite haplotypes were found in those oysters, but not in *S. forskali*, *Striostrea mytiloides*, *Saccostrea* sp. groups 1 and 3, as previously analyzed by RAPD analysis.

Distribution patterns of composite haplotypes in *C. belcheri*, *C. iredalei*, *S, cucullata*, *Crassostrea* sp., and *Saccostrea* sp. group 2 were different from those of *S. forskali*, *Striostrea mytiloides*, and *Saccostrea* groups 1 and 3. A common composite haplotype with a fixed (or high) frequency was observed in the former group, whereas the deficiency of fixed haplotypes and the presence of many unique haplotypes at low frequencies were observed in the latter group.

Notably, sample sizes of each species in this study were relatively small, but samples were collected from different geographic locations on different coastal sides of peninsular Thailand. This should be sufficient for identification of species-specific markers, but a larger number of specimens is still required for more accurate evaluation of genetic diversity of these oysters. The 18S rDNA haplotype is monomorphic within species and only variable between genera. As a result, including these within the composite rnitochondrial haplotypes is acceptable.

Haplotype and nucleotide diversity within *Saccostrea* and *Striostrea* oysters was greater than that within *Crassostrea* oysters, indicating high genetic diversity of small oysters. These results were concordant with those based on morphological studies (Brock, 1990), allozyme electrophoresis (Buroker et al, 1979; Hedgecock, 1995; Visootiviseth et al., 1998), and RAPD analysis (Klinbunga et al., 2001). Haplotype diversity of *Saccostrea* oysters in this study was as high as that previously reported in other mollusks, such as the American oyster *C. virginica* (0.80) from the Gulf of Mexico (Reeb and Avise, 1990) and the Japanese scallop *Patinopecten yessoensis* (0.66) (Boulding et al., 1993), based on restriction analysis of the entire mtDNA. Likewise, haplotype and nucleotide diversity of *C. belcheri* (0.2279 and 0.0945%) and *C. iredalei* (0.1857 and 0.0912%) were comparable to those of *C. virginica* (0.1079 and 0.1309%) analyzed by PCR-RFLP of the

Table 3. Molecular Markers for Taxonomy of Cupped Oysters in Thailand Based on RAPD and PCR-RFLP Analyses

Primers/restriction enzyme	*Genus and species*	*RAPD markers/ mitotype (bp)*	*Reference*
OPA09	*C. belcheri*	250	Klinbunga et al. (2001)
	C. iredalei	1150	"
OPB01	*C. belcheri*	2100, 1400, 1250, 650	"
	C. iredalei	700	"
OPB08	*C. belcheri*	1650, 1550, 835, 600	"
	C. iredalei	1250, 450	"
	S. cucullata	750	"
	Saccostrea sp. group 2	2300	Amparyup (1999)
UBC210	*Crassostrea* sp.	1400	"
UBC220	*S. cucullata*	1800	"
	Crassostrea sp.	2100	Amparyup (1999)
18S rDNA–*Hinf*I	*Crassostrea*	A: 550, 130, 120	Klinbunga et al. (2003)
	Saccostrea and *Striostrea* (*Parastriostrea*) *mytiloides*	B: 300, 250, 130, 120	"
	P. viridis	C: 550, 130, 90	"
COI-*Mbo*I	*C. belcheri*	A: 180, 150, 120, 80, 70, 55	"
		B: 290, 150, 80, 70, 55	"
	C. iredalei	C: 290, 160, 140, 60	"
		U: 420, 160, 60	"
	S. cucullata	D: 420, 78, 75, 60	"
		E: 495, 75, 60	"
	Crassostrea sp.	K: 420, 250	"
		L: 420, 190, 60	"
COI-*Mbo*I	*Saccostrea* sp. group 2	M: 600, 70	Klinbunga et al. (2003)
		S: 495, 130, 70	"
		V: 600, 95	"
COI-*Dde*I	C. *belcheri*	A: 420, 170, 120	"
	C. iredalei	B: 590, 125	"
	S cucullata	C: 320, 260, 125	"
	Crassostrea sp.	F: 430, 220, 60	"
	Saccostrea sp. group 2	G: 280, 250, 125	"
16S rDNA–*Acs*I	C. *belcheri*	A: 220, 160, 120, 60	This study
	C. iredalei	B: 380, 120, 60	"
	Saccostrea sp. group 2	J: 300, 150, 120	"
16S rDNA–*Alu*I	C. *belcheri*	A: 420, 80, 60	"
	S. cucullata	C: 250, 180, 70, 60	"
		D: 250, 180, 130	"
	Crassostrea sp.	I: 210, 170, 100, 80	"
	Saccostrea sp. group 2	J: 250, 180, 60, 50	"
16S rDNA–*Dde*I	*Crassostrea* sp.	H: 210, 105, 95, 80, 75	"
16S-*Dra*I	*C. iredalei*	B: 330, 220	"

