การสืบค้นเครื่องหมายทางพันธุกรรมระดับโมเลกุลสำหรับอนุกรมวิธานของหอยนางรมสกุล *CRASSOSTREA*, *SACCOSTREA* และ *STRIOSTREA* ในประเทศไทย

นางสาวณีรวรรณ คำน้ำทอง

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IDENTIFICATION OF MOLECULAR GENETIC MARKERS FOR TAXONOMY OF OYSTERS GENERA *CRASSOSTREA*, *SACCOSTREA* AND *STRIOSTREA* IN THAILAND

MISS NEERAWAN KHAMNAMTONG

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ณีรวรรณ คำน้ำทอง : การสืบค้นเครื่องหมายทางพันธุกรรมระดับโมเลกุลสำหรับอนุกรมวิธานของหอยนางรมสกุล *CRASSOSTREA*, *SACCOSTREA* และ *STRIOSTREA* ในประเทศไทย (IDENTIFICATION OF MOLECULAR GENETIC MARKERS FOR TAXONOMY OF OYSTERS GENERA *CRASSOSTREA*, *SACCOSTREA* AND *STRIOSTREA* IN THAILAND) อ.ที่ปรึกษา : รศ.ดร. เผดิมศักดิ์ จารยะพันธุ์, อ.ที่ปรึกษาร่วม : ดร. ศิราวุธ กลิ่นบุหงา 159 หน้า. ISBN 974-13-0262-2.

จากการศึกษาความหลากหลายทางพันธุกรรมของหอยนางรมในประเทศไทยจำนวน 5 ชนิดคือ *Crassostrea belcheri* (Sowerby, 1871), *C. iredalei* (Faustino, 1932), *Saccostrea cucullata* (Born, 1778), *S. forskali* (Chemnitz, 1785) และ *Striostrea (Parastriostrea) mytiloides* (Lamarck, 1819) โดยเทคนิค PCR-RFLP ของยีน 16S rDNA (mitochondrial DNA) ด้วย *Acs* I, *Alu* I, *Dde* I, *Dra* I, *Rsa* I และ *Taq* I ของยีน COI (mitochondrial DNA) ด้วย *Acs* I, *Dde* I และ *Mbo* I และยีน 18S rDNA (nuclear DNA) ด้วย *Hin*f I พบจำนวน composite haplotype ทั้งหมด 54 haplotypes โดยพบ composite haplotypes ที่จำเพาะต่อหอยนางรมที่มีการเพาะเลี้ยงในเชิงพาณิชย์ (*C. belcheri*, *C. iredalei* และ *S. cucullata*) เมื่อทำการวิเคราะห์ค่าความห่างทางพันธุกรรมระหว่างคู่ composite haplotypes พบว่าภายในสปีชีส์เดียวกันมีค่าความห่างทางพันธุกรรมต่ำกว่าระหว่างสปีชีส์ และจากการสร้างแผนภูมิความสัมพันธ์ในเชิงวิวัฒนาการโดยใช้วิธี neighbor-joining ที่สร้างจากค่า nucleotide divergence ระหว่างสปีชีส์พบว่าสามารถแยกหอยนางรมในสกุล *Crassostrea* และ *Saccostrea* (รวมทั้ง *S. mytiloides*) ออกจากกัน และพบว่าภายในหอยนางรมสกุลเดียวกันจะมีความสัมพันธ์ใกล้ชิดกันมากกว่าระหว่างสกุลที่ต่างกัน

เมื่อทำการวิเคราะห์พันธุกรรมหอยนางรมที่ไม่สามารถจำแนกชนิดได้แน่ชัดด้วยสัณฐานวิทยาจำนวน 4 กลุ่ม (*Crassostrea* sp., *Saccostrea* sp. กลุ่ม 1, 2 และ 3) พบว่า *Crassostrea* sp. และ *Saccostrea* sp. กลุ่ม 2 แสดงเครื่องหมายที่จำเพาะต่อสปีชีส์ทั้ง single และ composite haplotypes ในขณะที่ *Saccostrea* sp. กลุ่ม 3 แสดง composite haplotypes ที่พบใน *S. forskali* และ *S. mytiloides* และพบว่า *Saccostrea* sp. กลุ่ม 1 มี composite haplotypes DFGABABAHP ซึ่งพบใน *S. forskali* สำหรับ haplotypes ที่เหลือของสปีชีส์นี้ไม่พบในหอยนางรมชนิดอื่นๆแต่ haplotypes เหล่านี้มีความถี่ต่ำ

ทำการโคลนและหาลำดับเบสยีน COI จากตัวอย่างหอยนางรมชนิด *C. belcheri* ที่มี haplotype AAAAAAAAAA, ตัวอย่าง *C. iredalei* ที่มี haplotype แบบ BBBBAAAABC และตัวอย่าง *S. cucullata* ที่มี haplotype แบบ CDCCBBBBCD จากลำดับเบสที่ได้แสดงให้เห็นว่าชิ้นดีเอ็นเอที่นำมาโคลนนั้นเป็นยีน COI แน่นอนเนื่องจากมีความเหมือนกับลำดับเบสของยีน COI ของหอยนางรมอื่นๆในสกุล *Crassostrea*, *Saccostrea* และ *Ostrea* ใน GenBank อย่างมีนัยสำคัญทางสถิติ ($p < 0.0001$) แสดงว่าชิ้นดีเอ็นเอ COI จากหอยนางรมชนิดต่างๆที่ใช้ในการศึกษาเป็น homologous กัน ทำการออกแบบรีเวิร์สไพรเมอร์จำนวน 2 ไพรเมอร์ (R301 และ R353) นำมาใช้กับไพรเมอร์ HCO2198 เพื่อเพิ่มปริมาณดีเอ็นเออย่างจำเพาะด้วยเทคนิคพีซีอาร์ พบว่าผลผลิตพีซีอาร์ที่ได้จากทั้ง 2 ไพรเมอร์ใหม่มีความจำเพาะที่ดีกว่าการใช้ไพรเมอร์คู่เดิม โดยสามารถเพิ่มปริมาณดีเอ็นเอได้เฉพาะหอยนางรมชนิด *C. belcheri* และ *C. iredalei* แต่ไพรเมอร์ R353 ให้ผลดีกว่า R301 เมื่อตัดผลผลิตพีซีอาร์ที่ได้จาก R353 ด้วยเอนไซม์ตัดจำเพาะ *Mbo* I พบว่าสามารถจำแนกหอยนางรมชนิด *C. belcheri* และ *C. iredalei* ออกจากกันได้

หลักสูตร.....เทคโนโลยีทางชีวภาพ.....ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีทางชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2543.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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NEERAWAN KHAMNAMTONG : IDENTIFICATION OF MOLECULAR GENETIC MARKERS FOR TAXONOMY OF OYSTERS GENERA *CRASSOSTREA*, *SACCOSTREA* AND *STRIOSTREA* IN THAILAND. THESIS ADVISOR : ASSOC. PROF. PADERMSAK JARAYABHAND, Ph.D., THESIS CO-ADVISOR : SIRAWUT KLINBUNGA, Ph.D. 159 pp. ISBN 974-13-0262-2.

Genetic diversity of 5 oyster species of genera *Crassostrea* and *Saccostrea*; *Crassostrea belcheri* (Sowerby, 1871), *C. iredalei* (Faustino, 1932), *Saccostrea cucullata* (Born, 1778), *S. forskali* (Chemnitz, 1785) and *Striostrea (Parastriostrea) mytiloides* (Lamarck, 1819), were analyzed by PCR-RFLP of 16S (*Acs* I, *Alu* I, *Dde* I, *Dra* I, *Rsa* I and *Taq* I) and 18S (*Hinf* I) rDNAs and COI (*Acs* I, *Dde* I and *Mbo* I). A total of 54 composite haplotypes were observed. No overlapping haplotypes were found between different oyster species. Species-diagnostic composite haplotypes were specifically found in each commercially cultured oyster species (*C. belcheri*, *C. iredalei* and *S. cucullata*). Genetic distances between pairs of composite haplotypes within a particular species were lower than those between species. A neighbor-joining tree constructed from nucleotide divergence between pairs of species indicated large genetic differentiation between *Crassostrea* and *Saccostrea* (including *S. mytiloides*) but closer relationships were observed within each genus.

Four unidentified oyster groups (*Crassostrea* sp., *Saccostrea* sp. group 1, 2 and 3) were also genetically analyzed. Results from restriction analysis indicated that *Crassostrea* sp. and *Saccostrea* sp. group 2 showed unique species-diagnostic markers for both single and composite haplotypes whereas *Saccostrea* sp. group 3 showed composite haplotypes commonly found in *S. forskali* and *S. mytiloides*. The *Saccostrea* sp. group 1 showed composite haplotypes DFGABABAHP which was also found in *S. forskali*. The remaining haplotypes of this species were not found in other species but were available at low frequencies.

The amplified COI from a representative individual of *C. belcheri* showing AAAAAAAAAA haplotype, that of *C. iredalei* showing BBBBAAAABC haplotype and that of *S. cucullata* showing CDCCBBBBCD haplotype, were cloned and sequenced. The nucleotide sequences indicated that these cloned DNA fragment were COI as they exhibited significant similarity with COI sequences of other *Crassostrea*, *Saccostrea* and *Ostrea* oysters previously deposited in the GenBank ($p < 0.0001$). This further confirmed that the amplified COI fragments from different oyster species used in this study were homologous. Two reverse primers (R301 and R353) were designed and used with the primer HCO2198. Only a single fragment showing greater amplification specificity was observed. Although both primer set provided a specific amplification fragment for *C. belcheri* and *C. iredalei*, HCO2198+R353 gave more consistent results than did HCO2198+R301. Digestion of the HCO2198+R353 product could differentiate *C. belcheri* and *C. iredalei* unambigously.

Pragram……Biotechnology……Student's signature……
Field of study……...Biotechnology……Advisor's signature……..……...
Academic year……..2000……Co-advisor's signature……..

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LIST OF ABBREVIATIONS

ATP	=	adenosine triphosphate
bp	=	base pair
^{O}C	=	degree celcius
cm	=	centimetre
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine triphosphate
dTTP	=	deoxythymidine triphosphate
DNA	=	deoxyribonucleic acid
HCl	=	hydrochloric acid
IPTG	=	isopropyl-thiogalactoside
kb	=	kilobase
KCl	=	potassium chloride
L	=	length
$MgCl_2$	=	magnesium chloride
mg	=	milligram
ml	=	millilitre
mm	=	millimetre
M	=	molar
mtDNA	=	mitochondrial DNA
ng	=	nanogram
OD	=	optical density
PCR	=	polymerase chain reaction
pg	=	picogram
RAPD	=	randomly amplified polymorphic DNA
RFLP	=	restriction fragment length polymorphism

RNase A	=	ribonuclease A
rpm	=	revolution per minute
scnDNA	=	single copy nuclear DNA
SDS	=	sodium dodecyl sulfate
Tris	=	tris (hydroxy methyl) aminomethane
μg	=	microgram
μl	=	microlitre
μM	=	micromolar
U	=	unit
UV	=	ultraviolet
W/V	=	weight/volume

CHAPTER I
INTRODUCTION

Oysters are benthic marine species inhabiting near-shore, shallow water, bay and estuaries. They are economically important shellfish species. Nine species of Ostreoidea oysters including *Hyotissa hyotis* (Linneaus, 1758), *Parahyotissa (Parahyotissa) imbricata* (Lamark, 1819), *Lopha cristagalli* (Linnaeus, 1758), *Dendostrea folium* (Linnaeus, 1758), *Crassostrea belcheri* (Sowerby, 1871), *C. iredalei* (Faustino, 1932), *Saccostrea cucullata* (Born, 1778), *S. forskali* (Gmelin, 1791) and *Striostrea (Parastriostrea) mytiloides* (Lamarck, 1819), belonging to the subfamily Crassostreinae, were found in Thai waters (Yoosukh and Duangdee,1999). Nevertheless, only *Crassostrea*, *Saccostrea* and *Striostrea* oysters are commercially important.

Differentiation of these oysters at the genus level can be carried out using the chromata (ridges or tubercles along the margin of the shell). Oysters having this morphological character are regarded as members of genera *Saccostrea* or *Striostrea* whereas those do not exhibit this character are *Crassostrea* oysters. The latter can be further divided into *C. iredalei* (common Thai name, takrom kram dum) and *C. belcheri* (takrom kram khao) by the color of adductor muscle scars. If the scar is black, it is regarded as *C. iredalei*. The white scar oyster is *C. belcheri* (Visootiviseth et al., 1998). The small oysters (*Saccostrea* and *Striostrea*) are less economically important. The common Thai names for small oysters are hoy-joe, hoy-pakjip or hoy-tieb.

Culture of oysters in Thailand has been carried out for at least 50 years. Among local species, only *C. belcheri*, *C. iredalei* and *S. cucullata* are being cultured commercially. The production of these oysters (Table 1.1) was estimated to be approximately 1500 tons annually between 1986-1990 (Department of Fisheries, 1999a). The annual production of cultivated oysters had been limited owing to a lack of appropriate growing-out techniques and limitation and seasonal inconsistency of seed supply (Jarayabhand and Thavornyutikarn, 1995).

Laboratory scale production of *S. cucullata* (Jarayabhand et al., 1985) and large scale hatcheries of *C. belcheri* (Sahavacharin et al., 1988) were successfully developed. These provided the possibility to develop a sustainable breeding program to increase culture and management efficiency of Thai oysters, particularly *C. belcheri*, *C. iredalei* and *S. cucullata*.

Farming of oysters in Thailand has shown rapid development during the last few years. Although the oyster production from the aquaculture sector has been increased to approximately 20,000 tons annually (cultivated areas of 8,127 rai in 1996, see Table 1.2) since 1994, it still account for only 35% of the total production (Department of Fisheries, 1999b).

The highest production of cultured oysters was from Suratthani (mainly, *C. belcheri*) followed by Chonburi (mainly, *Saccostrea* species), Chanthaburi, Phang-nga and Trat (Table 1.2). The remaining cultured areas provide only 4-82 tons of cultured oysters annually.

Table 1.1 Production and value of oysters in Thailand between 1986-1996

Year	Production (tons)	Value (Mbaht)
1986	580	10.997
1987	1,483	17.486
1988	1,858	25.434
1989	1,399	23.466
1990	1,370	24.755
1991	3,311	49.291
1992	3,774	54.053
1993	17,810	576.383
1994	19,273	522.623
1995	23,037	591.983
1996	23,420	655.512

Source: Fisheries Statistics and Information Technology Sub-Division, Department of Fisheries, 1999a

Table 1.2 Cultured area, production and values of oysters from the aquaculture section in 1996

Province	Area (Rai)	Production (Tons)	Value (Mbaht)
Total	**8,127**	**23,420**	**655.512**
Coastal Zone 1 (Eastern part of the Gulf of Thailand)	2,006	2,886	32.156
Trat	776	670	18.111
Chanthaburi	1,230	2,216	14.045
Rayong	-	-	-
Coastal Zone 2 (Inner part of the Gulf of Thailand)	1,697	7,744	182.665
Chonburi	1,697	7,744	182.665
Chachoengsao	-	-	-
Samutprakan	-	-	-
Bangkok	-	-	-
Samutsakon	-	-	-
Samutsongkram	-	-	-
Phetchaburi	-	-	-
Coastal Zone 3 (Central part of the Gulf of Thailand)	4,357	10,888	390.653
Prachuapkhirikhan	18	82	2.375
Chumphon	299	24	0.122
Suratthani	4,040	10,782	388.156
Coastal Zone 4 (Southern part of the Gulf of Thailand)	3	13	0.081
Nakhon Si Thammarat	-	-	-
Songkhla	0	4	0.031
Phatthalung	-	-	-
Pattani	-	-	-
Narathiwat	3	9	0.050
Coastal Zone 5 (Andaman Sea)	64	1,889	49.957
Ranong	16	10	0.469
Phang-nga	48	1,879	49.488
Phuket	-	-	-
Krabi	-	-	-
Trang	-	-	-
Satun	-	-	-

Source: Fisheries Statistics and Information Technology Sub-Division, Department of Fisheries, 1999b

Knowledge on genetic variation levels of commercial oysters in Thailand is important for the construction of appropriate breeding programs and management scheme of Thai oysters. However, relative little is known about inter- and intraspecific genetic variability of oysters in Thai waters. Moreover, identification of genetic markers that would assist selection of appropriate broodstock species for aquaculture production and studies of larval distribution patterns and recruitment of Thai oysters should be carried out. This information would enhance aquaculture output without adversely affecting native populations leading to sustainable farming activity of these taxa.

At present, taxonomic difficulties oysters in Thai waters has limited culture efficiency and development of closed life-cycle culture of these species. Classification of these taxa has been carried out base principally on morphological characters. However, oysters are variable in forms. The external characteristics (e.g. shell morphology) are influenced by a variety of habitats and environmental conditions (Tack et al., 1992). Accordingly, two sympatric species may be morphologically similar and misidentified to be a single species (e.g. between *C. gigas* and *C. sikamea*). On the other hand, allopatric populations inhabiting different habitats may show variation in shell morphology but the species status is still questionable (e.g. between *S. commercialis* and *S. glomerata*). Thus, species-specific markers of commercially cultured oysters and phylogeny of oysters in Thailand are required at present.

1.1 Taxonomy of oysters

The taxonomic definition of Thai oysters used in this study is as follows (Vaught, 1989);

Phylum: Mollusca
Class: Bivalvia
Subclass: Pteriomorphia
Order: Ostreoida
Suborder: Ostreina
Superfamily: Ostreoidea
Family: Ostreidae (oysters)
Subfamily: Crassostreinae
Crassostrea belcheri (Sowerby, 1871)
Crassostrea iredalei (Faustino, 1932)
Crassostrea sp.
Saccostrea cucullata (Born, 1778)
Saccostrea forskali (Gmelin, 1791)
Striostrea (Parastriostrea) mytiloides (Lamarck, 1819), formerly called *Saccostrea echinata* (Torigoe, 1981)
Saccostrea sp. (group 1, 2 and 3)

1.2 Shell morphology

Taxonomy of oysters is based primarily on shell morphology (Figure 1.1). The shell characters that use to differentiate the species were general size, shape, radical ribs, commissural plication, hyote spines, chomata, commissural shelf, shape and color of adductor muscle scar, umbonal cavity and attachment areas of the left valve.

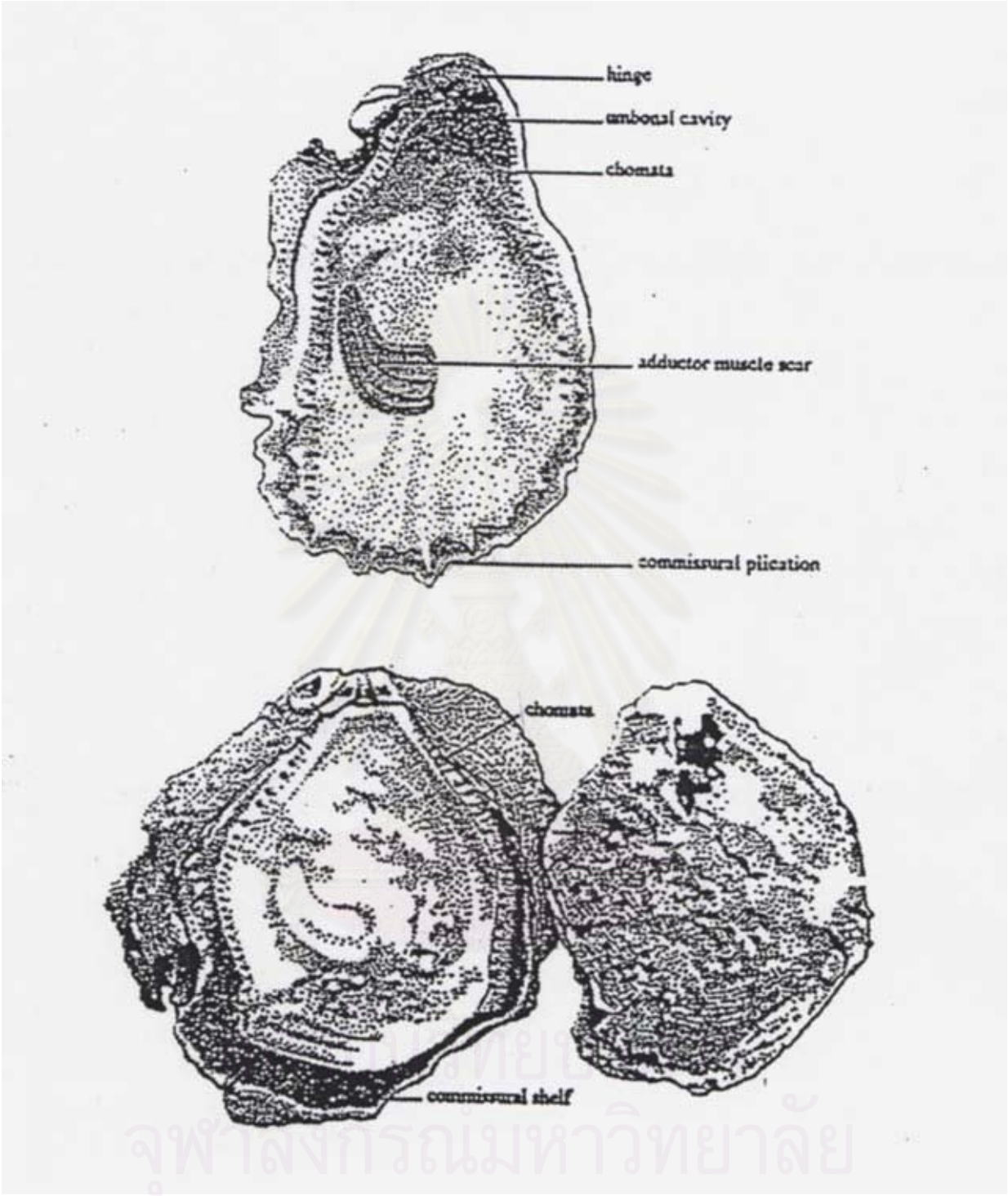

Figure 1.1 General shell morphology of oysters (Yoosukh, unpublished data)

1.2.1 *Crassostrea belcheri* (Figure 1.2A)

Synonym: *Ostrea belcheri* (Sowerby, 1871)

English common names: Belcher's oyster, white-scar oyster

Thai common name: Takrom kram khao

Characteristics: Shell is large (H: 13.0 cm x L: 9.0 cm), thick, oval to triangular. The external surface is lamellose. An interior chalky is white. The chomata is absent. The adductor muscle scar is white and cresent in shape. The commissural shelf is narrow. The attachment area is medium in size.

1.2.2 *Crassostrea iredalei* (Figure 1.2B)

Synonym: *Ostrea iredalei* (Fanstino, 1932)

Ostrea lugubris (Sowerby, 1871)

Crassostrea lugubris (Bernard et al., 1993)

English common names: Slipper-shaped oyster, black-scar oyster

Thai common name: Takrom kram dum

Characteristics: Shell is medium (H: 7.0 cm x L: 5.5 cm), round to elongate. The left valve is concave with shallow umbonal cavity. The right valve is almost flat covered with thin lamellae, scaly at margin. An inner surface is smooth, iridescent with patches of chalky white. The adductor muscle is kidney-shaped, purple in both valves, located near the posterior margin. The chomata is absent and the commissural shelf is narrow. The attachment area is small to medium.