16S rDNA with 10 restriction endonucleases (*Rsa*I, *Msp*I, *Hha*I, *Taq*I, *Mse*I, *Hae*III, *Mbo*I, *Hin*fI, *Hin*dIII, and *Hin*cII (Small and Chapman, 1997).

Saccostrea oysters in Thailand had been recognized as *S. cucullata* (Amornjaruchit, 1988). The existence of *S. glomerata* (Brohmanonda et al., 1988; Tookwinas, 1991) and *S. mordax* in Thailand had subsequently been reported (Yoosukh, 2000). Day et al. (2000) used allozymes and shell morphology to distinguish sympatric species of the rock oyster *Saccostrea* in Thailand and identified *S. glomerata*, *S. cucullata*, and *S. manilai*. Our results based on PCR-RFLP ($N = 6$) and RAPD analysis ($N = 12$, Klinbunga et al., 2001) of the Australian *S. glomerata* showed several fixed markers in *S. glomerata*, but these DNA markers were not found in any oyster of the present study, suggesting that the existence of *S. glomerata* in Thailand is still questionable.

Ó Foighil et al. (1998) used polymorphism of COI sequence (579 bp) to study molecular phylogeny of the Portuguese oyster (*C. angulata*). Results showed that *C. angulata* robustly clustered between Portuguese oyster haplotypes within a clade of congeneric Asian oysters and was close, but not identical, to *C. gigas* from Japan. The data first indicated that Portuguese oysters are genetically different from geographically representative samples of Japanese Pacific oysters, which is consistent with a recent introduction of *C. angulata* to Europe either from a non-Japanese Asian source population or from subsequent displacement of the Japanese source population.

Table 4. Frequencies of Composite Haplotypes of 16S and 18S rDNAs and COI Across Investigated Oyster Species and Outgroup Reference *P. viridis*[a]