1.2.3 *Saccostrea cucullata* (Figure 1.3A)

Synonym: *Ostrea cucullata* (Born, 1780); (Hamley, 1856); (Küster & Kobelt, 1868)

Ostrea mordax (Saville-Kent, 1891)

Saxostrea amasa (Iredale, 1939)

Crassostrea amasa (Thomson, 1954); (Carreon, 1969)

Saxostrea mordax (Kira, 1965)

Saxostrea parasitica (Habe & Kosuge, 1979)

Saxostrea mordax (Torigoe, 1981)

English common names: Hooded oyster, rock oyster

Thai common names: Hoy nang rom pak jip, hoy tieb, hoy joh

Characteristics: The shell is small size, thick, outline subtriangular to elongate. The shell margin is crenulated, and the hinge line is straight and short. The left valve is usually completely attached and deeply cupped with distinct umbonal cavity. The right valve is rather flat. The exterior is dull white or light purple with purplish black streaks and may be eroded. The interior is opaque white with dark purple margin. The adductor muscle scar is elliptical, white or stained with purple. The muscle scar may be dark in the right valve and white in the left valve of the same specimen. The chomata is well developed, with rod-shaped, and may extend partially or completely around valves. The commissural shelf is not developed. The attachment area is large.

1.2.4 *Saccostrea forskali* (Figure 1.3B)

Synonym: *Ostrea forskali* (Chemnitz, 1785); (Gmelin, 1790); (Küster & Kobelt, 1868); (Sacco, 1897)

Ostrea cucullata var. *forskali* (Lynge, 1909)

Ostrea cucullata (Awati & Rai, 1931)

Saccostrea forskali (Arakawa, 1990)

English common names: Indian rock oyster, Bombay rock oyster

Thai common names: Hoy nang rom pak jip, hoy tieb, hoy joh

Characteristics: The shell is small to medium size, and variable in shape. The shell margin irregularly crenulate. The left valve is concave forming a cup shape, with a distinct umbonal cavity. The right valve has thin lamellae at periphery. The external color is purplish white. The interior is dirty white, with dark purple staining at margin. The adductor muscle scar is kidney-shaped, of alternate strips of white and brown. The chomata is appear as tubercles arranged in

a single line around the inner margin, often obscure at ventral margin. The commissural shelf is narrow. The attachment area is small to medium.

1.2.5 *Striostrea (Parastriostrea) mytiloides* (Figure 1.3C)

Synonym: *Ostrea mytiloides* (Lamarck, 1819); (Hanley, 1856); (Martens, 1897)
Ostrea echinata (Hamley, 1856)
Ostrea spinosa (Iredale, 1939)
Crassostrea echinata (Thomson, 1954)
Saccostrea echinata (Torigoe, 1981)

English common names: Black bordered oyster, black edge oyster

Thai common name: Hoy nang rom

Characteristics: The shell is medium size, variable in shape (subcircular to elongate), and generally attached by the almost entire area of the left valve. The shell margin is irregularly undulate. Both valves are flat. The shell margin of the left valve is raised vertically, especially at the ventral side. The right valve is foliose at periphery, mostly worn off in large shells. The interior is iridescent white, often with light purple. The adductor muscle scar is kidney-shaped, white, and close to posterior margin. The chomata is strong, extending on both sides of ligamental areas and often obscure at the ventral margin in small shells. The commissural shelf is dark purple and narrow. The attachment area is large.

1.3 Anatomy of Oysters

Oysters are members of Bivalvia. The anatomy of oysters is shown by figure 1.4. The hinge is the ligament that joins two shells and acts to open the shell. Conversely, the adductor muscle function in closing the shells. It is usually whitish. The mantle is the meaty body part. The entire soft part of the oyster, including the stomach and the gills, is eatable.

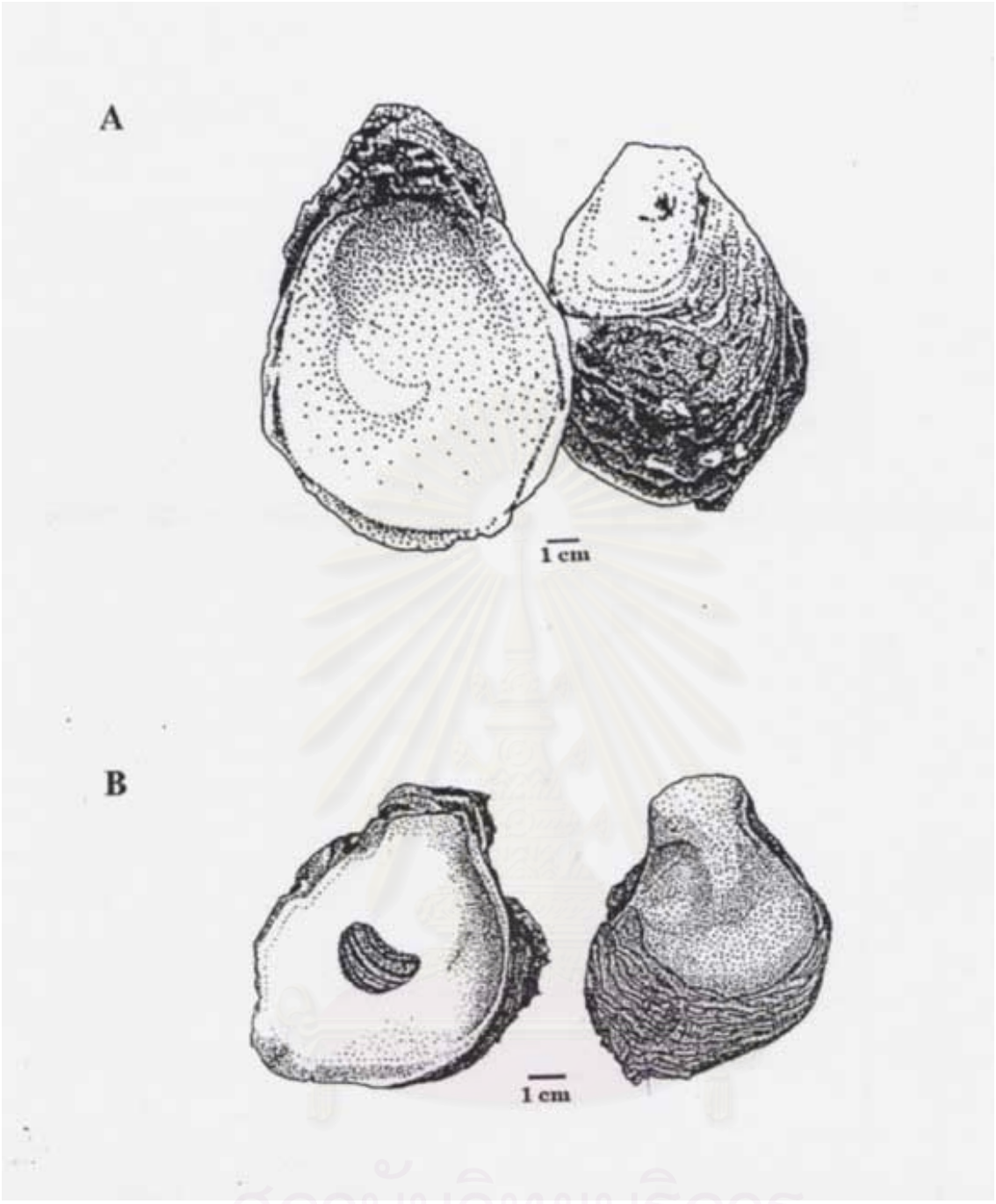

Figure 1.2 Shell morphology of *Crassostrea* oysters (Yoosukh and Duangdee, 1999)

A: White scar oyster; *C. belcheri*

B: Black scar oyster; *C. iredalei*

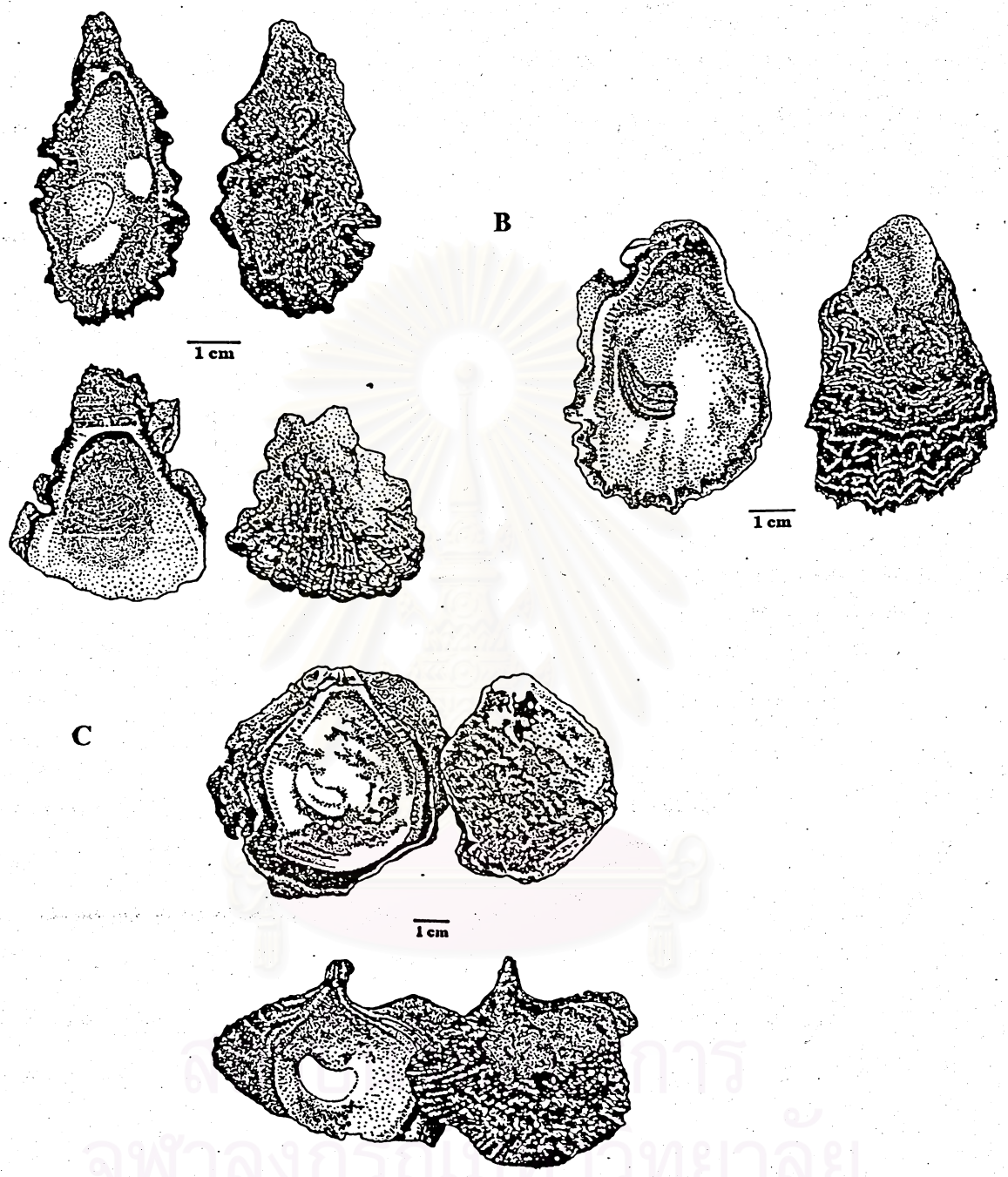

Figure 1.3 Shell morphology of *Saccostrea* oysters (Yoosukh and Duangdee, 1999)

A: *S. cucullata*

B: *S. forskali*

C: *Striostrea (Parastriostrea) mytiloides*

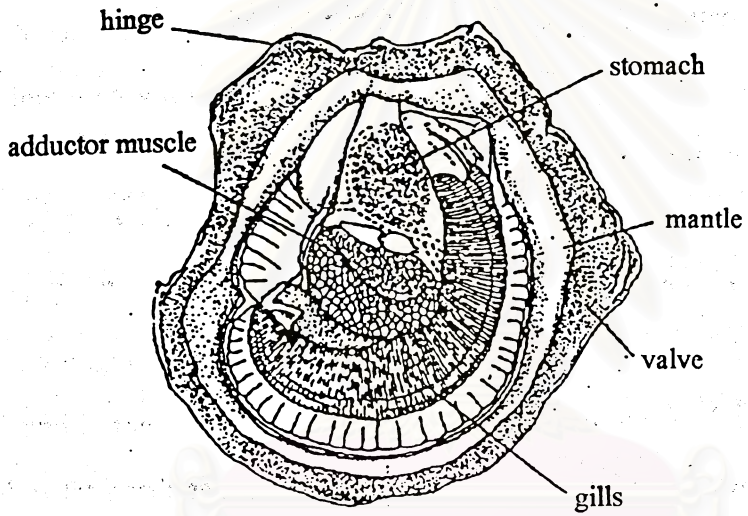

Figure 1.4 Anatomy of oysters (Dore, 1991)

1.4 Distribution of oysters

Oysters are widely distributed throughout tropical and subtropical areas ranging from 64^{O}N to 44^{O}S (Hedgecock, 1995). They are most diversified in warmer areas and do not occur in the Arctic or Antarctic zones (Harry, 1985). Oysters can grow in different salinity from full sea strength to intertidal stream. They are found abundantly in shallow water along rocky coastlines by attach to rocks and other substrates in intertidal streams. In the mangrove areas, oysters attach roots or other vegetative substrates. In Thailand, oysters are widely distributed in both the Gulf of Thailand and the Andaman Sea. Generally the small oysters distribute covering larger geographic areas than do the large oyster. For instance, *S. cucullata* and *S. mytiloides* are found in the tropical Indo-Pacific regions from the Red Sea and eastern Africa to Australia and as far north as Japan whereas *C. belcheri* and *C. iredalei* found in the either sides of the Malaysian Peninsular (Yoosukh and Duangdee, 1999).

1.5 Molecular genetic markers

Molecular genetic markers are useful for genetic and systematic studies of natural and cultured species. Genetic markers used for population genetic and systematic studies should exhibit suitable polymorphic levels for desired applications and be selectively neutral. Under this circumstance, the information from genotypes and allele frequencies is assumed to be primarily influenced by mutation, gene flow, genetic drift rather than by selection (Smith and Brown, 1998).

Molecular genetic markers are basically gathered to protein and DNA markers. An introduction of allozyme analysis in the 1960s allowed population geneticists using relatively simple approach to investigate a wealth of Mendelian data in various organisms. In addition, the use of DNA techniques (e.g., Southern blotting, DNA sequencing, cloning, restriction fragment length polymorphism and more recently the polymerase chain reaction) has allowed

the studies of genetic polymorphism at the DNA level with wider applications providing more detailed genetic information than that availible from the allozyme analysis alone.

Sources of genetic markers used for taxonomy of Thai oysters

Nuclear ribosomal DNA

The ribosomal DNA (rDNA) in the nuclear genome of eukaryotes usually exists as tandem repeated units (Figure 1.5). It is classified as one of the moderately repetitive gene families. Each repeat unit is composed of a highly conserved coding sequence (5.8S, 18S and 28S rRNA genes), more variable non-coding sequences (external transcribed spacers; ETS, internal transcribed spacers; ITSI and ITSII) and non-transcribed sequences (non-transcribed spacer; NTS, often called the intergenic spacers or IGS). The ribosomal tandem repeats may be located at one or several chromosome sites depending on species. The copy number per genome varies from a few to several hundred copies in insects, fish and mammals to many thousands in plants (Phillips, 1989; Avise, 1994).

Nuclear rDNA is particularly useful for phylogenetic and systematic studies because different rDNA regions evolve at different rates. Therefore rDNA is a versatile molecule for different applications. The main variability of rDNA is length polymorphism of the repeated units due to size heterogeneity of spacer regions. The genetic differences are also from substitutions in both coding regions and spacers. The coding regions (5.8S, 18S and 28S rRNA genes) are suitable for comparisons of distantly related species, whereas the internal and external transcribed regions that evolve more rapidly can be used for phylogenetic studies of more closely related species. Due to its rapid evolutionary rate, the IGS is typically used for genetic studies at the intraspecific level (Hills and Dixon, 1991; Phillips and Pleyte, 1991 and Phillips et al., 1992).

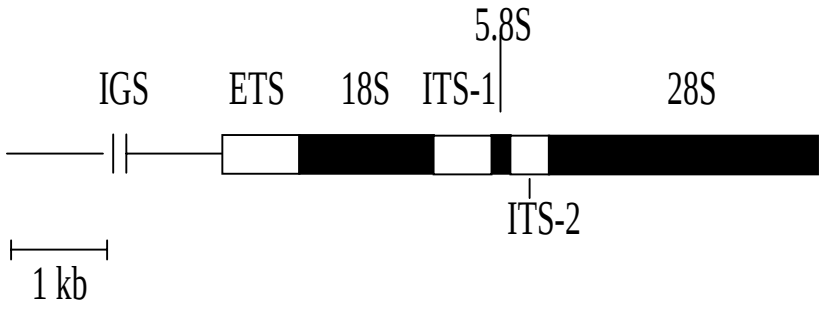

Figure 1.5 Structural features of the rDNA repeat module in eukaryotic organisms. The 5.8S, 18S and 28S regions coding for 5.8S, 18S and 28S rRNAs, respectively. The ITS-1 and ITS-2 are internal transcribed spacers and IGS is intergenic spacer region.

Animal mitochondrial DNA (mtDNA)

The mtDNA is a closed circular, double-stranded molecule ranging in size from 15.7 kb to 19.5 kb found in mitochondria. There are up to several thousand copies of the mitochondrial genomes per cell (Brown, 1983).

The animal mitochondrial genome is composed of 2 ribosomal RNAs coding genes (12S and 16S ribosomal rRNA), 22 transfer RNA (tRNA) coding genes, the control region (D loop) containing on initiation site for replication and transcription and 13 protein coding genes; NADH dehydrogenase (ND) subunits 1, 2, 3, 4, 4L, 5 and 6, cytochrome *b*, cytochrome *c* oxidase (COI, II and III) and two subunits of ATP synthetase (ATPase 6 and 8)(Avise, 1994).

Nucleotide substitution rates in mtDNA of higher vertebrates are approximately 5-10 times greater than that of single copy nuclear DNA (scnDNA) genes (Brown et al., 1979). Unlike nuclear DNA, animal mtDNA is maternally inherited in most species. Moreover, recombination in the mitochondrial genome is very rare (Smith and Brown, 1998). Thus, mtDNA offers several advantages for population and systematic studies. A smaller effective population size (assuming an equal sex ratio) comparing to that estimated from nuclear DNA results in a greater power for determination of genetic differentiation (due to genetic drift) and

it is more likely to provide population-specific markers (Ferguson et al., 1995). Typically, the number of gene contents and organization of gene order in the mitochondrial genome conserve within the same phylum.

Molecular approaches used in this study

Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) analysis is indirectly used to determine genetic variation at the DNA level by comparison of shared restriction fragments or sites. Basically, variation in restriction enzyme cleavage sites (usually detected by Southern blot hybridization) generates size differences of the resulting fragments.

Conventional RFLP analysis is carried out by digestion of genomic DNA with an appropriate restriction endonuclease followed by electrophoresis of digested DNA through the agarose gel. The fractionated DNA is then transferred to a supporting membrane (nylon or nitrocellulose) before hybridized with the specific labeled DNA probe. The results are visualized by autoradiography.

The hybridization-based RFLP approach has been widely used for population genetic studies in various species during the 1980s. Nevertheless, this technique is tedious and time consuming particularly when dealing with a large number of specimens. Accordingly, it has been increasingly replaced by various polymerase chain reaction (PCR)-based methods including PCR-based DNA fingerprinting, PCR-RFLP, Amplified fragment length polymorphism (AFLP) and microsatellites.

PCR-RFLP

The introduction of the polymerase chain reaction (PCR) by Millis et al. (1987) has opened a new approach for molecular genetic studies. PCR is a method for an *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. Million copies of the target DNA sequence can be synthesised from a low amount of starting DNA template within a few hours. The PCR reaction components are constituting of DNA template, a pair of primers for the target sequence, dNTPs (dATP, dCTP, dGTP and dTTP), buffer and heat-stable DNA polymerase (usually *Taq* polymerase). The amplification reaction consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the annealed primers by heat-stable DNA polymerase (Figure 1.6). The cycle is repeated for 30-40 times. The amplification product is determined by electrophoresis.

The PCR-amplified fragment can be further analysed by digestion with restriction endonucleases (restriction site or fragment length polymorphism). The restricted fragments are fractionated in an agarose gel (or polyacrylamide gel), stained with ethidium bromide and visualized by a UV transluminator. The incorporation of PCR to RFLP (or site) analysis reduces time consuming and risks from using radiolabelled probe from hybridization-based RFLP analysis. Accordingly, PCR-RFLP has increasingly utilized and has replaced the conventional RFLP method at present.