Composite haplotype		*Cb* (*N* = 17)	*Ci* (*N* = 21)	*Sc* (*N* = 23)	*Sf* (*N* = 46)	*Sm* (*N* = 17)	*Csp* (*N* = 9)	*S1SRE* (*N* = 8)	*S2RNW* (*N* = 9)	*S3SSE* (*N* = 5)	*Sglo* (*N* = 6)	*Pevi* (*N* = 5)
I	AAAAAAAAAA	0.882	—	—	—	—	—	—	—	—	—	—
II	AAAAAAAAAB	0.059	—	—	—	—	—	—	—	—	—	—
III	AABAAAAAAA	0.059	—	—	—	—	—	—	—	—	—	—
IV	BBBBACAABC	—	0.048	—	—	—	—	—	—	—	—	—
V	BBBBAAAABC[b]	—	0.904	—	—	—	—	—	—	—	—	—
VI	BBBBAAAABU	—	0.048	—	—	—	—	—	—	—	—	—
VII	CDCCBBBBCD	—	—	0.565	—	—	—	—	—	—	—	—
VIII	CCCCBBBBCE	—	—	0.087	—	—	—	—	—	—	—	—
IX	CCCCBBBBCD	—	—	0.261	—	—	—	—	—	—	—	—
X	CDCCBBBBCE	—	—	0.044	—	—	—	—	—	—	—	—
XI	DDCCBBBBCE	—	—	0.044	—	—	—	—	—	—	—	—
XII	EEDCBABCDF	—	—	—	0.022		—	—	—	—	—	—
XIII	DEDCBABCDF[c]	—	—	—	0.356	0.235	—	—	—	0.800	—	—
XIV	DEDABABCDF	—	—	—	0.156	0.059	—	—	—	0.200	—	—
XV	GFGCBABCJF	—	—	—	0.022	—	—	—	—	—	—	—
XVI	GFGCBABAJF	—	—	—	0.022	—	—	—	—	—	—	—
XVII	FFFCBBBAJF	—	—	—	0.067	—	—	—	—	—	—	—
XVIII	DFFABBBAIR	—	—	—	0.022	—	—	—	—	—	—	—
XIX	FFFABBBAJF	—	—	—	0.022	—	—	—	—	—	—	—
XX	IFFCBBBCEH	—	—	—	0.022	—	—	—	—	—	—	—
XXI	DEECBABCDF	—	—	—	0.022	—	—	—	—	—	—	—
XXII	DEDABABDDF	—	—	—	0.022	—	—	—	—	—	—	—
XXIII	DFGABABAHP	—	—	—	0.022	—	—	0.111	—	—	—	—
XXIV	DFGABBBAIR	—	—	—	0.022	—	—	—	—	—	—	—
XXV	DFDABABADF	—	—	—	0.022	—	—	—	—	—	—	—
XVI	DFDABABCDF	—	—	—	0.022	—	—	—	—	—	—	—
XVII	DFFCBBBAHR	—	—	—	0.022	—	—	—	—	—	—	—
XVIII	DKFABBBAHR	—	—	—	0.022	—	—	—	—	—	—	—
XXIX	DKFCBABAHR	—	—	—	0.022	—	—	—	—	—	—	—
XXX	DEDCBABAHF	—	—	—	0.022	—	—	—	—	—	—	—
XXXI	DFFCBABCHF	—	—	—	0.022	—	—	—	—	—	—	—
XXXII	DFFCBABAHF	—	—	—	0.022	—	—	—	—	—	—	—
XXXIII	DKFCBBBAHF	—	—	—	0.022	—	—	—	—	—	—	—
XXXIV	DEDCBABCDI	—	—	—	—	0.059	—	—	—	—	—	—
XXXV	DFFDBBBAKJ	—	—	—	—	0.059	—	—	—	—	—	—
XXXVI	HHFDBBBAKJ	—	—	—	—	0.059	—	—	—	—	—	—
XXXVII	IHFDBBBAKJ	—	—	—	—	0.118	—	—	—	—	—	—
XXXVIII	DGFDBBBAKJ	—	—	—	—	0.059	—	—	—	—	—	—
XXXIX	LHFDBBBAKJ	—	—	—	—	0.235	—	—	—	—	—	—
XL	DBDCBABCDF	—	—	—	—	0.059	—	—	—	—	—	—
XLI	DBDABABCDF	—	—	—	—	0.059	—	—	—	—	—	—
XLII	DIHABCAAFK	—	—	—	—	—	0.778	—	—	—	—	—
XLIII	CIHABCAAFL	—	—	—	—	—	0.222	—	—	—	—	—
XLIV	JJFCBBBBGM	—	—	—	—	—	—	—	0.778	—	—	—
XLV	JJFCBBBBGS	—	—	—	—	—	—	—	0.111	—	—	—
XLVI	JJFCBBBBGV	—	—	—	—	—	—	—	0.111	—	—	
XLVII	DFGCBBBAHQ	—	—	—	—	—	—	0.111	—	—	—	
XLVIII	DKGCBBBAHP	—	—	—	—	—	—	0.111	—	—	—	
XLIX	DFFCBBBAIG	—	—	—	—	—	—	0.333	—	—	—	
L	DKFCBBBAIG	—	—	—	—	—	—	0.111	—	—	—	
LI	DFFCBBBAIO	—	—	—	—	—	—	0.222	—	—	—	
LII	LFCCBBBELN	—	—	—	—	—	—	—	—	—	0.833	
LIII	LFCEBBBELN	—	—	—	—	—	—	—	—	—	0.167	
LIV	KLIFCDCFMT	—	—	—	—	—	—	—	—	—	—	1.000

[a]Composite haplotypes were constructed from each restriction pattern and arranged from that of 16S rDNA–*Acs*I, *Alu*I, *Dde*I, *Dra*I, *Taq*I, and *Rsa*I; 18S rDNA-*Hin*fI; and COI-*Acs*I, *Dde*I, and *Mbo*I, respectively. Cb indictaes *C. belcheri*, Ci, *C. iredalei*; Sc, *S. cucullata*; Sf, *S. forskali*; Sm, *Striostrea* (*Parastriostrea*) *mytiloides*; Csp, *Crassostrea* sp.; S1SRE, *Saccoatrea* sp. group 1; S2RNW, *Saccostrea* sp. group 2; S3SSE, *Saccostrea* sp. group 3; Sglo, *S. glomerata*; and Pevi, *P. viridis*.