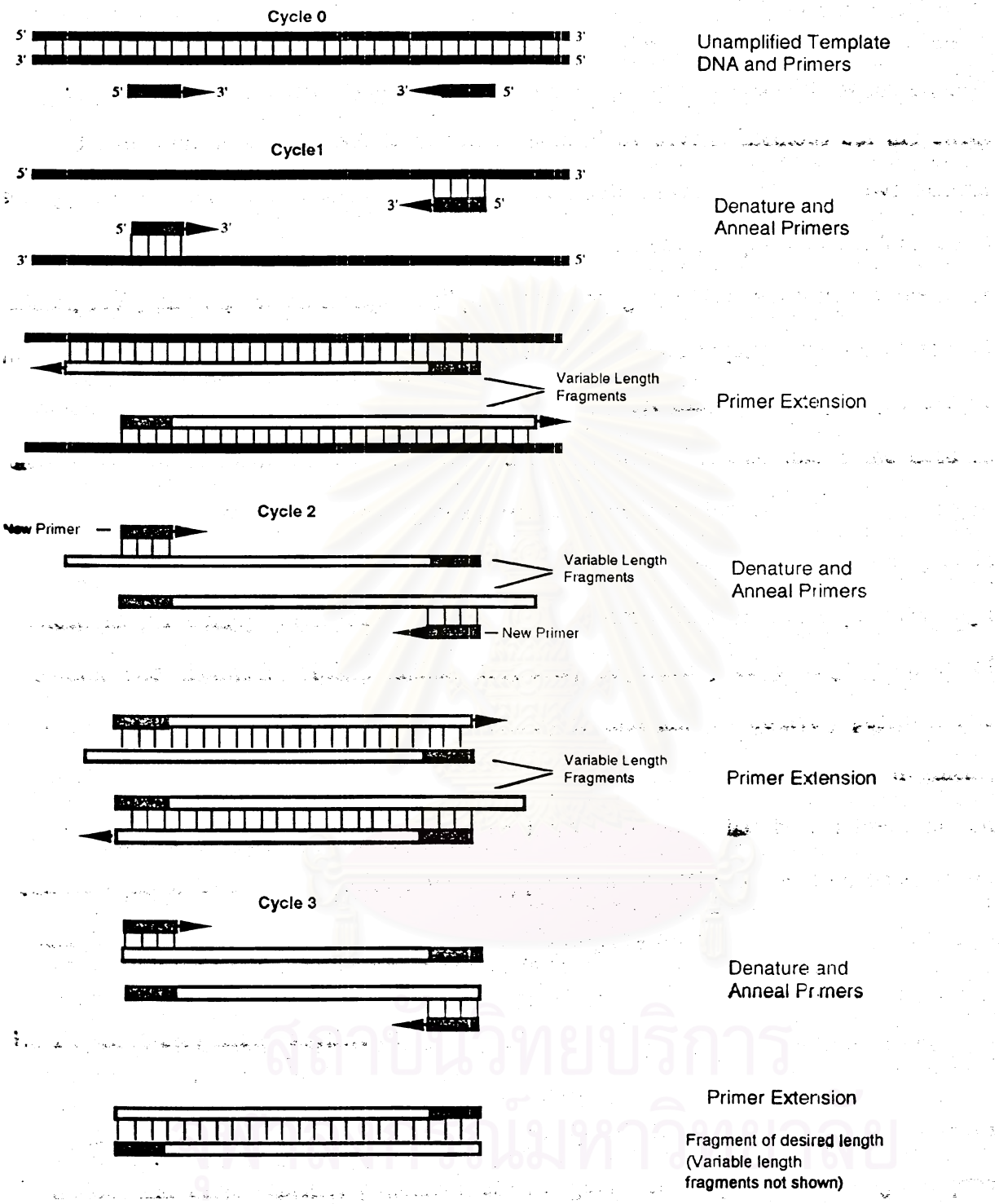

Figure 1.6 Diagram illustrating the PCR reaction for amplification of DNA (Gibthai Training Center, 2000)

DNA sequencing

Polymorphism at the DNA level can be studied by several methods but the direct strategy is determination of nucleotide sequences of a defined region. There are two general methods for sequencing of DNA segments: the “chemical cleavage” procedure (Maxam and Gilbert, 1977) and the “chain termination” procedure (Sanger, 1977). Nevertheless, the latter method is more popular because chemical cleavage procedure requires the use of several hazardous substances. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library.

DNA sequencing is the most optimal method for several population genetic applications particularly phylogenetic studies of organisms. This technique provides high resolution and facilitating interpretation. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The sequencing method has been facilitated by the direct and indirect use of DNA fragments generated through PCR. At present, automatic DNA sequencing has been introduced and commonly used. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

1.6 Population genetic studies in oysters

Buroker (1979a) estimated genetic diversity of *C. gigas* and *S. commercialis* populations and a representative population of *C. angulata* and *S. glomerata* using allozyme analysis. The level of heterozygosity ranged from 0.20 - 0.22 for *C. gigas* and 0.17 - 0.19 for *S. commercialis*. The genetic similarity between different geographic population of *C. gigas* and *S. commercialis* was approximately 99% based on the genetic distance approach. The Portuguese oyster (*C. angulata*) should be regarded as a recent colonized isolation of *C. gigas* whereas *S. glomerata* should be recognized as a subspecies of *S. commercialis*.

Buroker et al. (1979b) determined levels of genetic variation of six oyster species; *C. belcheri* (*N = 36*), *C. iredalei* (*N = 154*), *C. gigas* (*N = 96*), *C. rivularis* (*N = 108*), *C. rhizophoreae* (*N = 160*) and *C. virginica* (*N = 160*) using allozyme analysis (25 to 34 putative loci). The levels of heterozygosity was 0.06 - 0.22 and 0.17 - 0.19 for *Crassostrea* and *Saccostrea* species. Coincidentally, four (*Ald*, *Idh-1*, *Mdh-1* and *To-2*) and six species-specific (fixed) alleles (*Acp- 3*, *Ald*, *Aat -1*, *Mp-1*, *To-1* and *Xdh*) were found in *C. belcheri* and *C. iredalei*, respectively.

Visootiviseth et al. (1998) used electrophoretic and morphometric analyses to differentiate small oysters, *Saccostrea* spp. in Thailand. Samples were collected from Ko Chang (Trat), Sri Racha (Chonburi), Samseab and Ko Talu (Chumphon), Ko Prab (Suratthani), Tubtieng (Trang) and Ko Kaoyai (Satun). These oysters were analyzed for allozyme variation at 9 loci (Pgi, Lap, Pgm, Mpi-2, Ap, Est-2, Aat-2, Mdh-2 and Idh-1). All oysters could be split into “A”, “B” or “C” groups depending on their multilocus genotypes at loci Pgi, Pgm, Mpi-2, Lab and Idh-1. The shells of *Saccostrea* oysters were grouped according to the electrophoretic results and morphometric analysis. Although, these oysters were differentiated into 3 groups, the scientific name was recognized under *S. cucullata*. The UPGMA dendrograms constructed from similarity indices of gene frequencies and morphometric analysis exhibit well comparable results. The fixed Idh-1 allele was observed in the C oysters whereas different alleles were found in groups A and B. Several other unique alleles (found in only one species) of a particular locus were found in the group C but, unlike that of Idh-1, they could not be used as diagnostic marker due to their relatively low frequencies.

More recently, Day et al. (2000) used allozymes and shell morphology to distinguish sympatric species of the rock oysters (*Saccostrea*) in Thailand. Three hundred and fifty-five individuals of *Saccostrea* oysters were 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). Oysters were electrophoretically determined for allozyme variation at eight allozyme loci (Aat-1, Ap, Est-2, Lap, Mdh-1, Mpi, Pgi and Pgm). The allelic variation at Lap,

Mpi, Pgm and Pgi were observed. The principle component analysis (PCA) of these loci identified two principle components (PC1 and PC2) which allocate individuals into three discrete clusters corresponding to different species. Using diagnostic alleles at four marker loci and comparisons of observed genotype frequencies in putative single-species samples, oysters were identified as *Saccostrea commercialis*, *S. cucullata* and *S. manilai*. They reported that *S. manilai* and *S. commercialis* were sympatrically found in coastal and estuarine sites throughout the Gulf of Thailand whereas *S. cucullata* was restricted to offshore isles. Using shell morphometrics, the most useful character for species identification was the size of the umbo cavity, which is large in *S. cucullata* and *S. commercialis*, but almost non-existent in *S. manilai*. The mean umbo depths were 1.49 cm for *S. cucullata*, 0.7 cm for *S. commercialis* and 0.42 cm for *S. manilai*. Seventy-five percent of *S. cucullata* have black adductor scars, while those of the other two species were white or brown.

Reeb and Avise (1990) examined genetic discontinuity of the American oyster (*C. virginica*) collected from the Gulf of St. Lawrence (Canada) to Brown ville (Texas) using mtDNA polymorphism. Eighty-two haplotypes were found from analysis of 212 individuals with 13 restriction endonucleases (*Ava* I, *Ava* II, *BgI* I, *BgI* II, *Cla* I, *Eco*R I, *Hinc* II, *Hin*d III, *Msp* I, *Nde* I, *Pvu* II, *Spe* I and *Stu* I). MtDNA haplotypes were allocated into two distinguishable clusters having approximately 2.6 % nucleotide sequence divergence. A large genetic differences (genetic break) between *C. virginica* collected from northern and southern regions of the Atlantic mid-coast of Florida were observed.

Banks et al. (1993) developed molecular discriminatory techniques to discriminate closely related Pacific oyster species (between *C. gigas and C. sikamea*) using a large subunit of rRNA coding gene (16S rDNA). Three different technique providing species-specific discrimination between *C. gigas* and *C. sikamea* were developed. These included a multiplex species-specific PCR (319 bp for *C. gigas*, 319 and 246 bp for *C. sikamea*), dot-blot hybridization of immobilized PCR products with specific oligonucleotide probes of each species and digestion of amplified 16S rDNA of *C. gigas* with *Dra* I. The molecular

discriminatory techniques could be directly applicable to eliminate *C.gigas*-like oysters from the commercial production of *C. sikamea*.

Small and Chapman (1997) examined population structure and intraspecific genetic variation of *C. virginica* for the Atlantic coast and the Gulf of Mexico using PCR-RFLPs of 16S rDNA. Eleven composite haplotypes were generated from digestion of a 560-bp fragment with 10 restriction enzymes (*Rsa* I, *Msp* I, *Hha* I, *Taq* I, *Mse* I, *Hae* III, *Mbo* I, *Hin*f I, *Hin*d III, *Hinc* II). The most common haplotype (AAAAAAAAAA) was found in 387 (95%) of overall tested individuals (*N = 410*). The second common haplotype (AAAAAABAAA) was exhibited by 9 individuals, whereas the haplotype AAAACAAAAA was found in only 4 individuals. The remaining haplotypes were represented by either one or two individuals. No genetic structure was found between different populations within a region and between regions ($P > 0.05$). Haplotype diversity and nucleotide diversity within populations were low in all populations (average haplotype and nucleotide diversity were 0.1079 ± 0.00048 and 0.001309, respectively). The level of nucleotide divergence between paired populations indicated a lack of population differentiation of *C. virginica* from the Atlantic coast and the Gulf of Mexico.

Littlewood (1994) studied molecular phylogenetics of cupped oysters (*C. belcheri*, *C. gigas*, *C. rhizophorae*, *C. rivularis*, *C. virginica*, *S. commercialis*, *S. cucullata* and *O. edulis*) and the mussel (*Mytilus edulis*) using partial sequences of the large subunit of nuclear ribosomal DNA (28S rDNA). A total of 90 sites were phylogenetically informative for parsimony analysis. Phylogenetic trees using the maximum parsimony (MP) and the maximum-likelihood (ML) approaches showed identical topology and could differentiate these taxa into 3 groupings; *C. belcheri*, *C. gigas* and *C. rivularis* (group A), *C. virginica*, *C. rhizophorae*, *S. commercialis* and *S. cucullata* (group B) and *O. edulis* (group C). Molecular data support a later divergence of the tropical Pacific *Saccostrea* from a common ancestor of the Atlantic *Crassostrea* species. The close relationships of the group B oysters are not unexpected due to complex superspecies status between *C. virginica* and *C. rhizophorae* and *S. commercialis* and *S. cucullata*.

Folmer et al. (1994) developed “universal” DNA primers for the polymerase chain reaction (PCR) to amplify a 710-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene from 11 representative species of invertebrate phyla (Echinodermata, Mollusca, Annelida, Pogonophora, Arthropoda, Nemertinea, Echiura, Sipuncula, Platyhelminthes, Tardigrada and Coelenterata). The primers were LCO1490 (5′-GGTCAAC AAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAA TCA-3′). These primers generated amplificable and informative sequences for phylogenetic analysis at higher taxonomic levels. Moreover, they can be successfully used for amplification of COI from *Crassostrea* and *Ostrea* oysters, scallops (*Placopecten*), hard clams (*Mercenaria*), archaeogastropod limpets, arachnids and marine hydrozoans.

The taxonomic status of the Portuguese oyster (*C. angulata*) and the Pacific oyster (*C. gigas*) has been questioned because no morphological or genetic information between these species had been reported. Boudry et al. (1998) used mtDNA-RFLP analysis to investigated genetic differentiation between populations of these taxa. Restriction analysis of cytochrome *c* oxidase subunit I (COI) of *C. gigas* (*N = 203*) and *C. angulata* (*N = 50*) with *Taq* I, *Sau*3A I, *Hha* I and *Mse* I illustrated six composite haplotypes composing of A (ccab), B (cdab), C (dcad), D (dcab), E (dcbd) and J (acab). The C (dcad) and A (ccab) haplotypes were found in 76 % and 88 % of *C. gigas* and *C. angulata*, respectively. Therefore, these mtDNA markers offered partial differentiation between *C. angulata* and *C. gigas*.

PCR-based assays using anonymous scnDNA were also used for population genetic studies. Karl and Avise (1992 and 1993) developed oligonucleotide primers for analysis of anonymous scnDNA polymorphism in the American oyster (*C. virginica*). They found significant differences of allele frequencies of populations originating from the Atlantic coast and the Gulf of Mexico at four polymorphic loci (CV-07, CV-19, CV-32 and CV-195). The co-dominant segregation pattern of these nuclear DNA provides useful genetic markers for differentiation studies of this species where allozyme analysis has failed to provide informative results.

Hu and Foltz (1996) used PCR-RFLP of five anonymous single-copy nuclear DNA (CV-07, CV-19, CV-32, CV-195 and CV-233) to examine genetic polymorphism of juvenile *C. virginica* bred from a selected disease-resistant stock. Two types of RFLP were observed including simple presence or absence of a restriction site (e.g. CV-32-*Nsi* I and CV-195-*Ava* II) resulting in a diallelic system and multiple polymorphic restriction sites resulting in three or more allele (e.g. four alleles in CV-07-*Hin*f I and three alleles in CV-233-*Eco*R I). Restriction patterns of the locus CV-19 digested with *Bst*N I, *Stu* I and *Xba* I were more complicated. Strict Mendelian inheritance was observed in two scnDNA loci (CV-07 and CV-32). An overall of 7% among 174 paired-cross progeny from 11 crosses showed non-Mendelian genotypes.

1.7 Objectives

The objectives of this thesis are to determine genetic variation levels of commercially important oysters in Thailand and to identify species-specific RFLP markers to assist taxonomic identification of *Crassostrea*, *Saccostrea* and *Striostrea* oysters in Thailand using restriction analysis of mitochondrial (16S rDNA and COI) and nuclear genes (18S rDNA). The results obtained are used to construct phylogenetic relationships of these oysters. The knowledge obtained can be applied for the construction of breeding programs and for identification of seed and broodstock species of commercial cultured oysters in Thailand.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

- Automatic micropipettes P10, P20, P40, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)
- Camera Pentax K1000, Asahi Opt.Co.,LTD.
- Gene pulser (Bio-RAD Laboratories, USA)
- Laminar Flow Cabinet, model NU-440-300E (Nuaire)
- Microcentrifuge, model Micro Gen 13D (Herolab)
- PCR thermal cycler : PCR sprint (Hybaid)
- Power supply (Bio-RAD Laboratories, USA)
  : Power PAC 300
  : Power PAC Junior
  : model 200/2.0
- Refrigerated centrifuge, model 3K18 (Sigma Osterode and Harz, Germany)
- Refrigerated microcentrifuge : Kubota 1300 (Kubota, Japan)
- Shaking waterbath SBS30 (Stuart Scientific, UK)
- Spectrophotometer, model Spectronic GeneSys 5 (Milton Roy)
- UV transilluminator, model M-26 (UVP, USA)

2.1.2 Chemicals

- Absolute ethanol (BDH, England)
- Agarose gel (FMC Bioproducts, USA)
  : MetaPhor Agarose
  : Seakem LE Agarose
- Bacto-agar (Oxoid, England)
- Bacto-yeast extract (Oxoid, England)
- Bacto-tryptone (Oxoid, England)
- Boric acid (Merck, Germany)
- Bromophenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- λDNA (Promega Corporation Medison, Wisconsin)
- 100 base-pair DNA ladder (Promega Co., USA)
- 100 mM dATP, dCTP, dGTP, dTTP
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka Chemika, Switzerland)
- Ficoll, type 400 (Sigma Chemical Co., USA)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)
  : 10xPCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)
  : 25 mM $MgCl_2$
- D - Glucose (Sigma Chemical Co., USA)
- Hydrochloric acid (Merck, Germany)
- 8 - Hydroxy Quinoline (Sigma Chemical Co., USA)
- Isoamyl alcohol (Sigma Chemical Co., USA)
- 2 - Mercaptoethanol (Sigma Chemical Co., USA)
- Phenol, redistilled (Aldrich Chemical Co., USA)

- Prep-A-Gene^{R} DNA Purification Kit (Bio-RAD Laboratories, USA)
- Potassium chloride (Merck, Germany)
- Sodium acetate (Merck, Germany)
- Sodium chloride (APS Chemicals Limited CAN.)
- Sodium dodecyl sulfate
- Sodium hydroxide pellets (Merck, Germany)
- Spermidine trihydrochloride
- Tris (USB, Amersham Life Science, England)

2.1.3 Enzymes

- Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA)
- Proteinase K
- RNase A
- T4 DNA ligase (Pharmacia, USA)
- Restriction endonucleases
  : *Acs* I (Boehringer Mannheim, Germany)
  :*Eco*R I (New England Biolabs)
  :*Alu* I, *Ase* I, *Bam*H I, *Bfr* I, *Dde* I, *Dra* I, *Hae* III, *Hin*d III, *Hin*f I, *Mbo* I, *Nde* I, *Rsa* I, *Swa* I and *Taq* I (Promega Corporation Medison, Wisconsin)

2.1.4 Bacterial strain

- *Escherichia coli* : strain XL1 Blue
  (F′:: Tn*10proA*$^{+}$*B*$^{+}$*lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96(Nal*^{r}*) thi hsdR17 (* r_k^- m_k^+ *) supE44 relA1 lac)*

2.1.5 Cloning vector

- pGEM^{R}-T easy vector (Promega)

2.2 Sampling

Indigenous oysters in Thai waters including *C. belcheri* (*N = 17*), *C. iredalei* (*N = 21*), *S. cucullata* (*N = 23*), *S. forskali* (*N = 46*) and *S. mytiloides* (*N = 17*) and those having taxonomic difficulties, *Crassostrea* sp. (*N = 9*), unidentified *Saccostrea* sp. Group 1 (*N = 8*), unidentified *Saccostrea* sp.group 2 (*N = 9*) and unidentified *Saccostrea* sp. Group 3 (*N = 5*) were collected during April 1998 to December 1999. Geographic locations and abbreviations of sampling sites are shown by Figure 2.1 and Table 2.1. Oysters were collected and maintained on ice during the sampling schedule. Specimens were transported back to Marine Biotechnology Research Unit, Chulalongkorn University. The Australian oyster, *S. commercialis* (*N = 6*), and the mussel, *P. viridis* (*N = 5*), collected from Brisbane (Australia) and Chonburi (eastern Thailand) were included as an ingroup and an outgroup references, respectively. Each oyster was kept in a -30^{O}C freezer until required. Alternatively, the adductor muscle was dissected out individually and kept in a -80^{O}C freezer until used for DNA extraction.

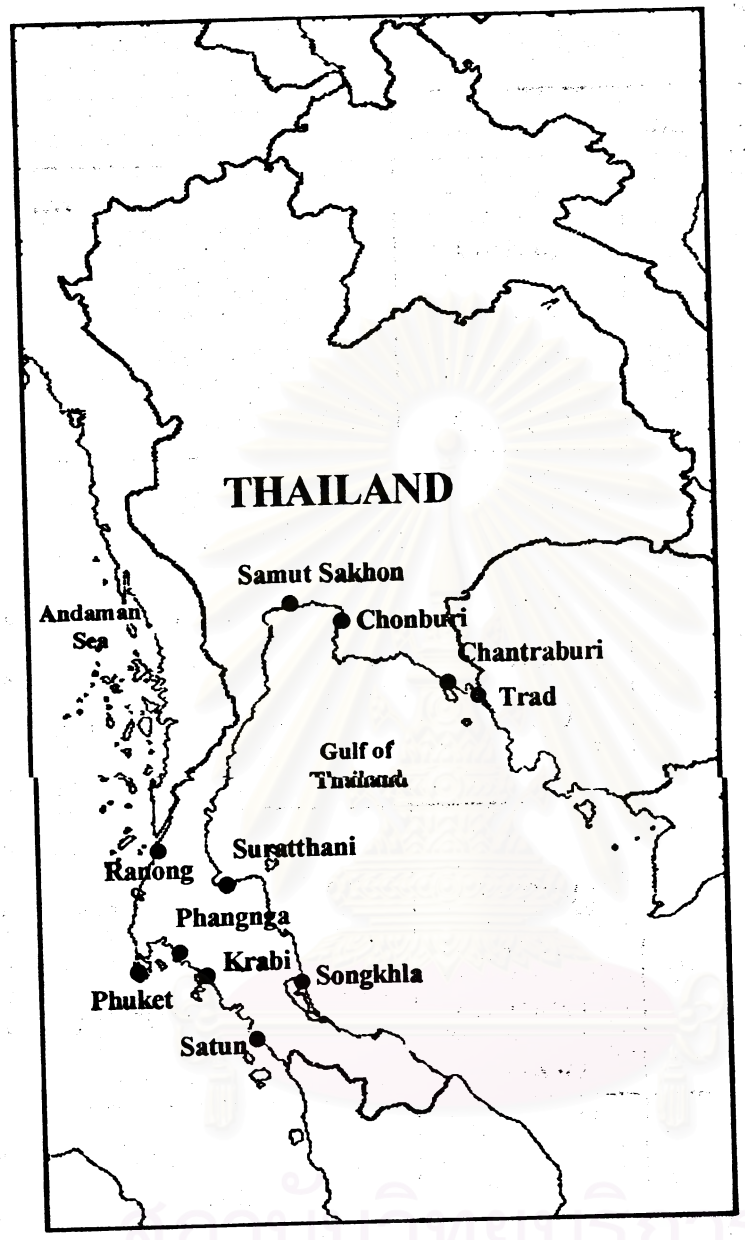

Figure 2.1 Map of peninsular Thailand indicating sample collection sites of oysters (*C. belcheri*, *C. iredalei*, *S. cucullata*, *S. mytiloides*, *S. forskali*, *Crassostrea* sp., *Saccostrea* sp. group 1, *Saccostrea* sp. group 2, *Saccostrea* sp. group 3) used in this study. Detailed information and abbreviations of sample sites are shown by Table 2.1.

Table 2.1 Sampling locations and sample sizes of oyster specimens used in this study

Sample	Abbreviation	Sample size (N)
Local oyster: *C. belcheri*		
Suratthani (east of PT)	CbSR(E)	8
Songkhla (east of PT)	CbSK(E)	3
Ranong (west of PT)	CbRN(W)	3
Krabi (west of PT)	CbKB(W)	3
Local oyster: *C. iredalei*		
Chonburi (Gulf of Thailand, east)	CiCB(E)	6
Prachuapkririkhan (east of PT)	CiPJ(E)	4
Songkhla (east of PT)	CiSK(E)	6
Phangnga (west of PT)	CiPN(W)	4
Ranong (west of PT)	CiRN(W)	1
Local oyster: *S. cucullata*		
Trad (Gulf of Thailand, east)	ScTD(E)	5
Chantraburi (Gulf of Thailand, east)	ScCT(E)	6
Ranong (west of PT)	ScRN(W)	6
Phuket (west of PT)	ScPK(W)	6
Local oyster: *S. forskali*		
Chantraburi (Gulf of Thailand, east)	SfCT(E)	12
Chonburi, Angsila (Gulf of Thailand, east)	SfCBA(E)	4
Chonburi, Sichang Island (Gulf of Thailand, east)	SfCBS(E)	10
Prachuapkririkhan (east of PT)	SfPJ(E)	3
Suratthani (east of PT)	SfSR(E)	3
Songkhla (east of PT)	SfSK(E)	5
Ranong (west of PT)	SfRN(W)	5
Satun (west of PT)	SfST(W)	4

Table 2.1 (continue)

Local oyster: *S. mytiloides*		
Chantraburi (Gulf of Thailand, east)	SmCT(E)	2
Phuket (west of PT)	SmPK(W)	3
Ranong (west of PT)	SmRN(W)	6
Samut Sakhon (Gulf of Thailand, east)	SmSS(E)	6
Unidentified local species: *Crassostrea sp.*		
Krabi (west of PT)	CsKB(W)	9
Unidentified local species: *Saccostrea sp.* group 1		
Suratthani (east of PT)	S1SR(E)	8
Unidentified local species: *Saccostrea sp.* group 2		
Ranong (west of PT)	S2RN(W)	9
Unidentified local species: *Saccostrea sp.* group 3		
Samut Sakhon (Gulf of Thailand, east)	S3SS(E)	5
An ingroup ref erence: *S. commercialis*		
Brisbane, Australia	Scom	6
An outgroup ref erence: *P. viridis*		
Chonburi, Thailand	Pevi	5
Total (N)		**166**

Abbreviation; PT = peninsular Thailand

2.3 DNA extraction

Genomic DNA was previously extracted from the adductor muscle of each oyster using a proteinase K/phenol-chloroform method by Ampayup (1999). A piece of adductor muscle was dissected out from a specimen. The tissue was placed in a prechilled microcentrifuge tube containing 600 μl of extraction buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 200 mM Sucrose, 50 mM EDTA, pH 8.0) and briefly homogenized with a micropestle. A 40% SDS solution was added to a final concentration of 1.0% (w/v). The resulting mixture was then incubated at 65^{O}C for 1 hour following by an addition of 15 μl of a proteinase K solution (20 mg/ml) and 10 μl of RNase A solution (10 mg/ml). The mixture was further incubated at the same temperature for 3-4 hours. DNA was extracted by a standard phenol-chloroform method. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) were added and mixed gently for 15 minutes. The mixture was centrifuged at 12000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube. The phenol-chloroform extraction was repeated. The resulting upper phase was further extracted with an equal volume of chloroform-isoamyl alcohol (24:1). One-tenth volume of 3 M sodium acetate (pH 5.5) was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80^{O}C for 30 minutes. The precipitated DNA was recovered by centrifugation at 12000 rpm for 15 minutes at room temperature and briefly wash twice with 70% ethanol. The DNA pellet was air-dried and resuspended in 100 μl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The DNA solution was incubated at 37^{O}C for 1-2 hours for complete solubilization and kept at 4^{O}C until further needed.

2.4 Measuring concentrations of extracted DNA using spectrophotometry and electrophoresis

The concentration of extracted DNA was estimated by spectrophotometrically at 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 μg/ml double stranded DNA. Therefore, the concentration of DNA samples is estimated in μg/ml by multiplying an OD_{260} value with a dilution factor and 50. The purity of DNA samples can be evaluated by a ratio of OD_{260}/OD_{280}. The ratio that much lower than 1.8 indicates contamination of residual proteins or organic solvents in the DNA solution (Kirby, 1992).

Electrophoresis can also be used for rough estimation of DNA on the basis of the direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining. DNA was run in a 0.8% agarose gel prepared in 1xTBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) at 100 V. The gel was stained with ethidium bromide. DNA concentration was estimated by comparing the fluorescent intensity of a given band with that of λ–*Hin*d III.

2.5 Screening of primers for population genetic study of Thai oysters

Seven primers; COI, ND5, 12S rDNA, 16S rDNA (1 forward primer and 3 reverse primers) and 18S rDNA, were screened for the amplification success against a representative individual of taxonomically-described Thai oysters (Table 2.2). Three reverse primers of 16S rDNA were tested with a universal forward primer. Although both 16SF/R1 and 16SF/R2 primers yielded the amplification success, primer 16SF/R2 gave higher yield and consistent results. These primers were then used throughout this thesis.

Table 2.2 Sequences of PCR primers screened for genetic analysis of Thai oysters

Primer	Sequence	Reference
12S rDNA	F: 5′ AAACTAGGATTATATACCCTATTA 3′ R:5′ AAGAGGGACGGGCGATTTGT 3′	Palumbi et al. (1991)
16S rDNA	F: 5′ CGCCTGTTTAACAAAAACAT 3′ R(1): 5′CCGGTCTGAACTCAGATCATGT 3′	Palumbi et al. (1991)
	R(2):5′GGTCTGAACTCAGATCAGATCACGT3′ R(3):5′CCGGTCTGAACTCAGATCAGATCACGT3′	Small and Chapman (1997)
COI	LCO1490:5′ GGTCAACAAATCATAAAGATATTGG 3′ HCO2198:5′TAAACTTCAGGGTGACCAAAAAATCA3′	Folmer et al. (1994)
ND5	F:5′ ATAGAGCGTTGCATTGAAGC 3′ R:5′ TCAAGGAGCATTAGAGTGAG 3′	GenBank (*P. notolis*)
18S rDNA	F:5′ TGGATCCGGGCAAGTCTGGTGCC 3′ R:5′ TGAAGTCAAGGGCATCACAGACC 3′	Aoki, T. (personal communication)

2.6 PCR amplification

Three different regions composing of 16S rDNA and COI (mitochondrial genome) and 18S rDNA (nuclear genome) of each oyster were separately amplified by PCR. The amplification reaction was set up in a 50 μl reaction volume containing 50 ng template DNA, 1xPCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 1 mM dNTPs, 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 and 1 unit of *Taq* DNA polymerase (Perkin Elmer Cetus). The reaction mixture was performed in a PCR sprint thermal cycler (Hybaid). The amplification cycles for each gene region are illustrated by Table 2.3. Five microlitres of the amplification reaction were electrophoresed through 1% agarose gel to determine whether the PCR reaction was successful. Specimens showing expected product sizes were subjected to restriction analysis.

Table 2.3 The amplification cycles for each gene region.

	16S rDNA	**18S rDNA**	**COI**
1. predenaturation	1 cycle: 94^{O}C, 3 min.	1 cycle: 94^{O}C, 3 min.	1 cycle: 94^{O}C, 3 min.
2.denaturation	5 cycles: 94^{O}C, 1min.	5 cycles: 94^{O}C, 1min.	10cycles:94^{O}C, 1min.
annealing	50^{O}C, 1min.	48^{O}C, 1min.	42^{O}C, 1min.
extension	72^{O}C, 1min.	72^{O}C, 1min.	72^{O}C, 1min.
3. denaturation	35cycles:94^{O}C, 1min.	40cycles:94^{O}C, 1min.	35cycles:94^{O}C, 1min.
annealing	60^{O}C, 1min.	53^{O}C, 1min.	53^{O}C, 1min.
extension	72^{O}C, 1min.	72^{O}C, 1min.	72^{O}C,1.5min.
4.final extension	1 cycle: 72^{O}C, 7 min.	1 cycle: 72^{O}C, 7 min.	1 cycle: 72^{O}C, 7 min.

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis separates DNA fragments on the basis of their molecular sizes. PCR products were analyzed by 1% standard agarose gels whereas digested products were electrophoretically analyzed using 2.5–3.5 % Metaphor agarose gels (FMC). An appropriate amount of agarose was weighed out and mixed with 1xTBE buffer (Maniatis et al., 1982). The solution was heated in a microwave oven until complete solubilization and allowed to cool below 60^{O}C before poured into the gel mould. The comb was then inserted. The gel was left to solidify at room temperature for 30-45 minutes. To achieve sieving ability, metaphor agarose gels were placed in a 4^{O}C refrigerator for approximately 30 minutes as recommended by the manufacturer (FMC). When needed, the comb was carefully removed. The gel was submerged in a chamber containing an enough amount of 1xTBE buffer covering the gel for approximately 0.5 cm.

PCR-amplified products or restriction enzyme digested products were mixed with the loading dye solution (0.25% bromophenol blue and 25% Ficoll 400 in H_2O). The mixture was carefully loaded into the well. DNA marker (λ-*Hin*d III and/or 100 bp ladder) were included as DNA standards. Electrophoresis was operated at 90 volts until bromophenol blue moved to approximately 2 cm from the bottom of the gel. The electrophoresed gel was stained with a 0.5 μg/ml ethidium bromide solution for 15 minutes and destained twice to remove unbound ethidium bromide in distilled water for 15 minutes each. DNA fragments were visualized under a UV transilluminator.

2.8 Recover of the amplified COI product from agarose gels

The single positive fragment was observed when amplified 16S and 18S rDNA of oysters. However, additional fragments were observed when the COI gene region was amplified. Therefore, the COI products were fractionated through a 1.5 % agarose gel. The 710 bp fragment was excised from the electrophoresed gel and placed into a pre-weight 1.5 ml

microcentrifuge tube. DNA was then eluted from agarose gels using Prep-A-Gene DNA purification kit (BioRad). Three volumes of DNA purification binding buffer (sodium perchlorate) was added to the microcentrifuge tube containing a gel slice. The tube was placed in a 50^{O}C waterbath for 5-10 minute with occasional agitating for complete dissolving of the agarose. 7.5 μl of Prep-A-Gene matrix were added and mixed by briefly vortexing. The mixture was incubated at room temperature for 10 minutes to allow binding of DNA to the matrix. The mixture was mixed by inversion of the tube every 2 minutes during this period and centrifuged at 7,000xg for 30 seconds at room temperature. The supernatant was removed. DNA binding buffer (equivalent to 25 volumes of the added matrix) was added and vortexed. The mixture was centrifuged at the same speed for 30 seconds. The supernatant was discarded. The pellet was washed twice with 25 volumes of wash buffer. After the final wash, the trace of liquid in the tube was carefully removed. 15 μl of the elution buffer was added. The tube was incubated at 37^{O}C for 5 minutes and spun as above. The eluted DNA solution was transferred to a new microcentrifuge tube. The elution process was carried out again using 10 μl elution buffer. The eluted DNA was kept at 4^{O}C until further needed.

2.9 Restriction endonuclease digestion

The amplified 16S, 18S rDNAs and COI of representative *C. belcheri* and *S. forskali* were screened against 13 restriction endonucleases; *Alu* I(AGCT), *Hae* III(GGCC), *Mbo* I (GATC), *Rsa* I(GTAC), *Taq* I(TCGA), *Dde* I(CTNAG), *Hin*f I(GANTC), *Ase* I(ATTAAT), *Bfr* I(CTTAAG), *Dra* I(TTTAAA), *Nde* I(CATATG), *Acs* I(AorGAATTTorC) and *Swa* I (ATTTorAAAT). The digestion was performed in a 15 μl-volume composing of 6 μl of the PCR product, 1x of restriction enzyme buffer, 0.1 μg/ml BSA, 4 mM spermidine trihydrochloride, 1 unit of a restriction endonucleases and appropriate amount of sterile deionized water. The mixture was incubated at 37^{O}C for 3 hours except *Taq* I where the appropriate temperature is at 65^{O}C. At the end of incubate period, 2 μl of a loading dye was added and mixed. The digestion was then electrophoretically analysed as described previously.

Results of restriction enzyme digestion were recorded by photographed through a red filter using the formapan film.

2.10 Data analysis

2.10.1 Restriction patterns analysis

The restriction patterns generated from each restriction endonuclease were given letter designations in order of their frequencies. Haplotype A refers to the most common digestion pattern in overall investigated specimens. The remaining alphabetical profile names (B, C, etc.) indicate variant patterns reflecting their frequencies in order. The fragment sizes of all restriction profiles were estimated in comparison with those of a 100 bp DNA marker. The 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 fragment of each oyster were recorded in a binary matrix and subjected to statistical analyses using Restriction Enzyme Analysis Package (REAP), version 4.0 (McElroy, 1991).

2.10.1.1 Genetic distance between composite haplotypes

The relationships of composite haplotype were observed by genetic distance values (*d*) that could be estimated as the number of nucleotide substitution per site following the equation:

$$d = -(2/r)\ln G$$

where r is the number of recognized sequences at the restriction site and G is $[F(3-2G_1)]^{1/4}$ and repeatly calculated until $G = G_1$. The $G_1 = F^{1/4}$ is recommended to initial trial value.

F is the similarity between haplotype patterns estimated by

$$F = 2m_{xy} / (m_x + m_y)$$

where m_x and m_y are the numbers of restriction fragments in the x^{th} and y^{th} haplotypes, respectively, and m_{xy} is the number of shared fragments between two haplotypes.

2.10.1.2 Haplotype and nucleotide diversity

Genetic diversity within population was estimated from the haplotype and nucleotide diversity. The haplotype diversity (h) was estimated by

$$h = n(1-\Sigma x_i^2) / (n-1)$$

where n is the number of individuals investigated and x_i is the frequency of the i^{th} haplotype. The nucleotide diversity (d) within population is the average number of nucleotide substitution within a population estimated by

$$d_x = \frac{n_x}{n_x-1} \Sigma_{ij} x_i x_j d_{ij}$$

where n_x is the number of sequences sampled and d_{ij} is the number of nucleotide substitutions per site between the i^{th} and j^{th} haplotype. The x_i and x_j values are the sample frequencies of the i^{th} and j^{th} haplotypes in population X.

2.10.1.3 Nucleotide diversity and nucleotide divergence between populations

The nucleotide diversity is the average number of nucleotide substitutions between DNA haplotypes from populations X and Y estimated by

$$d_{xy} = \Sigma_{ij} x_i y_j d_{ij}$$

where d_{ij} is the nucleotide substitutions between the i^{th} and j^{th} haplotype from populations X and Y, respectively.

The nucleotide divergence is the average number of nucleotide substitution per site where effects of within population polymorphism have been substracted. This value was then calculated by

$$d_A = d_{xy} - (d_x + d_y) / 2$$

2.10.2 Phylogenetic reconstruction

Phylogenetic relationships between investigated samples of Thai oysters were constructed based on genetic distance (*d*) between composite haplotypes and interpopulation divergence (d_A) using a neighbor-joining method (Saitou and Nei, 1987) implemented in Phylip, version 3.56c (Felsenstein, 1993).

2.11 Cloning of amplified COI fragment

2.11.1 Amplification of COI from *C. belcheri*, *C. iredalei* and *S. cucullata*

The COI segment was amplified from representative individuals of *C. belcheri*, *C. iredalei* and *S. cucullata*, using conditions described in section 2.4. After electrophoresis, a 710 bp band was excised and isolated DNA from agarose gel as described in section 2.6.

2.11.2 Purification of eluted COI

Taq DNA polymerase remains bound to DNA molecules after *in vitro* amplification of DNA. Digestion of PCR products with proteinase K significantly increases cloning efficiency by several times (Crowe, 1991). As a result, a proteinase K solution was added to the eluted DNA to a final concentration of 50 μg/ml in the presence of 0.5% SDS. The mixture was incubated for 60 minutes at 65^{O}C. After cooling to room temperature, the mixture was extracted once with phenol-chloroform and once with chloroform. DNA was recovered by ethanol precipitation.

2.12 Ligation of COI to the T-vector

Taq I polymerase have a terminal transferase activity which results in the non-template addition of nucleotides to the 3' end of PCR products for which deoxyadenosine is almost preferentially added. This allows cloning of PCR–amplified fragments to the modified vector containing a single 3'-overhang thymidine residue (T-A cloning method).

A 710 bp COI fragment that was ligated to pGEM^{R}-T easy vector (Promega) in a 10 μl ligation reaction constituting of 5 μl of 2x rapid ligation buffer (60 mM Tris-HCl, pH7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol; MW 8000), 3 weiss units of T4 DNA ligase, 25 ng of pGEM^{R}-T easy vector and 25 ng of DNA insert. The reaction mixture was incubated overnight at 4^{O}C before electrotransformed to *E. coli* XL1-Blue.

2.13 Transformation of ligated products to *E. coli* by electroporation (Dower et al., 1988)

2.13.1 Preparation of host cells

A single colony of *E.coli* XL1-BLUE was inoculated into 15 ml of LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) plus tetracycline and incubated with shaking at 37^{O}C overnight. The starting culture was transferred into 1 liter of LB-broth and continued culture at 37^{O}C with shaking to the OD_{600} of 0.5 to 0.8. The cells were chilled briefly on ice for 15 to 30 minutes and transferred into a microcentrifuge tube and harvested by centrifugation in a prechilled rotor at 4,000xg for 15 minutes at 4^{O}C. The pellets were resuspended in 1 liter of prechilled sterile water and recentrifuged as above. The pellets were resuspended in 0.5 liter of prechilled sterile water. After centrifugation, the pellets were resuspended in 20 ml of 10% glycerol and recentrifuged. Finally, the pellets were resuspended in 2 - 3 ml of 10% glycerol and devided to 45 μl aliquots. These concentrated cells could be used immediately or stored at –70^{O}C until needed.

2.13.2 Electrotransformation

The competent cells were thawed on ice for 5 minutes. Approximately, 1 - 2 μl of the ligation product was added. The mixture was gently mixed by pipetting and left on ice for 1 minute. The mixture was electroporated in a prechilled 0.2 cm cuvette using a Gene pulser (BioRad) with the setting parameters of 25 μF, 200 Ω and 2.5 KV. After electroporation, the mixture was removed from the cuvette and added to a microcentrifuge tube containing 1 ml of SOC medium and incubated with shaking at 37^{O}C for 1 to 2 hours. Afterwards, 10 – 30 μl of cell suspension were spread on the LB agar plate containing 50 μg/ml of ampicillin, 25 μg/ml of IPTG and 20 μg/ml of X-Gal and further incubated at 37^{O}C overnight (Sambrook et al., 1989). The recombinant clones containing PCR products are usually white while those without PCR products are blue.

2.14 Isolation of recombinant plasmids DNA

Plasmid DNA was isolated using a modified alkaline lysis minipreparation procedure (Li et al., 1997). A single white colony was inoculated into 3 ml of LB medium supplementing with 50 μg/ml of ampicillin and incubated with vigorous shaking at 37^{O}C overnight. The culture was transferred into a 1.5 ml microcentrifuge tube and centrifuged for 30 seconds at 10,000xg. The supernatant was carefully poured off. One hundred microlitres of solution I (50 mM glucose, 10mM EDTA: pH8.0, 25mM Tris-HCl : pH8.0) was added. The cell pellet was resuspended by vortexing. Two hundred microlitres of freshly prepared solution II (0.2 N NaOH and 1% SDS) was added. The tube was gently inverted for 10 – 15 times. One hundred and fifty microlitres of solution III (3 M Sodium acetate: pH4.8) was added and mixed by inversion and flicking of the tube. The mixture was centrifuged for 30 seconds at 10,000xg to pellet cell debris. The supernatant was transferred into a new tube and extracted with phenol-chloroform. An equal volume of ice-cold absolute ethanol was added and mixed by inversion. The mixture was left at -80^{O}C for 30 minutes and centrifuged at 10,000xg for 10 minutes at

room temperature. The supernatant was discarded. The pellets were briefly washed twice with ice-cold 70% ethanol. The pellet was dried *in vacuo* for 5 minutes before resuspended in 50 μl of TE buffer and incubated at 65^{O}C for 10 minutes to inactivate any residual DNases. The mixture was centrifuged at 10,000xg for 5 minutes and transferred the supernatant to a new tube. RNase A was added to a final concentration of 200 μg/ml to digest contaminating RNA. The reaction mixture was incubated at 37^{O}C for 30 minutes. Plasmid DNA was stored at -20^{O}C.

2.15 Detection of recombinant clones

The recombinant clones containing insert DNA was examined by digest with *Eco*R I. The reaction was carried out in a 20 μl volume at 37^{O}C overnight. The resulting product was electrophoretically analysed by 1% agarose gel. The size of insert was compare with that of λ-*Hin*d III and 100 bp DNA ladder.

2.16 DNA sequencing

Three recombinant clones (pNKCb1, pNKCi1 and pNKSc4) were unidirectional sequenced using an automatic sequencer (ABI-PRISM, Medel 377) at the Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus. The obtained sequences were blasted against previously deposited sequences in the GenBank (NCBI) using BlastN.

2.17 Primer design

Sequences obtained were aligned using clustal X to search for any conserved region of the COI gene region. Two reverse primers (R301, CTTACTCTTATCATGGCGTTT and R353 TGTAGAAAAATGGGGTTGGGG) were designed using the Oligo 4.0.

2.18 PCR amplification and restriction analysis of the amplicon

PCR was carried out using HCO2198+R301 and HCO2198+R353 to identify the specific amplification product and to evaluate the possibility to developed selective amplification for *C. iredalei* using the conditions described in 2.6 and Table 2.3.

Subsequently, higher stringency of amplification conditions was performed at 60^{O}C and 62^{O}C annealing temperatures for 30 cycles. The amplification products were digested with *Mbo* I for 4 hours, electrophoretically fractionated through 3.0% Metaphor agarose, stained with ethidium bromide (0.5 μg/ml), and visualized using a UV transilluminator.

CHAPTER III
RESULTS

3.1 DNA Extraction

Genomic DNA of approximately 23.1 kb in size was obtained from the adductor muscle of oysters using a proteinase K-phenol-chloroform extraction method. However, partial degraded DNA was observed in most of the samples (Figure 3.1). The amount and purity of extracted DNA was examined spectrophotometrically. Approximately 50-100 μg of nucleic acids were obtained from 100 mg starting tissue. The ratio of OD_{260}/OD_{280} was 2.0-2.5 indicating a contamination of RNA in the extracted DNA samples. Nevertheless, the quality of degraded DNA and the contamination of RNA did not interfere subsequent PCR amplification.

3.2 PCR amplification

Seven sets of primers of mitochondrial and nuclear DNA genes were screened. Three pairs of primers including 16S and 18S rDNAs and COI (Figure 3.2) were successfully amplified in all oyster species and the outgroup reference, *P. viridis*. Notably, three reverse primers (16S-R1, 16S-R2 and 16S-R3) of 16S rDNA were tested with a fixed forward primer (16S-F). The 16S-R3 did not yield any amplification product whereas both 16S-R1 and 16S-R2 provide a specific amplification product at 560 bp in length but the latter generate greater amount of the PCR product.

Although the size of COI at approximately 710 bp was specifically amplified, several fragments were observed from amplification of COI from each oyster (Figure 3.2C). Alteration of the amplification profiles could not eliminate these nonspecific products. Nevertheless, the most intense amplification band at 710 bp was observed as expected. This band was then excised from the gel. The recovered COI DNA was then subjected to restriction analysis.