[b]Composite haplotypes possessed by Oys039 and Oys104.

[c]Composite haplotypes possessed by Oys30. Oys095 exhibited a BBBBBAAABC composite haplotype.

Table 5. Haplotype and Nucleotide Diversity Within Indigenous Oysters, Ingroup *S. glomerata*, and Outgroup *P. viridis* from Restriction Analysis of 16S rDNA, 18S rDNA, and COI

Species	*Haplotype diversity (h ± SE)*	*Nucleotide diversity (×100)*
C. belcheri	0.2279 ± 0.1295	0.0945
C. iredalei	0.1857 ± 0.1102	0.0912
S. cucullata	0.6285 ± 0.0872	0.3501
S. forskali	0.8545 ± 0.0454	2.8711
Striostrea mytiloides	0.9044 ± 0.0497	3.2388
Crassostrea sp.	0.3889 ± 0.1644	0.3538
Saccostrea sp. group 1	0.8889 ± 0.0910	1.0858
Saccostrea sp. group 2	0.4167 ± 0.1907	0.1447
Saccostrea sp. group 3	0.4000 ± 0.2373	0.1828
S. glomerata	0.3333 ± 0.2152	0.0862
P. viridis	0.0000 ± 0.00000	0.0000
Average	0.4753 ± 0.0085	0.7726 ± 0.0012

Haplotype and species neighbor-joining trees indicated clear differentiation between *Crassostrea* and *Saccostrea* oysters. Large genetic differences between each of *Crassostrea* oysters were observed. Classical taxonomy (Vaught, 1989; Yoosukh and Duangdee, 1999) indicated that *Crassostrea* sp. should have been the juvenile stages of either *C. belcheri* or *C. iredalei*. Nevertheless, these oysters possessed haplotypes XLII (DIHABCAAFK) and XLIII (CIHABCAAFL), which were not available in other Thai oysters. Additionally, species-specific composite haplotypes (this study) and RAPD markers (Klinbunga et al., 2001) of *C. belcheri* and *C. iredalei* were not found in *Crassostrea* sp., indicating that this group of oysters should have been a newly unidentified *Crassostrea* species in Thailand. The actual scientific name of this oyster should be further clarified by taxonomists.

Taxonomically complex status was observed in small oysters. While *S. cucullata* and *Saccostrea* sp. group 2 (S2RNW) were closely related phylogenetically, *S. forskali* and *Striostrea mytiloides* could be differentiated into several subgroups. The phylogenetic complexity of these species reflected difficulties with taxonomy of *Saccostrea* oysters based on morphology.

On the basis of 18S rDNA and COI polymorphism, Klinbunga et al. (2003) suggested that *Striostrea mytiloides* (formerly called *Saccostrea echinata*) should be recognized as a member of *Saccostrea* rather than *Striostrea*. Results of the present study further suggested that *S. forskali* and *Striostrea mytiloides* may not only be members of the same genus but also be conspecific. Further studies based on both morphological and molecular data must be extensively examined before this conclusion can be drawn unambiguously.

According to morphology, *Saccostrea* sp. group 1 was a *S. forskali*–like oyster exhibiting composite haplotypes closely related to those of *S. forskali* but did not show any fixed marker. Therefore, it should be regarded as *S. forskali* exhibiting ecomorphological variation. Conversely, 3 composite haplotypes (XLIV, XLV, and XLVI) were specifically observed in *Saccostrea* sp. group 2. The 750-bp (OPB08) and 1800-bp (UBC220) RAPD fragments were not observed in these specimens (Klinbunga et al., 2001), indicating that they are not *S. cucullata*. Several fixed RFLP patterns (this study) and RAPD fragments (Amparyup, 1999) were observed, suggesting that it should have been a newly unidentified *Saccostrea* species in Thailand.