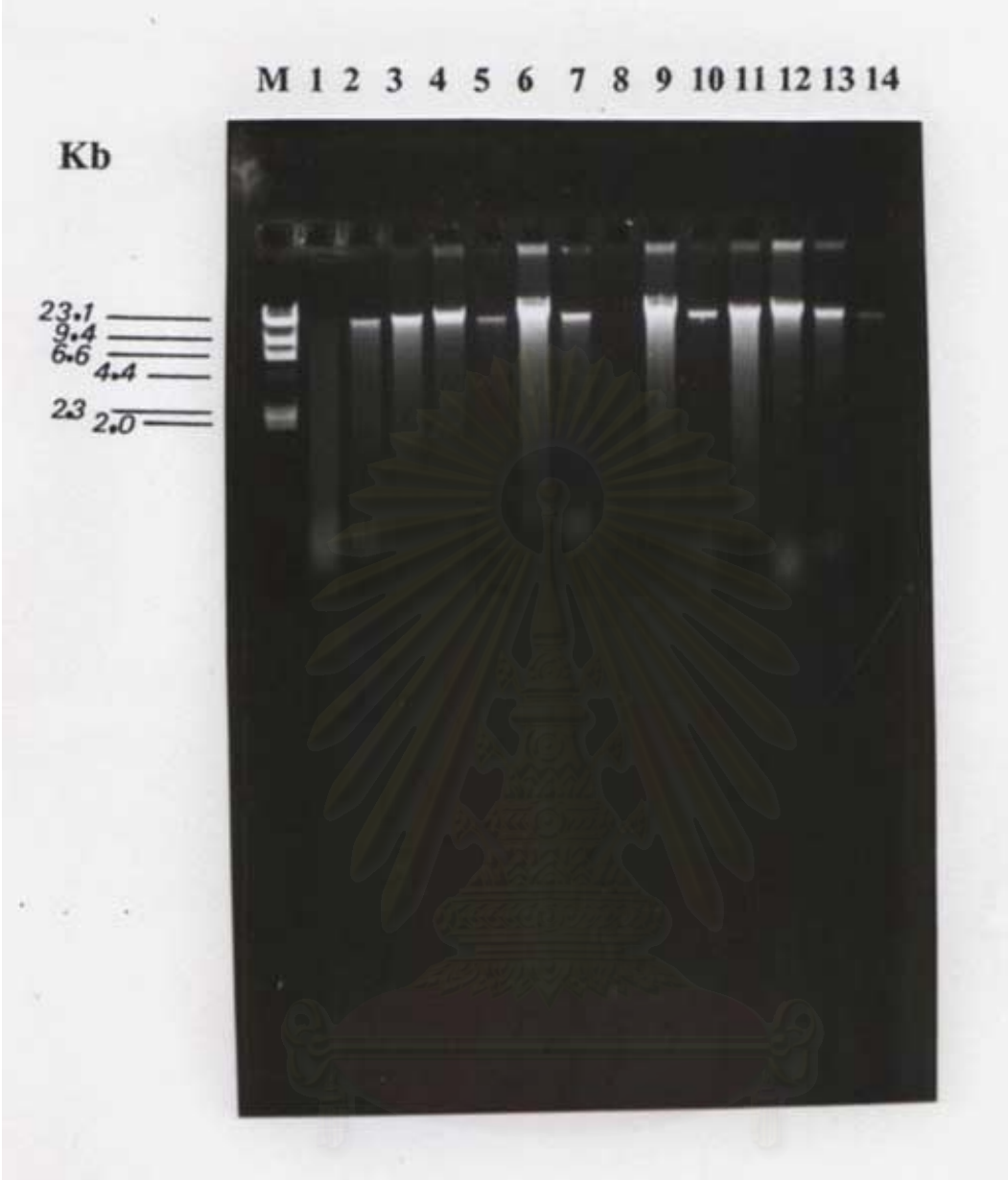

Figure 3.1 Genomic DNA extracted from the adductor muscle of oysters (lanes 1-14). The extracted DNA was electrophoresed through a 1.0 % agarose gel and stained with ethidium bromide. A λ-*Hin*d III was used as a DNA marker (lane M).

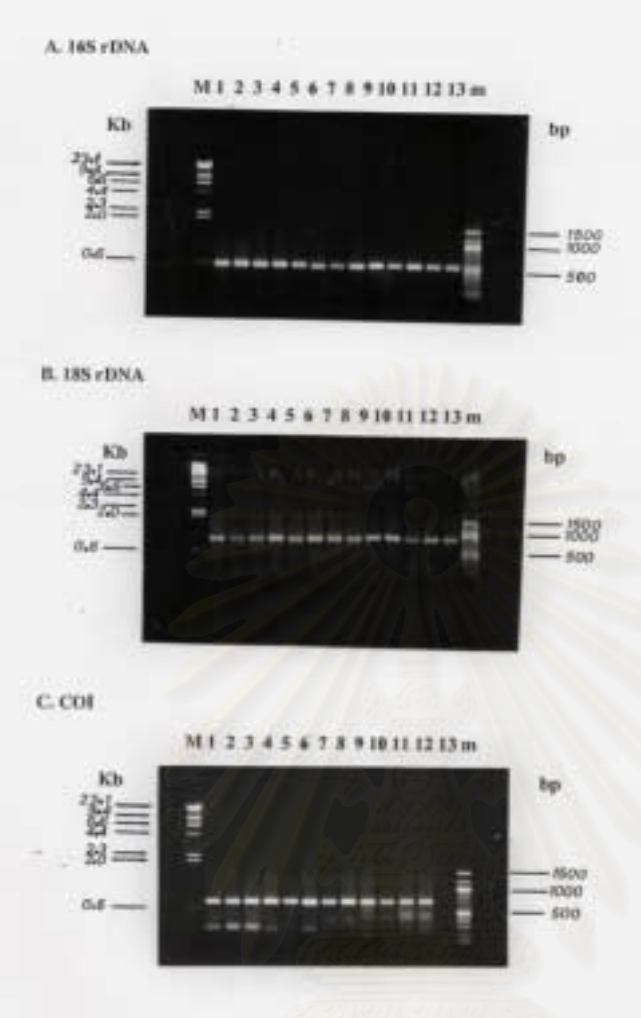

Figure 3.2 Electrophoresis of amplified 16S (A), 18S rDNA (B) and COI (C) of *C. belcheri* (lane 1-3), *C.iredalei* (lane 4-6), *S.cucullata* (lane7-9), *S. forskali* (lane 10-11 in A and B and lane 10 in C) and *S. mytiloides* (lane 12-13 in A and B and lane 11-12 in C). Lane 13 (C) was a negative control for COI. A λ-*Hin*d III (lane M) and a 100 bp ladder (lane m) were used as a DNA marker.

3.3 Restriction analysis of 16S rDNA, 18S rDNA and COI genes

The PCR-amplified 16S rDNA, 18S rDNA and COI of *C. belcheri* and *S. forskali* were digested with 13 restriction enzymes. Of these, the 16S rDNA restricted with *Acs* I, *Alu* I, *Dde* I, *Dra* I, *Rsa* I, *Taq* I, the 18S rDNA restricted with *Hin*f I and the COI restricted with *Acs* I, *Dde* I and *Mbo* I revealed polymorphism of restriction enzyme patterns. These enzymes were chosen for restriction analysis across overall specimens.

A total of 90 restriction patterns (single haplotypes) was observed from analysis of 166 investigated individuals with polymorphic restriction endonucleases (Figure 3.3-3.12 and Table 3.1). Digestion of 16S rDNA (560bp) with *Acs* I, *Alu* I, *Dde* I, *Dra* I, *Rsa* I and *Taq* I generated 12, 12, 9, 6, 4 and 3 patterns, respectively. Only three restriction patterns were observed when digested 18S rDNA with *Hin*f I. Digestion of amplified COI with *Acs* I, *Dde* I and *Mbo* I generated 6, 13 and 22 digestion patterns, respectively. Interconnection between COI-digested patterns (e.g. from *Acs* I) can be simply explained by loss and gain of restriction sites indicating that a 710 bp fragment from different species used for restriction analysis was homologous.

Differentiation of the species origin of three commercials oysters in Thailand (*C. belcheri*, *C. iredalei* and *S. cucullata*), unidentified *Crassostrea* sp., unidentified *Saccostrea* sp. group 2, *S. commercialis* and *P. viridis* could be carried out by at least one restriction enzyme (Table 3.2 and 3.3). Results from digestion of 18S rDNA with *Hin*f I could differentiate *Crassostrea* (pattern A), *Saccostrea* and *Striostrea* (pattern B) and the mussel (pattern C) unambiguously. As can be seen from Table 3.2, *C. belcheri* can be differentiate from other species in the present study by 16S rDNA digested with *Acs* I, *Alu* I and COI digested with *Dde* I and *Mbo* I.

The black scar oyster, *C. iredalei* could be identified by species-specific patterns from 16S rDNA digested with *Acs* I, *Dra* I and COI digested with *Dde* I and *Mbo* I. Species-specific single haplotypes for *S. cucullata* were observed from 16S rDNA digested with *Alu* I and COI digested with *Dde* I and *Mbo* I. A large number of restriction patterns were observed when

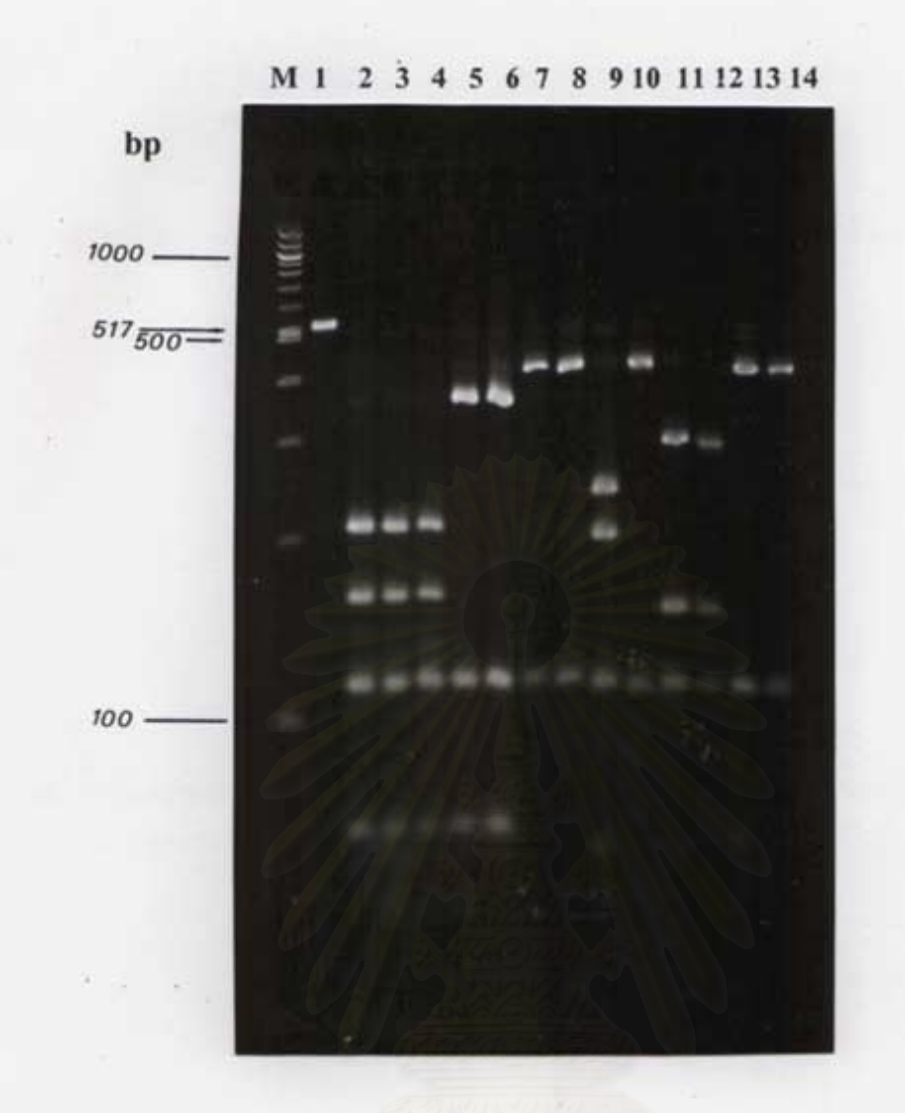

Figure 3.3 An example of restriction patterns of 16S rDNA digested with *Acs* I

Lane M = A 100 bp DNA ladder (Promega)
Lane 1 = An undigested 16S rDNA (560 bp in length)
Lanes 2 – 4 = *C. belcheri* (pattern A)
Lanes 5 – 6 = *C. iredalei* (pattern B)
Lanes 7 – 8 = *Crassostrea* sp. (pattern D)
Lane 9 = *S. cucullata* (pattern C)
Lane 10 = *S. forskali* (pattern D)
Lanes 11 – 12 = *Saccostrea* sp. group 2 (pattern J)
Lanes 13 – 14 = *Saccostrea* sp. group 1 (pattern D)

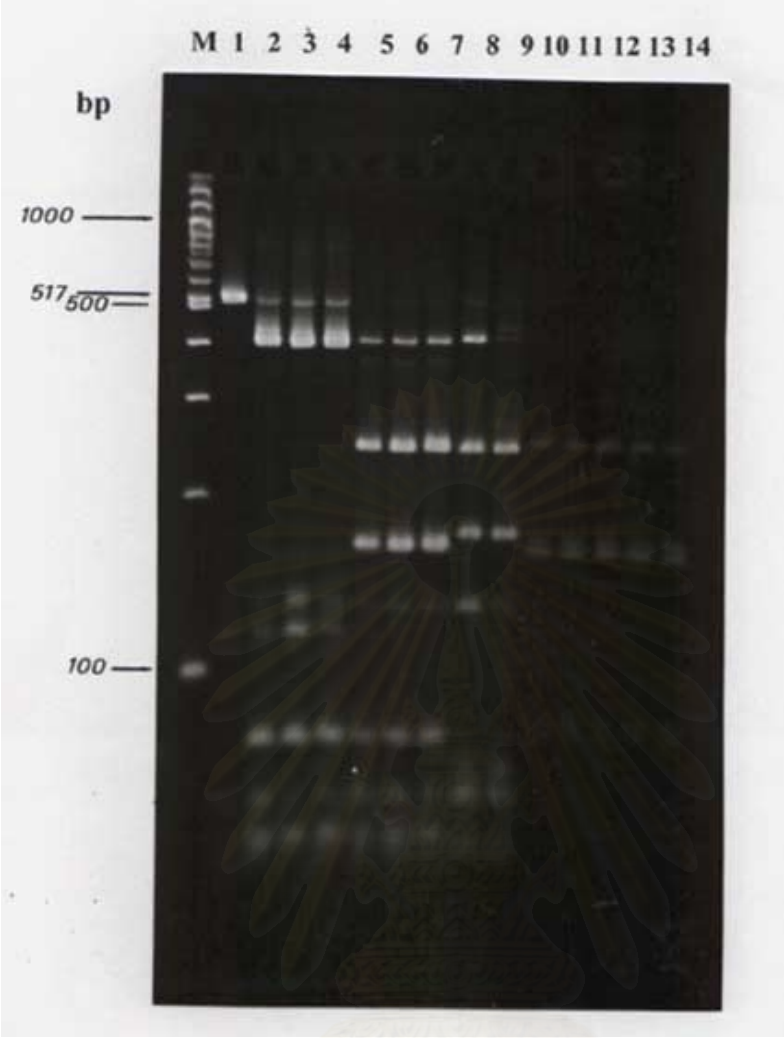

Figure 3.4 An example of restriction patterns of 16S rDNA digested with *Alu* I

Lane M = A 100 bp DNA ladder (Promega)
Lane 1 = An undigested 16S rDNA (560 bp in length)
Lanes 2 – 4 = *C. belcheri* (pattern A)
Lanes 5 – 7 = *C. iredalei* (pattern B)
Lanes 8 - 9 = *S. cucullata* (pattern C)
Lanes 10 - 13 = *S. forskali* (pattern E)
Lane 14 = *S. mytiloides* (pattern E)

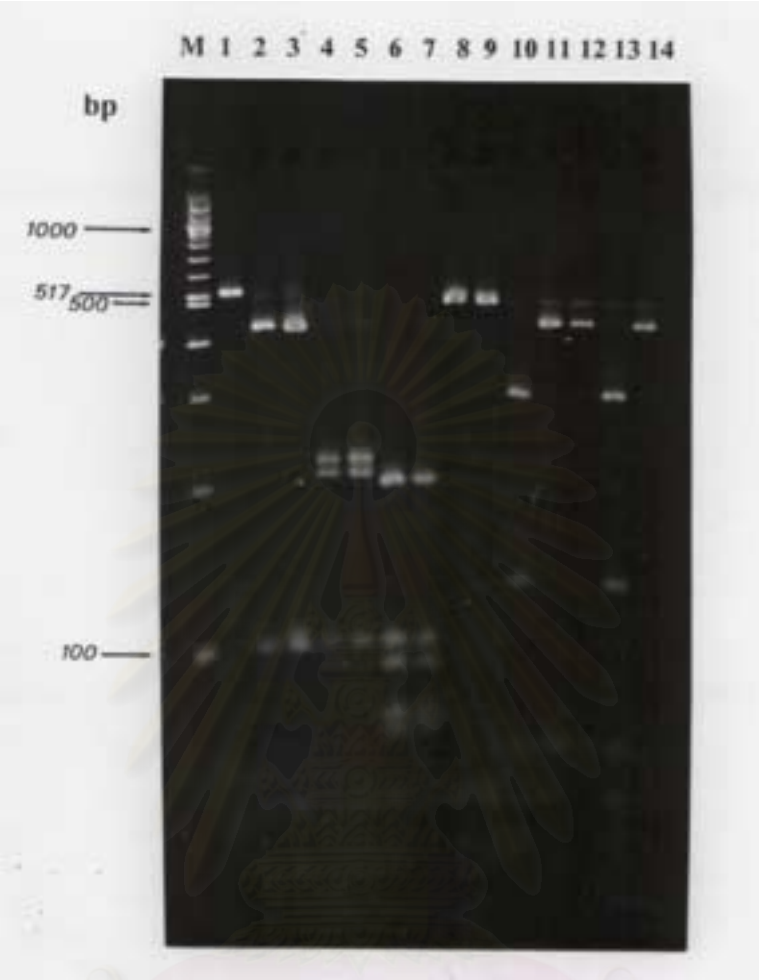

Figure 3.5 An example of restriction patterns of 16S rDNA digested with *Dde* I

Lane M	=	A 100 bp DNA ladder (Promega)
Lane 1	=	An undigested 16S rDNA (560 bp in length)
Lanes 2 – 3	=	*C. belcheri* (pattern A)
Lanes 4 – 5	=	*C. iredalei* (pattern B)
Lanes 6 – 7	=	*Crassostrea* sp. (pattern H)
Lanes 8 - 9	=	*S. cucullata* (pattern C)
Lane 10	=	*S. forskali* (pattern D)
Lanes 11 – 12	=	*Saccostrea* sp. group 2 (pattern F)
Lanes 13 – 14	=	*S. mytiloides* (pattern D and F, respectively)

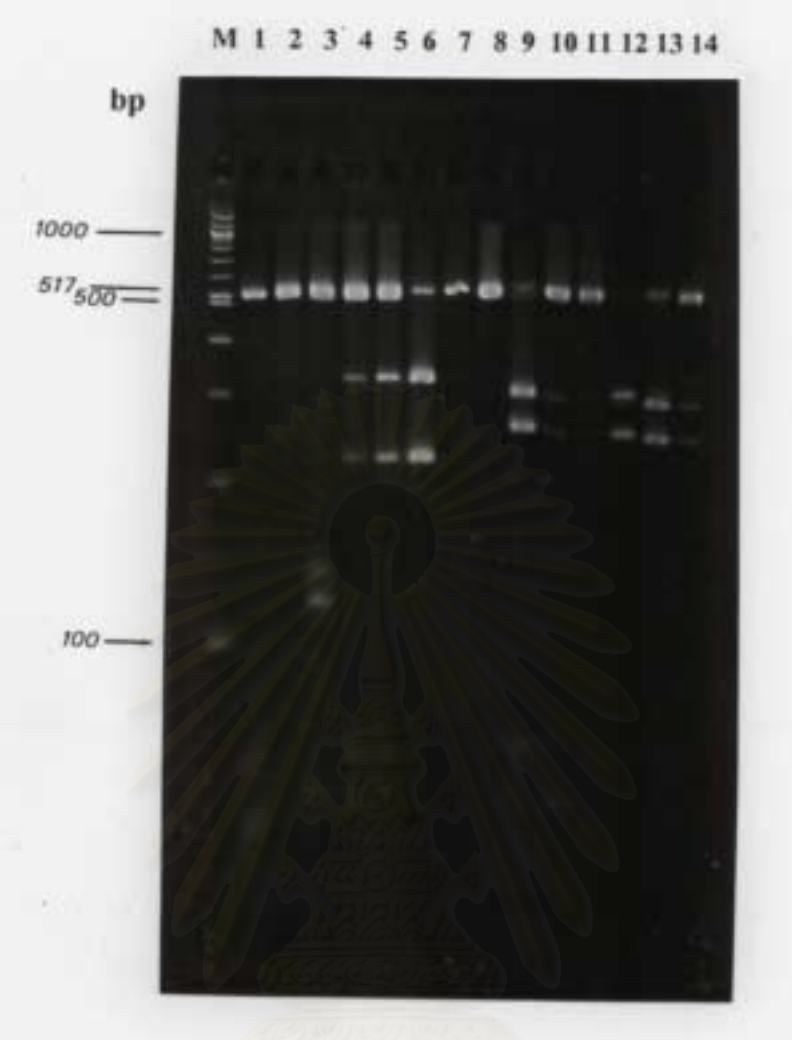

Figure 3.6 An example of restriction patterns of 16S rDNA digested with *Dra* I

Lane M = A 100 bp DNA ladder (Promega)
Lane 1 = An undigested 16S rDNA (560 bp in length)
Lanes 2 – 3 = *C. belcheri* (pattern A)
Lanes 4 – 6 = *C. iredalei* (pattern B)
Lanes 7 – 8 = *Crassostrea* sp. (pattern A)
Lane 9 = *S. cucullata* (pattern C)
Lanes 10 - 11 = *S. forskali* (pattern C)
Lane 12 = *Saccostrea* sp. group 2 (pattern C)
Lanes 13 – 14 = *S. mytiloides* (pattern C)

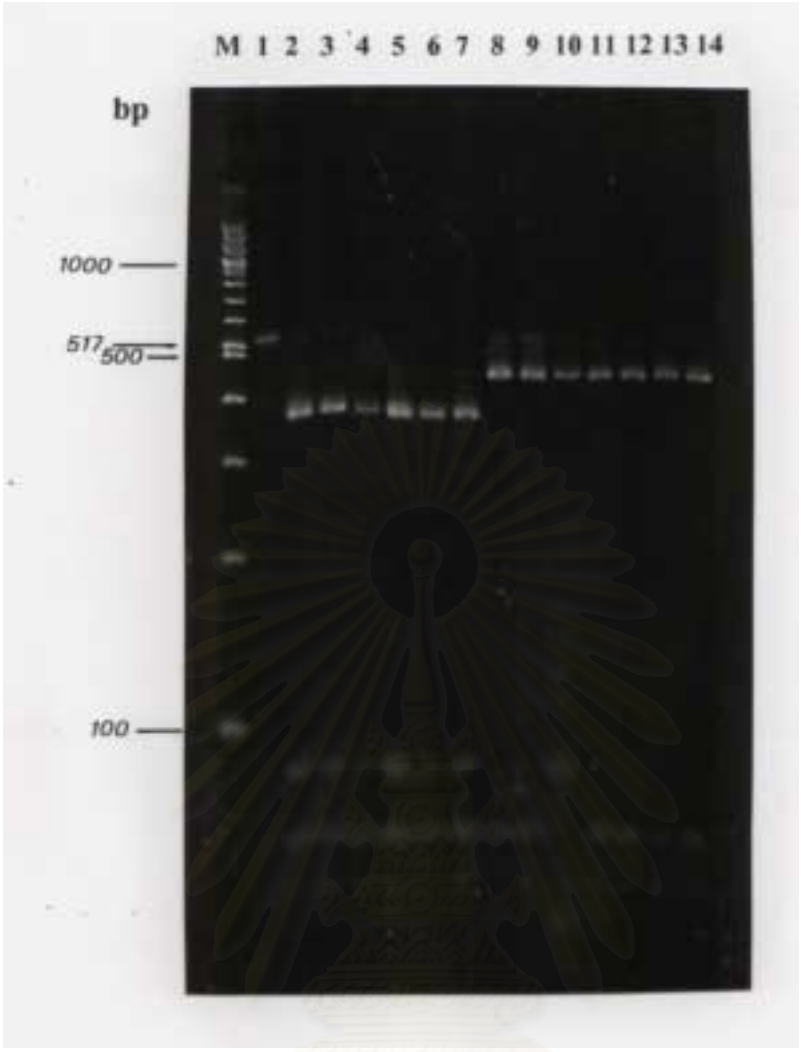

Figure 3.7 An example of restriction patterns of 16S rDNA digested with *Taq* I

Lane M	=	A 100 bp DNA ladder (Promega)
Lane 1	=	An undigested 16S rDNA (560 bp in length)
Lanes 2 – 4	=	*C. belcheri* (pattern A)
Lanes 5 – 7	=	*C. iredalei* (pattern A)
Lane 8	=	*Crassostrea* sp. (pattern B)
Lanes 9 - 12	=	*S. forskali* (pattern B)
Lanes 13 – 14	=	*S. mytiloides* (pattern B)

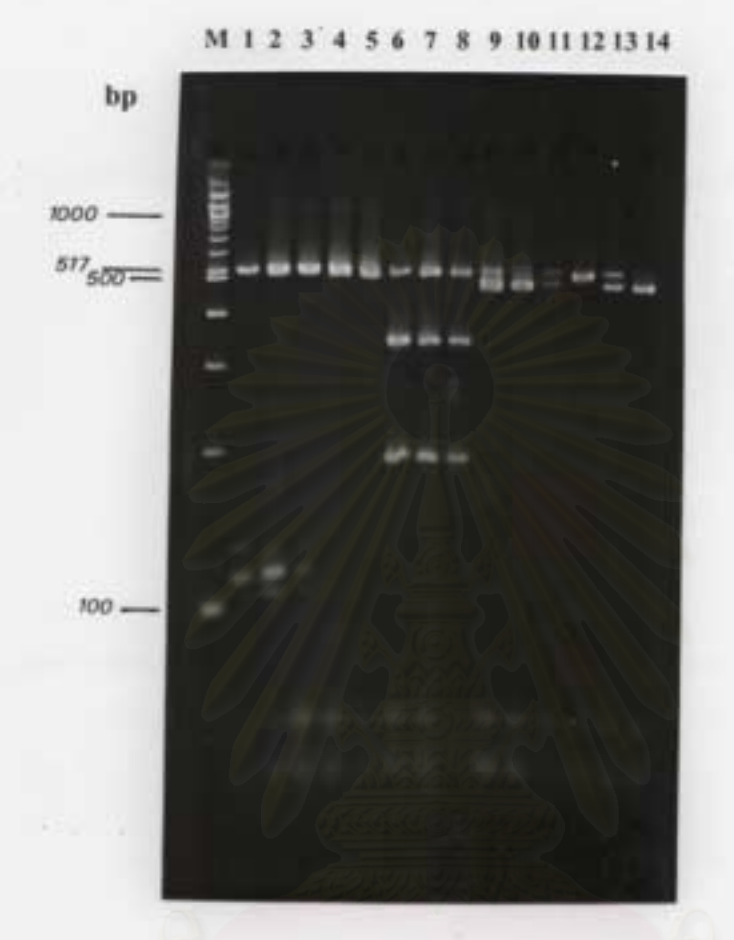

Figure 3.8 An example of restriction patterns of 16S rDNA digested with *Rsa* I

Lane M = A 100 bp DNA ladder (Promega)
Lane 1 = An undigested 16S rDNA (560 bp in length)
Lanes 2 – 3 = *C. belcheri* (pattern A)
Lanes 4 – 5 = *C. iredalei* (pattern B)
Lanes 6 – 8 = *Crassostrea* sp. (pattern C)
Lane 9 = *S. cucullata* (pattern B)
Lanes 10 - 11 = *Saccostrea* sp. group 2 (pattern B)
Lane 12 = *S. forskali* (pattern A)
Lanes 13 – 14 = *S. mytiloides* (pattern B)

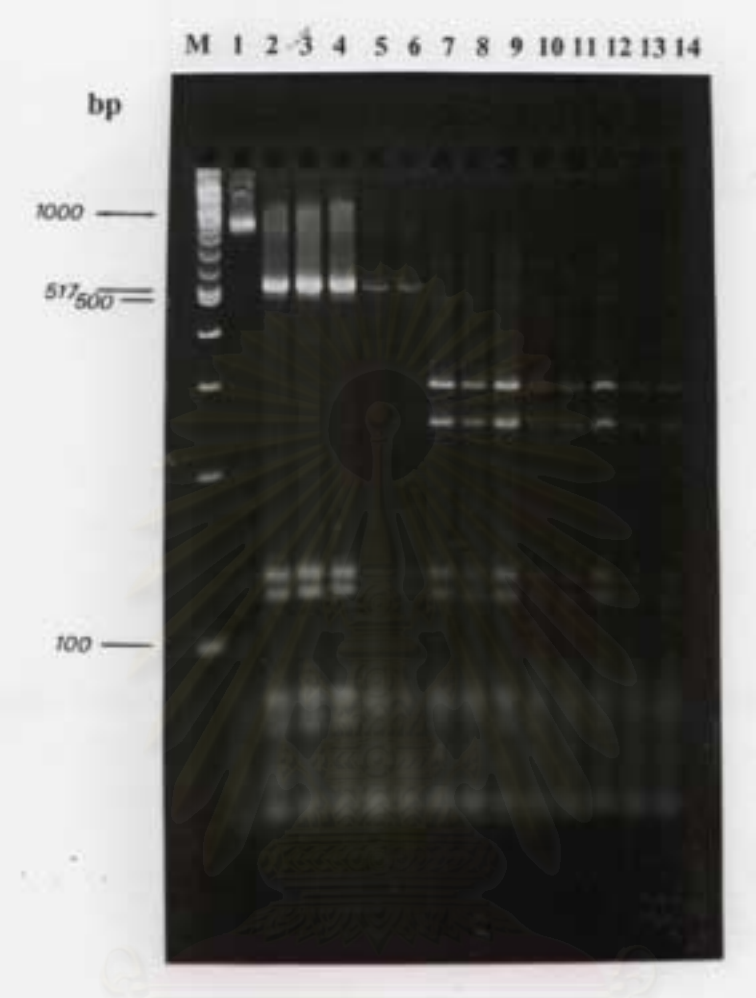

Figure 3.9 An example of restriction patterns of 18S rDNA digested with *Hinf* I

Lane M	=	A 100 bp DNA ladder (Promega)
Lane 1	=	An undigested 18S rDNA (800 bp in length)
Lanes 2 – 3	=	*C. belcheri* (pattern A)
Lanes 4 – 5	=	*C. iredalei* (pattern A)
Lane 6	=	*Crassostrea* sp. (pattern A)
Lanes 7 - 9	=	*S. cucullata* (pattern B)
Lanes 10 - 12	=	*S. forskali* (pattern B)
Lanes 13 – 14	=	*S. mytiloides* (pattern B)

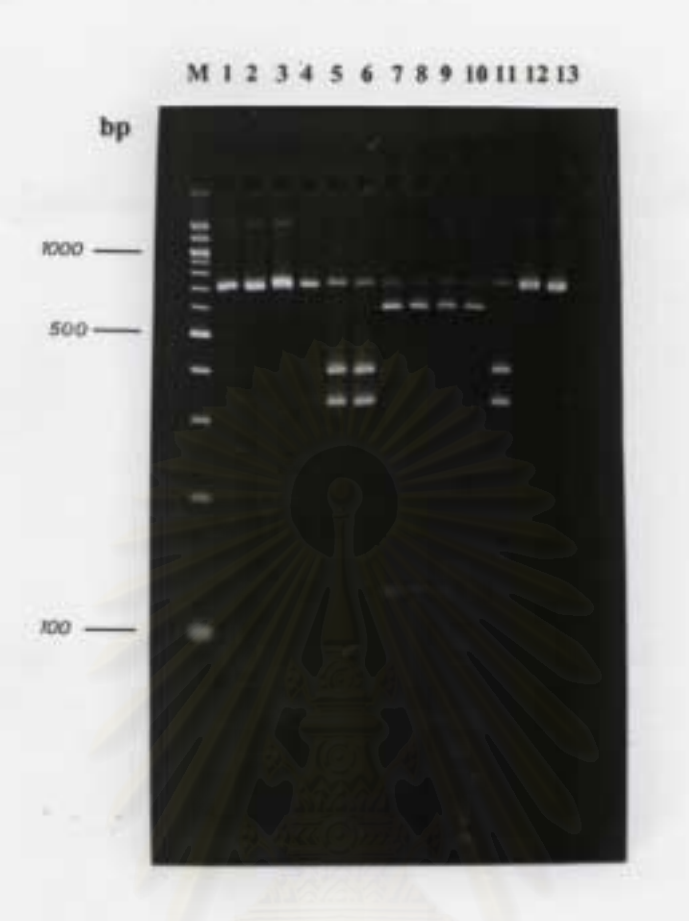

Figure 3.10 An example of restriction patterns of COI digested with *Acs* I

Lane M	=	A 100 bp DNA ladder (Promega)
Lane 1	=	An undigested COI (710 bp in length)
Lane 2	=	*C. belcheri* (pattern A)
Lane 3	=	*C. iredalei* (pattern A)
Lane 4	=	*Crassostrea* sp. (pattern A)
Lanes 5 - 6	=	*S. cucullata* (pattern B)
Lanes 7 - 10	=	*S. forskali* (pattern C)
Lane 11	=	*Saccostrea* sp. group 2 (pattern B)
Lane 12	=	*Saccostrea* sp. group 1 (pattern A)
Lane 13	=	oys122 (pattern A)

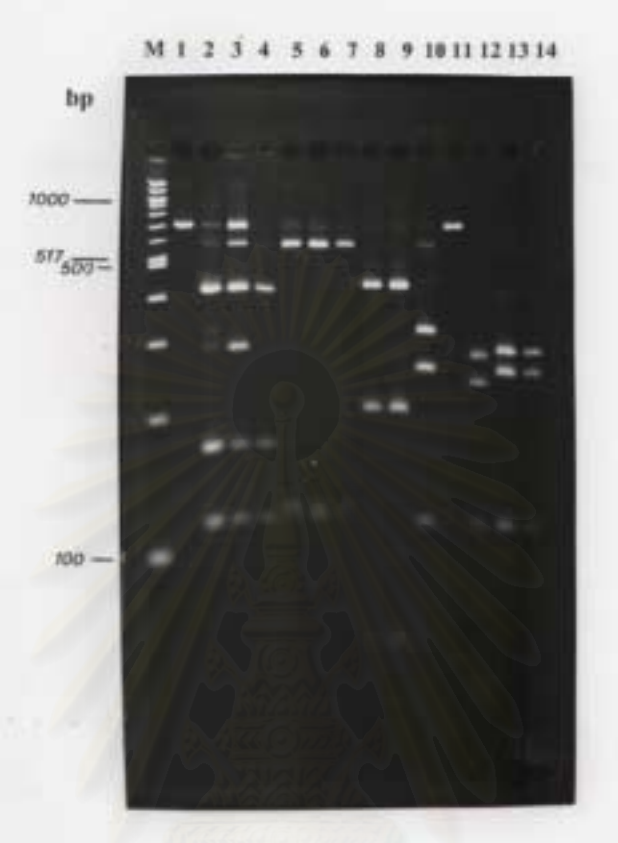

Figure 3.11 An example of restriction patterns of COI digested with *Dde* I

Lane M	=	A 100 bp DNA ladder (Promega)
Lane 1	=	An undigested COI (710 bp in length)
Lanes 2 – 4	=	*C. belcheri* (pattern A)
Lanes 5 – 7	=	*C. iredalei* (pattern B)
Lanes 8 – 9	=	*Crassostrea* sp. (pattern G)
Lane 10	=	*S. cucullata* (pattern C)
Lane 11	=	*S. forskali* (pattern D)
Lane 12	=	*Saccostrea* sp. group 2 (pattern H)
Lane 13	=	*Saccostrea* sp. group 1 (pattern J)
Lane 14	=	oys122 (pattern J)

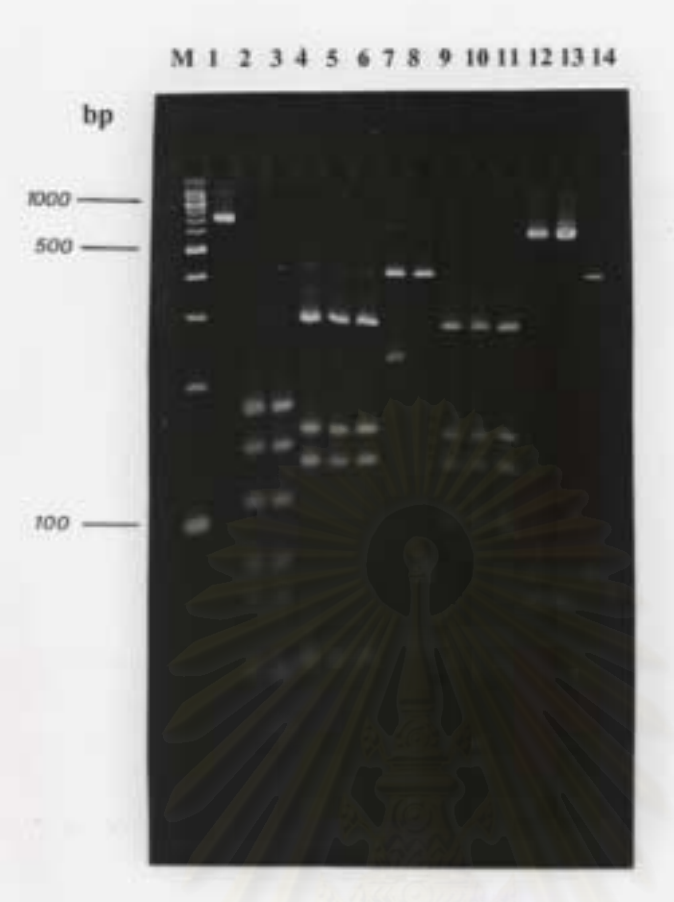

Figure 3.12 An example of restriction patterns of COI digested with *Mbo* I

Lane M	=	A 100 bp DNA ladder (Promega)
Lane 1	=	An undigested COI (710 bp in length)
Lanes 2 – 3	=	*C. belcheri* (pattern A)
Lanes 4 – 6	=	*C. iredalei* (pattern C)
Lane 7	=	*Crassostrea* sp. (pattern K)
Lane 8	=	*S. cucullata* (pattern D)
Lanes 9 - 11	=	*S. forskali* (pattern F)
Lanes 12 – 13	=	*Saccostrea* sp. group 2 (pattern M)
Lane 14	=	*Saccostrea* sp. group 1 (pattern G)

Table 3.1 Restriction fragment patterns from digestion of the 16S rDNA, 18S rDNA and COI genes of Thai oysters and *P. viridis* with restriction endonucleases used in this study. Numbers in bracket indicate missing bands. When possible, a maximum number of a sum of those bands was inferred.

Restriction analysis	Genotype observed (bp)					
16S rDNA – *Acs* I	A	B	C	D	E	F
	220	380	240	430	560	280
	160	120	200	120		150
	120	60	120			80
	60					(50)
	G	H	I	J	K	L
	320	230	280	300	400	260
	150	150	150	150	140	150
	80	120	120	120		120
		(50)				(20)

Table 3.1 (continue)

Restriction analysis	Genotype observed (bp)					
16S rDNA – *Alu* I	A	B	C	D	E	F
	420	250	250	250	170	250
	80	170	180	180	165	170
	60	80	70	130	85	130
		60	60		80	
					60	
	G	H	I	J	K	L
	250	250	210	250	250	250
	170	90	170	180	140	170
	70	80	100	60	130	115
	(60)	70	80	50		
		(60)				

Table 3.1 (continue)

Restriction analysis 16S rDNA – *Dde* I	Genotype observed (bp)					
	A	B	C	D	E	F
	430	230	500	300	300	430
	105	220	50	135	190	70
		105		70	70	(50)
				(50)		
	G	H	I			
	430	210	430			
	65	105	60			
	(50)	95	50			
		80				
		75				

Restriction analysis 16S rDNA – *Dra* I	Genotype observed (bp)					
	A	B	C	D	E	F
	560	330	295	250	250	320
		220	250	210	240	205
				80		

Restriction analysis 16S rDNA – *Rsa* I	Genotype observed (bp)			
	A	B	C	D
	560	500	350	210
		(60)	200	160
				80

Table 3.1 (continue)

Restriction analysis	Genotype observed (bp)		
16S rDNA – *Taq* I	A	B	C
	360	430	425
	85	70	70
	70	(60)	60
	(60)		

Restriction analysis	Genotype observed (bp)		
18S rDNA – *Hinf* I	A	B	C
	530	300	530
	130	250	130
	120	130	90
		120	

Restriction analysis	Genotype observed (bp)					
COI – *Acs* I	A	B	C	D	E	F
	710	400	600	400	390	520
		330	130	200	320	80
				130		70

Table 3.1 (continue)

Restriction analysis COI – *Dde* I	Genotype observed (bp)					
	A	B	C	D	E	F
	420	600	330	710	300	430
	170	130	270		250	230
	120		130		200	60
	G	H	I	J	K	L
	300	300	305	405	400	300
	255	270	270	195	150	295
	130	120	130	130	120	120
	M					
	450					
	190					
	70					

Table 3.1 (continue)

Restriction analysis	Genotype observed (bp)					
COI – *Mbo* I	A	B	C	D	E	F
	180	290	290	420	495	290
	150	150	160	78	75	160
	120	80	140	75	60	140
	80	70	60	60		100
	70	55	(40)			
	55					
	G	H	I	J	K	L
	420	290	290	295	420	420
	80	140	210	195	250	190
	60	120	100	140		60
		75	(85)	(60)		
		55				
	M	N	O	P	Q	R
	600	520	420	420	420	420
	70	75	80	80	75	75
		60	60	75	60	55x2
		55		60		
	S	T	U	V		
	495	600	420	600		
	130	60	160	95		
	70		60			

Table 3.2 Distribution frequencies of single haplotype of each oyster species and the mussel (*P. viridis*) resulted from digestion of 16S rDNA with *Acs* I, *Alu* I, *Dde* I, *Dra* I, *Taq* I and *Rsa* I, 18S rDNA with *Hin*f I and COI with *Acs* I, *Dde* I and *Mbo* I

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Acs* I:											
A	17(1.0)	-	-	-	-	-	-	-	-	-	-
B	-	21(1.0) Oys039 Oys095 Oys104	-	-	-	-	-	-	-	-	-
C	-	-	22(0.96)	-	-	2(0.22)	-	-	-	-	-
D	-	-	1(0.04)	37(0.82) Oys030 Oys121 Oys122	10(0.59)	7(0.78)	9(1.0)	-	5(1.0)	-	-
E	-	-	-	1(0.02)	-	-	-	-	-	-	-

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Acs* I:											
F	-	-	-	3(0.07)	-	-	-	-	-	-	-
F(D+F)*	-	-	-	1(0.02)	-	-	-	-	-	-	-
G	-	-	-	2(0.04)	-	-	-	-	-	-	-
H	-	-	-	-	1(0.06)	-	-	-	-	-	-
I	-	-	-	1(0.02)	1(0.06)	-	-	-	-	-	-
I(D+I)*	-	-	-	-	1(0.06)	-	-	-	-	-	-
J	-	-	-	-	-	-	-	9(1.0)	-	-	-
K	-	-	-	-	-	-	-	-	-	-	5(1.0)
L(L+I)*	-	-	-	-	3(0.17)	-	-	-	-	6(1.0)	-
L(D+L)*	-	-	-	-	1(0.06)	-	-	-	-	-	-

* specimens showing mixed patterns of digestion implying their hybrid status. Only the dominant pattern of a particular specimen was inferred and used for further analysis

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Alu* I:											
A	17(1.0)	-	-	-	-	-	-	-	-	-	-
B	-	21(1.0) Oys039 Oys095 Oys104	-	-	2(0.118)	-	-	-	-	-	-
C	-	-	8(0.35)	-	-	-	-	-	-	-	-
D	-	-	15(0.65)	-	-	-	-	-	-	-	-
E	-	-	-	27(0.60) Oys030	6(0.353)	-	-	-	5(1.0)	-	-
F	-	-	-	15(0.33) Oys121 Oys122	1(0.059)	-	7(0.78)	-	-	6(1.0)	-

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Alu* I:											
G	-	-	-	-	1(0.059)	-	-	-	-	-	-
H	-	-	-	-	7(0.411)	-	-	-	-	-	-
I	-	-	-	-	-	9(1.0)	-	-	-	-	-
J	-	-	-	-	-	-	-	9(1.0)	-	-	-
K	-	-	-	3(0.07)	-	-	2(0.22)	-	-	-	-
L	-	-	-	-	-	-	-	-	-	-	5(1.0)

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Dde* I:											
A	16(0.94)	-	-	-	-	-	-	-	-	-	-
B	1(0.06)	21(1.0) Oys039 Oys095 Oys104	-	-	-	-	-	-	-	-	-
C	-	-	23(1.0)	-	-	-	-	-	-	6(1.0)	-
D	-	-	-	28(0.62) Oys030	8(0.47)	-	-	-	5(1.0)	-	-
E	-	-	-	1(0.02)	-	-	-	-	-	-	-
F	-	-	-	12(0.27)	9(0.53)	-	6(0.67)	9(1.0)	-	-	-

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Dde* I:											
G	-	-	-	4(0.09) Oys121 Oys122	-	-	3(0.33)	-	-	-	-
H	-	-	-	-	-	9(1.0)	-	-	-	-	-
I	-	-	-	-	-	-	-	-	-	-	5(1.0)

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Dra* I:											
A	17(1.0)	-	-	15(0.33) Oys121 Oys122	2(0.12)	9(1.0)	1(0.11)	-	1(0.20)	-	-
B	-	21(1.0) Oys039 Oys095 Oys104	-	-	-	-	-	-	-	-	-
C	-	-	23(1.0)	30(0.67) Oys030	6(0.35)	-	8(0.89)	9(1.0)	4(0.80)	5(0.83)	-
D	-	-	-	-	9(0.53)	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	1(0.17)	-
F	-	-	-	-	-	-	-	-	-	-	6(1.0)