In addition, *Saccostrea* sp. group 3 exhibited both *S. forskali* and *Striostrea mytiloides* morphological characters. Lack of species-specific RAPD and RFLP markers in *S. forskali* and *Striostrea mytiloides* prevented accurate assignment of the species origin for this oyster. Phylogenetic analyses did not differentiate *Saccostrea* sp. group 3 from *S. forskali* and *Striostrea mytiloides*. Geographic heterogeneity analysis revealed significant genetic differences be-

Table 6. Percentage of Nucleotide Divergence (Below Diagonal) Between Species from Restriction Analysis of 16S rDNA, 18S rDNA, and COI of Local Oysters, Ingroup *S. glomerata* and Outgroup *P. viridis*

	Cb	*Ci*	*Sc*	*Sf*	*Sm*	*Csp*	*S1SRE*	*S2RNW*	*S3SSE*	*Sglo*	*Pevi*
Cb	—										
Ci	5.004	—									
Sc	11.190	8.877	—								
Sf	7.913	5.628	4.777	—							
Sm	7.130	5.410	4.285	0.716	—						
Csp	7.836	7.154	9.601	7.323	7.339	—					
Ssp 1	9.017	7.295	2.214	2.672	2.430	7.267	—				
Ssp 2	9.475	9.300	2.869	4.037	3.334	11.272	3.223	—			
Ssp 3	9.088	6.482	6.819	0.432	1.450	8.359	5.040	5.812	—		
Scom	10.398	9.433	3.898	4.539	3.714	9.914	3.134	4.175	6.653	—	
Pevi	13.051	10.823	11.730	10.746	10.243	12.340	9.327	10.998	12.891	11.119	—

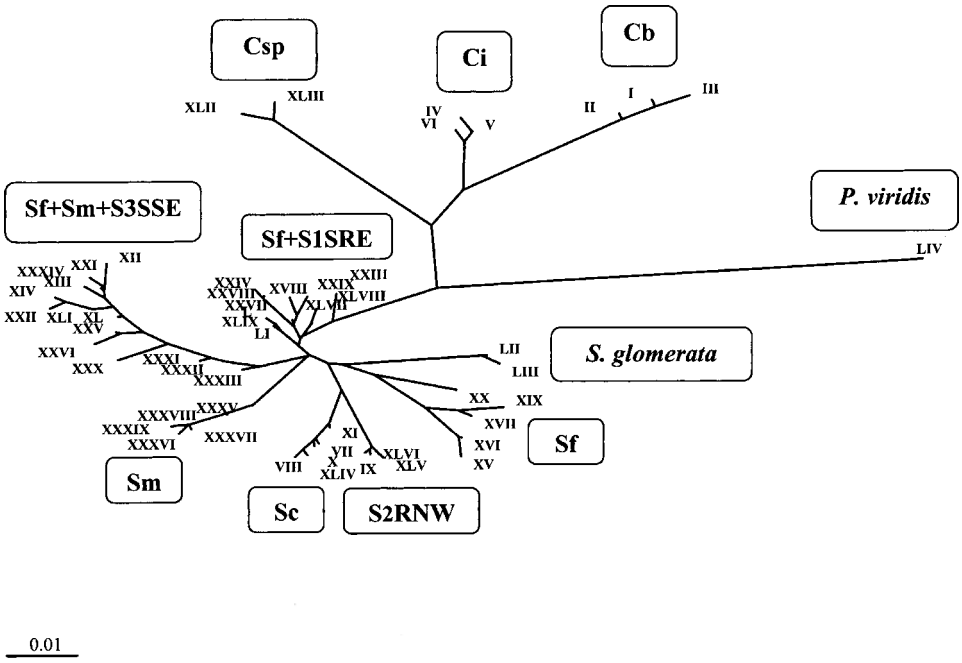

Fig. 3. Neighbor-joining tree summarizing genetic relationships of composite haplotypes of Thai oysters, an ingroup reference (*S. glomerata*), and an outgroup reference (*P. viridis*), constructed from the average genetic distance between pairs of composite haplotypes.

tween *Saccostrea* sp. group 3 and *S. forskali* ($P < 0.0001$) and *Striostrea mytiloides* from the Gulf of Thailand ($P < 0.0001$), but not that from the Andaman Sea ($P = 0.5475$). Therefore, the species status of *Saccostrea* sp. group 3 still cannot be concluded (for external morphology of oysters used in this study, see Yoosukh and Duangdee, 1999).