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Taq* I:											
A	17(1.0)	21(1.0) Oys039 Oys104	-	-	-	-	-	-	-	-	-
B	-	Oys095	23(1.0)	45(1.0) Oys030 Oys121 Oys122	17(1.0)	9(1.0)	9(1.0)	9(1.0)	5(1.0)	6(1.0)	-
C	-	-	-	-	-	-	-	-	-	-	5(1.0)

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
16S-*Rsa* I:											
A	17(1.0)	20(0.95) Oys039 Oys095 Oys104	-	35(0.78) Oys030	8(0.47)	-	1(0.11)	-	5(1.0)	-	-
B	-	-	23(1.0)	10(0.22) Oys121 Oys122	9(0.53)	-	8(0.89)	9(1.0)	-	6(1.0)	-
C	-	1(0.05)	-	-	-	9(1.0)	-	-	-	-	-
D	-	-	-	-	-	-	-	-	-	-	5(1.0)

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
18S-*Hin*f I:											
A	17(1.0)	21(1.0) Oys039 Oys095 Oys104	-	-	-	9(1.0)	-	-	-	-	-
B	-	-	23(1.0)	45(1.0) Oys030 Oys121 Oys122	17(1.0)	-	9(1.0)	9(1.0)	5(1.0)	6(1.0)	-
C	-	-	-	-	-	-	-	-	-	-	5(1.0)

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
COI-*Acs* I:											
A	17(1.0)	21(1.0) Oys039 Oys095 Oys104	-	15(0.33) Oys121 Oys122	9(0.53)	9(1.0)	9(1.0)	-	-	-	-
B	-	-	23(1.0)	-	-	-	-	9(1.0)	-	-	-
C	-	-	-	29(0.64) Oys030	8(0.47)	-	-	-	5(1.0)	-	-
D	-	-	-	1(0.02)	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	6(1.0)	-
F	-	-	-	-	-	-	-	-	-	-	5(1.0)

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
COI-*Dde* I:											
A	17(1.0)	-	-	-	-	-	-	-	-	-	-
B	-	21(1.0) Oys039 Oys095 Oys104	-	-	-	-	-	-	-	-	-
C	-	-	23(1.0)	-	-	-	-	-	-	-	-
D	-	-	-	28(0.62) Oys030	8(0.47)	-	-	-	5(1.0)	-	-
E	-	-	-	1(0.02)	-	-	-	-	-	-	-
F	-	-	-	-	-	9(1.0)	-	-	-	-	-
G	-	-	-	-	-	-	-	9(1.0)	-	-	-

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp.3	Scom	Pevi
COI-*Dde* I:											
H	-	-	-	8(0.18) Oys121	-	-	3(0.33)	-	-	-	-
I	-	-	-	2(0.04) Oys122	-	-	6(0.67)	-	-	-	-
J	-	-	-	6(0.13)	-	-	-	-	-	-	-
K	-	-	-	-	9(0.53)	-	-	-	-	-	-
L	-	-	-	-	-	-	-	-	-	6(1.0)	-
M	-	-	-	-	-	-	-	-	-	-	5(1.0)

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
COI-*Mbo* I:											
A	16(0.94)	-	-	-	-	-	-	-	-	-	-
B	1(0.06)	-	-	-	-	-	-	-	-	-	-
C	-	20(0.95) Oys039 Oys095 Oys104	-	-	-	-	-	-	-	-	-
D	-	-	19(0.83)	-	-	-	-	-	-	-	-
E	-	-	4(0.17)	-	-	-	-	-	-	-	-
F	-	-	-	38(0.84) Oys030	7(0.41)	-	-	-	5(1.0)	-	-
G	-	-	-	-	-	-	4(0.44)	-	-	-	-
H	-	-	-	1(0.02)	-	-	-	-	-	-	-

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
COI-*Mbo* I:											
I	-	-	-	-	1(0.06)	-	-	-	-	-	-
J	-	-	-	-	9(0.53)	-	-	-	-	-	-
K	-	-	-	-	-	7(0.78)	-	-	-	-	-
L	-	-	-	-	-	2(0.22)	-	-	-	-	-
M	-	-	-	-	-	-	-	7(0.78)	-	-	-
N	-	-	-	-	-	-	-	-	-	6(1.0)	-
O	-	-	-	Oys122	-	-	2(0.22)	-	-	-	-
P	-	-	-	1(0.02) Oys121	-	-	2(0.22)	-	-	-	-
Q	-	-	-	-	-	-	1(0.11)	-	-	-	-

Table 3.2 (continue)

Haplotype	Frequency										
	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
COI-*Mbo* I:											
R	-	-	-	5(0.11)	-	-	-	-	-	-	-
S	-	-	-	-	-	-	-	1(0.11)	-	-	-
T	-	-	-	-	-	-	-	-	-	-	5(1.0)
U	-	1(0.05)	-	-	-	-	-	-	-	-	-
V	-	-	-	-	-	-	-	1(0.11)	-	-	-

Abbreviation: Cb = *Crassostrea belcheri*, Ci = *C. iredalei*, Sc = *Saccostrea cucullata*, Sf = *S. forskali*, Sm = *Striostrea* (*Paratriostrea*) *mytiloides*, Csp = *Crassostrea* sp., Ssp 1 = *Saccostrea* sp. group 1 from Suratthani, Ssp 2 = *Saccostrea* sp. group 2 from Ranong, Ssp 3 = *Saccostrea* sp. group 3 from Samutsakhon, Scom = *S. commercialis* and Pevi = *Perna viridis*

Table 3.3 A molecular taxonomic key for three commercially cultured oysters in Thailand

DNA marker	Species
1. 18S rDNA-*Hinf* I	
Pattern A (530,130,120)	*Crassostrea*
Pattern B (300,250,130,120)	*Saccostrea* and *Striostrea (Paratriostrea) mytiloides*
Pattern C (530,130,90)	*P. viridis*
2. COI-*Dde* I	
Pattern A (420,170,120)	*C. belcheri*
Pattern B (600,130)	*C. iredalei*
Pattern C (330,270,130)	*S. cucullata*
3. COI-*Mbo* I	
Patterns A (180,150,120,80,70,55) and B (290,150,80,70,55)	*C. belcheri*
Pattern C (290,160,140,60(40))	*C. iredalei*
Patterns D (420,78,75,60) and E (495,75,60)	*S. cucullata*
4. Does not show above patterns	*S. forskali* or *S. mytiloides*

S. forskali and *S. mytiloides* were analyzed. As a result, species-specific pattern was not found in these species. However, fixed patterns were observed in *S. commercialis* (COI-*Acs* I, *Dde* I and *Mbo* I) and *P. viridis* (16S rDNA-*Acs* I, *Alu* I, *Dde* I, *Dra* I, *Taq* I and *Rsa* I and COI-*Acs* I, *Dde* I and *Mbo* I).

For unidentified species, *Crassostrea* sp. showed species-specific patterns when analyzed with both 16S rDNA (*Alu* I and *Dde* I) and COI (*Dde* I and *Mbo* I). Likewise, *Saccostrea* sp. group 2 exhibited its specific patterns from digestion of 16S rDNA with *Acs* I and *Alu* I and COI digested with *Dde* I and *Mbo* I. On the other hand, *Saccostrea* sp. group 1 and 3 did not showed any species-specific pattern for all restriction enzymes used in this study. They, however, showed some patterns that found in *S. forskali* and/or *S. mytiloides* indicating that these oysters could be genetically regarded to *S. foskali* and *S. mytiloides*.

Fifty-four composite haplotypes were generated from combination of each single haplotypes (Table 3.4). Disregarding effects influenced by different sample sizes, the number of composite haplotypes found in *C. belcheri*, *C. iredalei*, *S. cucullata*, *S. forskali*, *S. mytiloides*, *S. commercialis* and *P. viridis* was 3, 3, 5, 22, 10, 2 and 1 composite haplotypes, respectively. The unidentified *Crassostrea* sp., *Saccostrea* sp. group 1, *Saccostrea* sp. group 2 and *Saccostrea* sp. group 3 exhibited 2, 6, 3 and 2 composite haplotypes, respectively.

Species-specific composite haplotypes with relatively high frequencies were observed in *C. belcheri* (I, AAAAAAAAAA; II, AAAAAAAAAB and III, AABAAAAAAA with the frequency of 0.882, 0.059 and 0.059, respectively), *C. iredalei* (IV, BBBBACAABC; V, BB BBAAAABC; VI, BBBBAAAABU with the frequency of 0.048, 0.904 and 0.048, respectively) and *S. cucullata* (VII, CDCCBBBBCD; VIII, CCCCBBBBCE; IX, CCCCBB BBCD; X, CDCCBBBBCE and XI, DDCCBBBBCE with the frequency of 0.565, 0.087, 0.261, 0.0435 and 0.0435, respectively) but were not found in *S. forskali* and *S. mytiloides*. The Australian oyster (*S. commercialis*) showed composite haplotypes LII (LFCCBBBELN) and LIII (LFCEBBBELN) with the frequency of 0.833 and 0.167, respectively. The mussel (*P. viridis*) showed a composite haplotype LIV (KLIFCDCFMT). The *Saccostrea* sp. group 3 showed composite haplotypes commonly found in *S. forskali* and *S. mytiloides*. *Crassostrea*

Table 3.4 Composite haplotypes found from restriction analysis of 16S and 18S rDNAs and COI of indigenous oysters, the Australian oyster (*S. commercialis*) and the mussel (*P. viridis*)

	Genotype	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi	Total(N)
I	AAAAAAAAAA	15	-	-	-	-	-	-	-	-	-	-	15
II	AAAAAAAAAB	1	-	-	-	-	-	-	-	-	-	-	1
III	AABAAAAAAA	1	-	-	-	-	-	-	-	-	-	-	1
IV	BBBBACAABC	-	1	-	-	-	-	-	-	-	-	-	1
V	BBBBAAAABC	-	19 Oys039 Oys104	-	-	-	-	-	-	-	-	-	21
VI	BBBBAAAABU	-	1	-	-	-	-	-	-	-	-	-	1
VII	CDCCBBBBCD	-	-	13	-	-	-	-	-	-	-	-	13
VIII	CCCCBBBBCE	-	-	2	-	-	-	-	-	-	-	-	2
IX	CCCCBBBBCD	-	-	6	-	-	-	-	-	-	-	-	6
X	CDCCBBBBCE	-	-	1	-	-	-	-	-	-	-	-	1
XI	DDCCBBBBCE	-	-	1	-	-	-	-	-	-	-	-	1

Table 3.4 (continue)

	Genotype	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi	Total(N)
XII	EEDCBABCDF	-	-	-	1	-	-	-	-	-	-	-	1
XIII	DEDCBABCDF	-	-	-	16 Oys030	4	-	-	-	4	-	-	25
XIV	DEDABABCDF	-	-	-	7	1	-	-	-	1	-	-	9
XV	GFGCBABCJF	-	-	-	1	-	-	-	-	-	-	-	1
XVI	GFGCBABAJF	-	-	-	1	-	-	-	-	-	-	-	1
XVII	FFFCBBBAJF	-	-	-	3	-	-	-	-	-	-	-	3
XVIII	DFFABBBAIR	-	-	-	1	-	-	-	-	-	-	-	1
XIX	FFFABBBAJF	-	-	-	1	-	-	-	-	-	-	-	1
XX	IFFCBBBCEH	-	-	-	1	-	-	-	-	-	-	-	1
XXI	DEECBABCDF	-	-	-	1	-	-	-	-	-	-	-	1
XXII	DEDABABDDF	-	-	-	1	-	-	-	-	-	-	-	1
XXIII	DFGABABAHP	-	-	-	1	-	-	1	-	-	-	-	2
XXIV	DFGABBBAIR	-	-	-	1	-	-	-	-	-	-	-	1

Table 3.4 (continue)

Genotype		Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi	Total(N)
XXV	DFDABABADF	-	-	-	1	-	-	-	-	-	-	-	1
XXVI	DFDABABCDF	-	-	-	1	-	-	-	-	-	-	-	1
XXVII	DFFCBBBAHR	-	-	-	1	-	-	-	-	-	-	-	1
XXVIII	DKFABBBAHR	-	-	-	1	-	-	-	-	-	-	-	1
XXIX	DKFCBABAHR	-	-	-	1	-	-	-	-	-	-	-	1
XXX	DEDCBABAHF	-	-	-	1	-	-	-	-	-	-	-	1
XXXI	DFFCBABCHF	-	-	-	1	-	-	-	-	-	-	-	1
XXXII	DFFCBABAHF	-	-	-	1	-	-	-	-	-	-	-	1
XXXIII	DKFCBBBAHF	-	-	-	1	-	-	-	-	-	-	-	1
XXXIV	DEDCBABCDI	-	-	-	-	1	-	-	-	-	-	-	1
XXXV	DFFDBBBAKJ	-	-	-	-	1	-	-	-	-	-	-	1
XXXVI	HHFDBBBAKJ	-	-	-	-	1	-	-	-	-	-	-	1
XXXVII	IHFDBBBAKJ	-	-	-	-	2	-	-	-	-	-	-	2

Table 3.4 (continue)

Genotype		Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi	Total(N)
XXXVIII	DGFDBBBAKJ	-	-	-	-	1	-	-	-	-	-	-	1
XXXIX	LHFDBBBAKJ	-	-	-	-	4	-	-	-	-	-	-	4
XL	DBDCBABCDF	-	-	-	-	1	-	-	-	-	-	-	1
XLI	DBDABABCDF	-	-	-	-	1	-	-	-	-	-	-	1
XLII	DIHABCAAFK	-	-	-	-	-	7	-	-	-	-	-	7
XLIII	CIHABCAAFL	-	-	-	-	-	2	-	-	-	-	-	2
XLIV	JJFCBBBBGM	-	-	-	-	-	-	-	7	-	-	-	7
XLV	JJFCBBBBGS	-	-	-	-	-	-	-	1	-	-	-	1
XLVI	JJFCBBBBGV	-	-	-	-	-	-	-	1	-	-	-	1
XLVII	DFGCBBBAHQ	-	-	-	-	-	-	1	-	-	-	-	1
XLVIII	DKGCBBBAHP	-	-	-	-	-	-	1	-	-	-	-	1
XLIX	DFFCBBBAIG	-	-	-	-	-	-	3	-	-	-	-	3
L	DKFCBBBAIG	-	-	-	-	-	-	1	-	-	-	-	1

Table 3.4 (continue)

Genotype		Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi	Total(N)
LI	DFFCBBBAIO	-	-	-	-	-	-	2	-	-	-	-	2
LII	LFCCBBBELN	-	-	-	-	-	-	-	-	-	5	-	5
LIII	LFCEBBBELN	-	-	-	-	-	-	-	-	-	1	-	1
LIV	KLIFCDCFMT	-	-	-	-	-	-	-	-	-	-	5	5
LV	BBBBBAAABC	-	Oys095	-	-	-	-	-	-	-	-	-	1
LVI	DFGABBBAHP	-	-	-	Oys121	-	-	-	-	-	-	-	1
LVII	DFGABBBAIO	-	-	-	Oys122	-	-	-	-	-	-	-	1

Composite haplotypes were constructed from each single haplotypes (restriction patterns) arranged from that of 16S rDNA-*Acs* I, *Alu* I, *Dde* I, *Dra* I, *Taq* I and *Rsa* I, 18S rDNA-*Hin*f I and COI-*Acs* I, *Dde* I and *Mbo* I, respectively.

Table 3.5 Frequencies of composite haplotypes across investigated oyster species and the outgroup reference, *P. viridis*

	Genotype	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
I	AAAAAAAAAA	0.882	-	-	-	-	-	-	-	-	-	-
II	AAAAAAAAAB	0.059	-	-	-	-	-	-	-	-	-	-
III	AABAAAAAAA	0.059	-	-	-	-	-	-	-	-	-	-
IV	BBBBACAABC	-	0.048	-	-	-	-	-	-	-	-	-
V	BBBBAAAABC	-	0.904 Oys039 Oys104	-	-	-	-	-	-	-	-	-
VI	BBBBAAAABU	-	0.048	-	-	-	-	-	-	-	-	-
VII	CDCCBBBBCD	-	-	0.565	-	-	-	-	-	-	-	-
VIII	CCCCBBBBCE	-	-	0.087	-	-	-	-	-	-	-	-
IX	CCCCBBBBCD	-	-	0.261	-	-	-	-	-	-	-	-
X	CDCCBBBBCE	-	-	0.0435	-	-	-	-	-	-	-	-
XI	DDCCBBBBCE	-	-	0.0435	-	-	-	-	-	-	-	-

Table 3.5 (continue)

	Genotype	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
XII	EEDCBABCDF	-	-	-	0.0222	-	-	-	-	-	-	-
XIII	DEDCBABCDF	-	-	-	0.3555 Oys030	0.2353	-	-	-	0.80	-	-
XIV	DEDABABCDF	-	-	-	0.1555	0.0588	-	-	-	0.20	-	-
XV	GFGCBABCJF	-	-	-	0.0222	-	-	-	-	-	-	-
XVI	GFGCBABAJF	-	-	-	0.0222	-	-	-	-	-	-	-
XVII	FFFCBBBAJF	-	-	-	0.0667	-	-	-	-	-	-	-
XVIII	DFFABBBAIR	-	-	-	0.0222	-	-	-	-	-	-	-
XIX	FFFABBBAJF	-	-	-	0.0222	-	-	-	-	-	-	-
XX	IFFCBBBCEH	-	-	-	0.0222	-	-	-	-	-	-	-
XXI	DEECBABCDF	-	-	-	0.0222	-	-	-	-	-	-	-
XXII	DEDABABDDF	-	-	-	0.0222	-	-	-	-	-	-	-
XXIII	DFGABABAHP	-	-	-	0.0222	-	-	0.1111	-	-	-	-
XXIV	DFGABBBAIR	-	-	-	0.0222	-	-	-	-	-	-	-

Table 3.5 (continue)

Genotype		Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
XXV	DFDABABADF	-	-	-	0.0222	-	-	-	-	-	-	-
XXVI	DFDABABCDF	-	-	-	0.0222	-	-	-	-	-	-	-
XXVII	DFFCBBBAHR	-	-	-	0.0222	-	-	-	-	-	-	-
XXVIII	DKFABBBAHR	-	-	-	0.0222	-	-	-	-	-	-	-
XXIX	DKFCBABAHR	-	-	-	0.0222	-	-	-	-	-	-	-
XXX	DEDCBABAHF	-	-	-	0.0222	-	-	-	-	-	-	-
XXXI	DFFCBABCHF	-	-	-	0.0222	-	-	-	-	-	-	-
XXXII	DFFCBABAHF	-	-	-	0.0222	-	-	-	-	-	-	-
XXXIII	DKFCBBBAHF	-	-	-	0.0222	-	-	-	-	-	-	-
XXXIV	DEDCBABCDI	-	-	-	-	0.0588	-	-	-	-	-	-
XXXV	DFFDBBBAKJ	-	-	-	-	0.0588	-	-	-	-	-	-
XXXVI	HHFDBBBAKJ	-	-	-	-	0.0588	-	-	-	-	-	-
XXXVII	IHFDBBBAKJ	-	-	-	-	0.1176	-	-	-	-	-	-

Table 3.5 (continue)

Genotype		Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
XXXVIII	DGFDBBBAKJ	-	-	-	-	0.0588	-	-	-	-	-	-
XXXIX	LHFDBBBAKJ	-	-	-	-	0.2353	-	-	-	-	-	-
XL	DBDCBABCDF	-	-	-	-	0.0588	-	-	-	-	-	-
XLI	DBDABABCDF	-	-	-	-	0.0588	-	-	-	-	-	-
XLII	DIHABCAAFK	-	-	-	-	-	0.7778	-	-	-	-	-
XLIII	CIHABCAAFL	-	-	-	-	-	0.2222	-	-	-	-	-
XLIV	JJFCBBBBGM	-	-	-	-	-	-	-	0.7778	-	-	-
XLV	JJFCBBBBGS	-	-	-	-	-	-	-	0.1111	-	-	-
XLVI	JJFCBBBBGV	-	-	-	-	-	-	-	0.1111	-	-	-
XLVII	DFGCBBBAHQ	-	-	-	-	-	-	0.1111	-	-	-	-
XLVIII	DKGCBBBAHP	-	-	-	-	-	-	0.1111	-	-	-	-
XLIX	DFFCBBBAIG	-	-	-	-	-	-	0.3333	-	-	-	-
L	DKFCBBBAIG	-	-	-	-	-	-	0.1111	-	-	-	-

Table 3.5 (continue)

	Genotype	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
LI	DFFCBBBAIO	-	-	-	-	-	-	0.2222	-	-	-	-
LII	LFCCBBBELN	-	-	-	-	-	-	-	-	-	0.833	-
LIII	LFCEBBBELN	-	-	-	-	-	-	-	-	-	0.167	-
LIV	KLIFCDCFMT	-	-	-	-	-	-	-	-	-	-	1.00
LV	BBBBBAAABC	-	Oys095	-	-	-	-	-	-	-	-	-
LVI	DFGABBBAHP	-	-	-	Oys121	-	-	-	-	-	-	-
LVII	DFGABBBAIO	-	-	-	Oys122	-	-	-	-	-	-	-

Composite haplotypes were constructed from each single haplotypes (restriction patterns) and arranged from that of 16S rDNA-*Acs* I, *Alu* I, *Dde* I, *Dra* I, *Taq* I and *Rsa* I, 18S rDNA-*Hin*f I and COI-*Acs* I, *Dde* I and *Mbo* I, respectively.

sp. and *Saccostrea* sp. group 2 showed unique species-diagnostic markers for both single and composite haplotypes.

Several oyster individuals in this study could not be identified based on morphological characters (e.g. shell morphology, pigment of adductor muscle scar etc.). The Oys030 shows morphological characters as *S. forskali*-like oyster. This specimens also showed haplotype XIII (DEDCBABCDF) which are commonly found in *S. forskali*. The Oys095 and Oys104 were morphological classified as a hybrid between *C. belcheri* and *C. iredalei* and *C. iredalei*-like oyster, respectively. Analysis of mtDNA indicated that they carried the BBBBBAAABC and BBBBAAAABC haplotype which are specifically found in *C. iredalei*. Likewise, the Oys039 previously classified as the *C. iredalei*-like oyster carried the most common haplotype (BBBBAAAABC) found in *C. iredalei*. The Oys121 and Oys122 could be classified to *Saccostrea* but the exact species name could not be concluded. These oysters did not show composite haplotypes found in *S. cucullata*, *S. commercialis*, *Saccostrea* sp. group 1, *Saccostrea* sp. group 2 and *Saccostrea* sp. group 3 but they possessed new haplotypes closely related to those for *S. forskali* phylogenetically (see discussion for taxonomic identification of these oyster based on morphological, RAPD and mtDNA analyses).

3.4 Genetic distances between composite haplotypes and their phylogenetic relationships

Genetic distances (number of nucleotide substitutions per site) between pairs of composite haplotypes within a particular species were lower than those between species (Appendix C). The distances found within *Crassostrea* oysters were also lower than those within *Saccostrea* and *Striostrea* oysters.