Interspecific hybridization between *Crassostrea* oysters (e.g., between *C. gigas* and *C. rivularis*) resulted in viable and fertile progeny (Allen and Gaffney, 1993). In addition, intergeneric hybridization between *C. iredalei* and *S. cucullata* were also successfully carried out under laboratory conditions. Shell morphology of the hybrids was intermediate between parental species (Charoensit, 1995). This

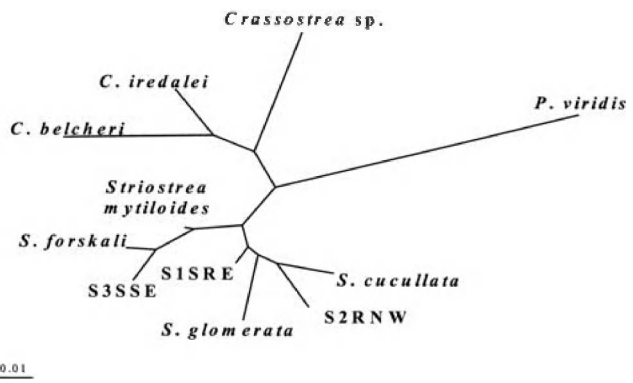

Fig. 4. Neighbor-joining tree summarizing genetic relationships of Thai oysters, an ingroup reference (*S. glomerata*), an outgroup reference (*P. viridis*), constructed from the percentage of nucleotide divergence between species.

information indicates further taxonomic difficulties created for oysters through hybridization.

Several oysters (Oys030, Oys039, Oys095, and Oys104) could not be clearly assigned their species origin (e.g., shell morphology, pigment of adductor muscle scar). Using RAPD and RFLP analysis, the species status of these specimens could be clarified. Oys030, Oys039, and Oys104, which were identified as *C. iredalei*–like oysters, showed *C. iredalei*–specific RAPD fragments (for species-specific RAPD markers of Oys30, see Figure 3, B, lane 8, in Klinbunga et al., 2001). The composite haplotype of Oys030 was XIII (DEDCBABCDF), which is commonly found in *S. forskali* and *Striostrea mytiloides*. This specimen was then regarded as an introgressive hybrid between *C. iredalei* and *S. forskali* (or *Striostrea mytiloides*) for which *S. forskali* (or *Striostrea mytiloides*) served as the maternal species.

Oys39, Oys95, and Oys104 possessed pattern A of *Hin*fI-digested 18S rDNA, which confirmed their taxonomic status as *Crassostrea* oysters. In addition, Oys39 and Oys104 exhibited haplotype V (BBBBAAAABC), which was restricted to *C. iredalei* and revealed all *C. iredalei*–specific RAPD markers (Klinbunga et al., 2001). As a result, they are considered as *C. iredalei* showing ecomorphological variation. Oys095 was morphologically classified as a hybrid between *C. belcheri* and *C. iredalei*. Genotyping indicated that Oys95 possessed the BBBBBAAABC haplotype, which has only one mutation step from the common haplotype V (BBBBAAAABC). RAPD analysis of this specimen revealed all *C. ire-*

dalei–specific RAPD markers, but no *C. belcheri*–specific markers, suggesting that it was a pure *C. iredalei* oyster.

The basic information on numbers of species or populations of exploited species in a particular area is important for broodstock selection and breeding programs (Carvalho and Hauser, 1994). Moreover, knowledge on the genetic diversity of oysters is essential for the construction of an appropriate management scheme in these taxa. Our results illustrate the potential of PCR-RFLP for determination of genetic diversity, identification of species-specific markers, and examination of interspecific hybridization of Thai oysters. Species-diagnostic RFLP patterns can be utilized for identification of seed species, and distribution and recruitment of oyster larvae, leading to increasing efficiency in management of local oysters in Thailand.

Acknowledgments

The authors thank colleagues from the Department of Fisheries, Ministry of Agriculture and Cooperatives, for helping us collect specimens used in this study. We also thank the National Center for Genetic Engineering and Biotechnology (BIOTEC) and the National Science and Technology Development Agency (NSTDA) for providing facilities required by our experiments. This research was subsidized by Thailand Research Funds (TRF) project RDG4120018.

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