A neighbor-joining tree constructed from genetic distances between composite haplotypes indicated large genetic differentiation between *Crassostrea* and *Saccostrea* (Figure 3.13)(including *S. mytiloides*). Within the *Crassostrea* oysters, haplotypes found in *C. belcheri*, *C. iredalei* and *Crassostrea* sp. were well differentiated between different species. Large genetic distances among composite haplotypes of different *Crassostrea* oysters composed to that of *Saccostrea* oyster were observed. This reflected clearly taxonomic identifiable of the *Crassostrea* oysters. Oysters showing taxonomic difficulties like *S. forskali* and *S. mytiloides* are genetically related. Shared genotypes of these oysters were also observed along with those of *Saccostrea* sp. group 3. The unidentified *Saccostrea* sp. group 1 from Suratthani is allocated into branches representing *S. forskali*. Interestingly, *S. cucullata* and *Saccostrea* sp. group 2 are closely related phylogenetically.

3.5 Haplotype and nucleotide diversity within oyster species, nucleotide divergence between species and phylogenetic relationships at the species levels

High haplotype and nucleotide diversity were observed in *Saccostrea* and *Striostrea* oysters in Thailand. The highest diversity within species was found in *S. mytiloides* (haplotype and nucleotide diversity were 0.9044 and 3.2388%, respectively) followed by *S. forskali* (0.8545 and 2.8711%) and *S. cucullata* (0.6285 and 0.3501).

Lower genetic diversity was found in both *C. iredalei* (0.1857 and 0.0912%) and *C. belcheri* (0.2279 and 0.0945%). Only one composite haplotype was observed in *P. viridis* resulting in a lack of haplotype and nucleotide diversity within these species. Unidentified *Saccostrea* oysters (haplotype diversity = 0.4000 – 0.8889 and nucleotide diversity = 0.1447 – 1.0858) also exhibited greater diversity than do *C. belcheri*, *C. iredalei* and *Crassostrea* sp. (0.3889 and 0.3538%, respectively). The *Saccostrea* sp. group 3 oyster possessed shared genotype with that of *S. forskali* and *S. mytiloides*. As a result, the low genetic diversity in this group of oyster was probably due to its small sample size (N = 5). The average haplotype and

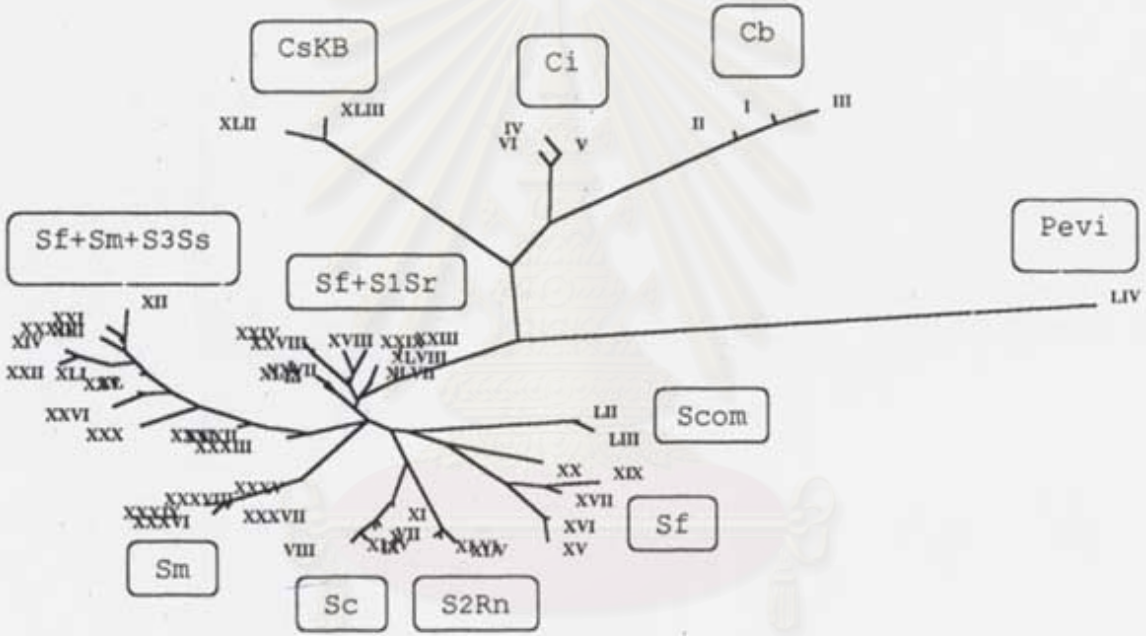

Figure 3.13 A neighbor–joining tree summarizing genetic relationships of composite haplotypes of oysters and the outgroup reference; *P. viridis*, constructed from average genetic distances between paired composite haplotypes

Table 3.6 Haplotype and nucleotide diversity within indigenous oyster species, the ingroup (*S. commercialis*) and the outgroup (*P. viridis*) references

Species	Haplotype diversity (h ± SE)	Nucleotide diversity (x100)
C. belcheri	0.2279 ± 0.12949	0.0945
C. iredalei	0.1857 ± 0.11022	0.0912
S. cucullata	0.6285 ± 0.08716	0.3501
S. forskali	0.8545 ± 0.04544	2.8711
S. mytiloides	0.9044 ± 0.04968	3.2388
Crassostrea sp.	0.3889 ± 0.16440	0.3538
Saccostrea sp. group 1	0.8889 ± 0.09100	1.0858
Saccostrea sp. group 2	0.4167 ± 0.19066	0.1447
Saccostrea sp. group 3	0.4000 ± 0.23732	0.1828
S. commercialis	0.3333 ± 0.21517	0.0862
P. viridis	0.0000 ± 0.00000	0.0000
Average	0.4753 ± 0.00846	0.7726 ± 0.00124

Table 3.7 Nucleotide diversity (above diagonal) and divergence (below) between species (including *S. commercialis* and *P. viridis*) resulted from restriction analysis of 16S rDNA, 18S rDNA and COI of specimens used in this study

	Cb	Ci	Sc	Sf	Sm	Csp	Ssp 1	Ssp 2	Ssp 3	Scom	Pevi
Cb	-	0.050973	0.114125	0.093959	0.087970	0.080604	0.096071	0.095941	0.092264	0.104880	0.130984
Ci	0.050044	-	0.090977	0.071090	0.070745	0.073767	0.078835	0.094179	0.066192	0.095216	0.108684
Sc	0.111902	0.088771	-	0.063875	0.060793	0.099533	0.029320	0.031169	0.070859	0.041160	0.119048
Sf	0.079131	0.056278	0.047769	-	0.037709	0.089358	0.046508	0.055451	0.019585	0.060174	0.121814
Sm	0.071304	0.054095	0.042849	0.007160	-	0.091350	0.045919	0.050259	0.031607	0.053766	0.118622
Csp	0.078362	0.071542	0.096013	0.073233	0.073387	-	0.079867	0.115208	0.086270	0.101341	0.125164
Ssp 1	0.090170	0.072951	0.022140	0.026724	0.024296	0.072669	-	0.038383	0.056745	0.037195	0.098699
Ssp 2	0.094745	0.092999	0.028694	0.040371	0.033341	0.112715	0.032230	-	0.059757	0.042904	0.110704
Ssp 3	0.090877	0.064822	0.068194	0.004316	0.014499	0.083587	0.050402	0.058119	-	0.067875	0.129820
Scom	0.103977	0.094329	0.038978	0.045388	0.037141	0.099141	0.031335	0.041750	0.066530	-	0.111616
Pevi	0.130511	0.108229	0.117298	0.107459	0.102428	0.123395	0.093270	0.109981	0.128906	0.111185	-

nucleotide diversity across overall investigated species were 0.4753 and 0.7726%, respectively.

Large nucleotide divergence was observed between *Crassostrea* oysters and small oysters (*Saccostrea* and *Striostrea*). The lowest divergence was 0.0043 (between *S. forskali* and S3Ss) whereas the highest divergence between those oysters was 0.1127 (between *Crassostrea* sp. and S2Rn).

A phylogenetic tree constructed from nucleotide divergence between species indicated comparable results as the haplotype tree. The isolated branch of *Crassostrea* sp. revealed that this oyster should be regarded as a new identified species in Thailand. The scientific name of this species should be further classified by taxonomists. Likewise, *Saccostrea* sp. group 2 from Ranong should also be regarded as a newly unidentified species. On the other hand, the species status of S3Ss and S1Sr are still questionable (but see discussion for conclusion of results from morphology, RAPD and mtDNA-RFLP analyses).

3.6 Cloning and sequencing of COI fragment

The COI DNA segment showed relatively high polymorphism (41 restriction patterns from 3 polymorphic enzymes) across overall investigated samples. Nevertheless, a 710 bp fragment were amplified along with other non-specific product. Accordingly, the amplification product of each oyster need to be fractionated electrophoretically, excised and recovered from the gel. Results from restriction analysis revealed the potential to use polymorphism of COI for phylogenetic studies in oysters. Therefore, more specific primers were need to be developed. The single COI product could have been directly digested with restriction enzymes eliminating an extra step for recovering of gel-purified COI. Additionally, haplotype-specific amplification of COI from different species may have been carried out. As a result, the amplified COI from *C. belcheri* showing AAAAAAAAAA haplotype, *C. iredalei* showing BBBBAAAABC haplotype and *S. cucullata* showing CDCCBBBBCD haplotype, were cloned using a TA cloning method.

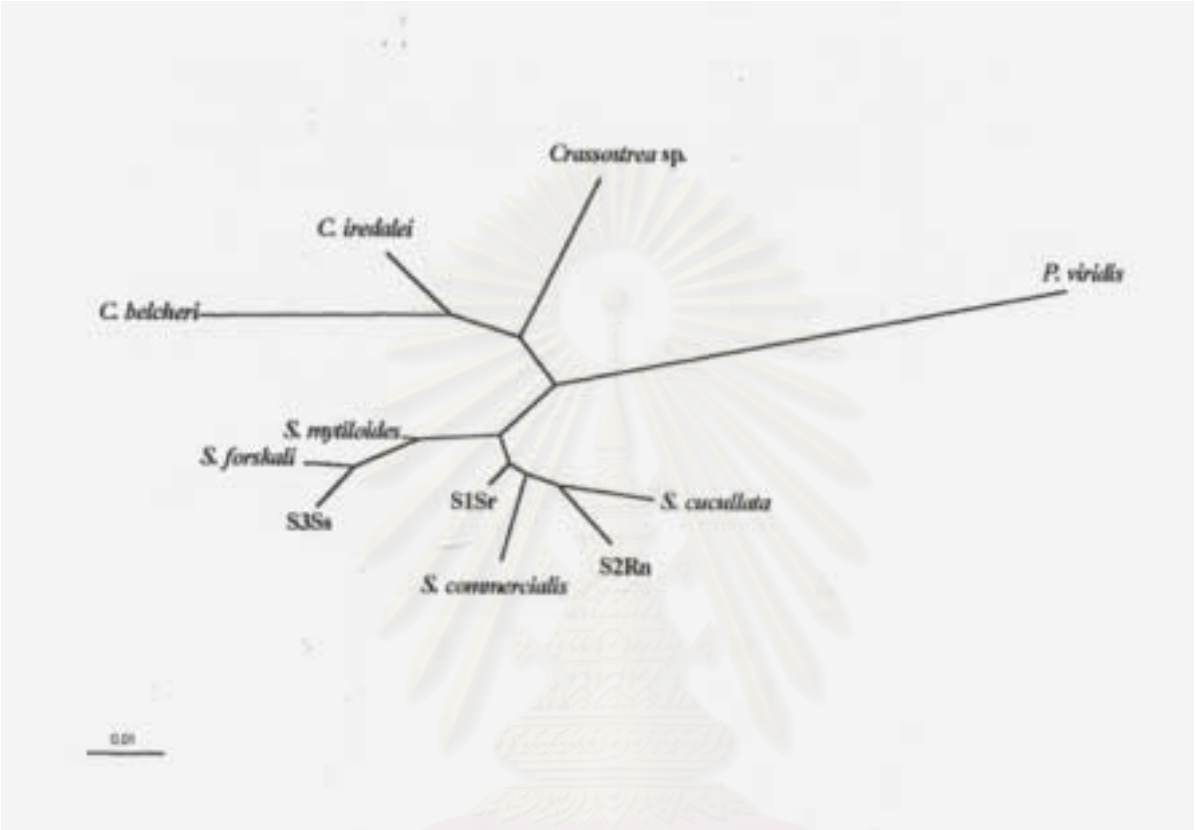

Figure 3.14 A neighbor–joining tree summarizing genetic relationship of oysters and the outgroup reference; *P. viridis*, constructed from the percentage nucleotide divergence between species

Three recombinant clones (pNKCb1, pNKCi1 and pNKSc4) were sequenced. The actual molecular length of pNKCb1, pNKCi1 and pNKSc4 was 681 bp, 687 bp and 704 bp, respectively (Figure 3.15). The nucleotide sequences of these recombinant plasmids were blasted to compare with those previously deposited in GenBank and indicated that these DNA fragment are actually COI (Appendix D) as they exhibited significant matching with COI sequences of oysters of genera *Crassostrea*, *Saccostrea* and *Ostrea* (P values between $4e^{-25}$ - e^{-106}). High genetic differences among COI of three oysters were observed. As can be seen from Figure 3.16, no conserved sequences longer than 20 bases were observed from the aligned COI sequences of *C. belcheri*, *C. iredalei* and *S.cucullata*. This limited the possibility to develop haplotype-specific PCR from these three species. The approach was then altered to develop more specific PCR primers generating a single fragment for this gene region. Two reverse primers (R301 and R353) were designed and used in coupling with the primer (HCO2198, TAAACTTCAGGGTGACCAAAAAATCA). Only a single expected fragment was obtained when amplified *C. belcheri* and *C. iredalei* DNA with each pair of primers (HCO2198+R301 and HCO2198+R353) indicating improved specificity of newly designed reverse primers (Figure 3.17). PCR was then carried out covering larger sample sizes. Only the HCO2198+R353 primer set yielded positive amplification results across all *C. belcheri* and *C. iredalei* individuals. Nevertheless, the intense amplification product was also observed in *S. cucullata* whereas *S. forskali* and *S. mytiloides* exhibited the fainted band at an identical fragment size (Figure 3.18 and 3.19). This eliminated the possibility to develop selectively amplified PCR for *C. iredalei*. Higher stringency PCR conditions were then carried out at the annealing temperature of 60^{O}C and 62^{O}C. The results showed specific amplification of the COI fragment in *C. belcheri* and *C. iredalei* at the latter annealing temperature (Figure 3.19). Digestion of the 353 bp product with *Mbo* I clearly differentiate *C. belcheri* and *C. iredalei* from one another.

A. pNKCb1

TAAACTTCAG GGGTGCCCAA AAATCAAAAT AAATGGTTGA AATAAAATAG 50
GGTCACTTCC TCCGACAGGA TCAAAAAAAG ATGTATTAAA ATGCGGGTCA 100
GTTAAAAGCA TCGTAAGGCC CCCAGCTAAA ACTGGAAGAG TGGTTAAAAG 150
CAGAAATGAA GTAACTTTAA TAGACCATGG GAATAGTGCT AACAGGTGGC 200
CCCCTACAGA TCGCATATTC AGTATGGTTA CTATGAAGTT AATTGATCTG 250
AAAATAGAAC TTAATACCCG CCAGATGTAG TCTCAAAATT GCAAGATCCA 300
TGCAAACCCC GTGATAGGAG TAAGTTGATA GAGAAGGGTA GATTGTTCAT 350
CCTGCCCCCA CCCCATTTTC TACAAGATTA GATATAAGTA TAAGATAAAG 400
CGATCCCGGT AATACTCAGA ACCTAAATGC ATTAAGTCGA GGAAATTGCA 450
TATGCGCAAC TTGAAGCATT AGAGGAATAA GTCAATTACC GAACCCGCCA 500
ATTATCACTG GCATAACAAA GAAAAAAATC ATAACTAATG CATGACTAGT 550
TACAACTGCG TTATAAGTAA CAGGATCTAA AAACTTAGCT CCTGGGTTAT 600
ACAACCTTCA ACGAATAAGA GATCTGAATC TTGTTCCAGC AAGAACAGCT 650
CAAAATCCAA ATACTATGTA AAACCTTCCA A 681

B. pNKCi1

TAAACTTCAG GGTGACCAAA AAATCAAAAT AAATGCTGAA ACAATACAGG 50
ATCGCCTCCG CCCACAGGAT CGAAAAAAGA CGTATTAAAA TGACGATCAG 100
TAAGAAGTAT AGTAAGGCCC CCTGCCAATA CTGGAAGAGT GGTTAAAAGT 150
AAAAATGATG TGACCTTAAT AGACCAGGGG AATAATGCTA GTAAATGCCC 200
GCCAACAGAC CGCATATTTC TAATAGNCAC TATAAAGTTA ATTGATCTAA 250
AAATAGAGCT AATACCGGCC AAATGCAGCC TTAAAATAGC AAGATCTATC 300
AAACGCCATG ATAAGAGTAA GGTGATAGCG GCNGGTAAAT AGTTCATTCA 350
GGCCCCAACC CCATTTTTCT ACAANATTAG ACATAAGTAT AAGATAAAGT 400
GAGCCTGGTA AAACCCAAAA TCTAAATGCA TTNAAGCGAG GAAACTGCAT 450
ATCTGCAACT TGGAAGTATT AATGGAATTA ATCANTTACC AAATCCCCCA 500
ATTATAACTG GGATAACNNT GAAGAAAATC ATAACTAATG CNTGGCTTGG 550
TACTACTGCN TTATATGGNA CANGGATCTA AGAACTTAAN CCCCGGGNTT 600
TACANCCTTT AACGGAATNA GANGAACTGA ATNTAGGNCC CCGNAAGGAC 650
ANNCCAAAAT CCANCCCCCC ANCTTGGAAC CTCCCAA 687

C. pNKSc4

```
TAAACTTCAG GGTGACCAAA AAATCAAAAT AGATGCTGAA ATAAAACCGG      50
ATCCCCACCA CCCACTGGAT CAAAAAATGA GGTATTAAAA TGTCGATCAG     100
TTAAAAGCAT AGTAAGTCCG CCGGCTAAAA CAGGTAAAGT TGTAAGCAAC     150
AAAAAAGAGG TTACTTTAAT AGATCATGGG AATAACCTTA ATAAATGACC     200
GTCTACAGAA CGTATATTTT TAATAGTCAC CATAAAGTTA ATTGATCTAA     250
AAATCGACCT AAGCCCAGCT AAATGCAGAC TTAAAATAGC CAAGTCCATA     300
CACATACCGT GATAAGAAAA GGTTGATAAC GGAGGGTAAA TAGTTCAACC     350
GGCCCCCACT CCATTTTCTA CAAACCCAGA AATTCCTATA AAATAAAGAG     400
ATGCAGGCAA AACTCAAAAC CTAAAAGCGT TAACTCGTGG GGAATTGCAT     450
ATCANGCACC TCAAGTATCA NCGGCACTAA TCAGTTTTCC AAATCCTNCA     500
ATTATCACAG GCATTACANA AAAGAAAATT ATGACCAAAG CGTGGTATTG     550
GGTACTACCG GATTATAACA CACANGGTCT AAAAACTTAA CTTNCTGGGA     600
TTAAAAAGGA CTTTCATCGG AATTTAACCG ACCTAAAATC TTTGNCCCCA     650
GCCCAAAACT GGATCAAAAA ACCCAAAAAA CTTNTGTTAA AAAACTTTTC     700
CAAT                                                      704
```

Figure 3.15 Nucleotide sequences of pNKCb1 (A), pNKCi1 (B) and pNKSc4 (C) specimens.

```
CB      TAAACTTCAGGGGTGCCCAAAAATCAAAATAAATGGTTGAAATAAAATAG
CI      TAAACTTCAGGGTGACCAAAAAATCAAAATAAATGCT-GAAACAATACAG
SC      TAAACTTCAGGGTGACCAAAAAATCAAAATAGATGCT-GAAATAAAACCG
        ************   ** ************* *** * **** ** *  *

CB      GGTCACTTCCTCCGACAGGATCAAAAAAAGATGTATTAAAATGCGGGTCAGTTAAAAGCA
CI      GATCGCCTCCGCCCACAGGATCGAAAAAAGACGTATTAAAATGACGATCAGTAAGAAGTA
SC      GATCCCCACCACCCACTGGATCAAAAAATGAGGTATTAAAATGTCGATCAGTTAAAAGCA
        * ** *  ** ** ** ***** ***** ** ***********  * ***** * *** *

CB      TCGTAAGGCCCCCAGCTAAAACTGGAAGAGTGGTTAAAAGCAGAAATGAAGTAACTTTAA
CI      TAGTAAGGCCCCCTGCCAATACTGGAAGAGTGGTTAAAAGTAAAAATGATGTGACCTTAA
SC      TAGTAAGTCCGCCGGCTAAAACAGGTAAAGTTGTAAGCAACAAAAAAGAGGTTACTTTAA
        * ***** ** ** ** ** ** ** * *** ** *  *  * *** ** ** ** ****

CB      TAGACCATGGGAATAGTGCTAACAGGTGGCCCCCTACAGATCGCATATTCAGTATGGTTA
CI      TAGACCAGGGGAATAATGCTAGTAAATGCCCGCCAACAGACCGCATATTTCTAATAGNCA
SC      TAGATCATGGGAATAACCTTAATAAATGACCGTCTACAGAACGTATATTTTTAATAGTCA
        **** ** *******    **  *  ** **  * ***** ** *****    ** *  *

CB      CTATGAAGTTAATTGATCTGAAAATAGAACTTAATACCCGCCAGATGTAGTCTCAAAATT
CI      CTATAAAGTTAATTGATCTAAAAATAGAGCT-AATACCGGCCAAATGCAGCCTTAAAATA
SC      CCATAAAGTTAATTGATCTAAAAATCGACCT-AAGCCCAGCTAAATGCAGACTTAAAATA
        * ** ************** ***** ** ** **  ** ** * *** ** ** *****
                  R301
CB      GCAAGATCCATGCAAACCCCGTGATAGGAGTAAGTTGATAGAGAAGGGTAGATTGTTCAT
CI      GCAAGATCTAT-C**AAACGCCATGATAAGAGTAAG**GTGATAGCGGCNGGTAAATAGTTCAT
SC      GCCAAGTCCATACACATACCGTGATAAGAAAAGGTTGATAACGGAGGGTAAATAGTTCAA
        ** *  ** ** ** *  ** ***** **  * * *****  *   **** ** *****
              R353
CB      CCTGCCCCCA-CCCCATTTT-CTACAAGATTAGATATAAGTATAAGATAAAGCGATCCCG
CI      TCAGG**CCCCAACCCCATTTTTCTACA**ANATTAGACATAAGTATAAGATAAAGTGAGCCTG
SC      CCGGCCCCCA-CTCCATTTT-CTACAAACCCAGAAATTCCTATAAAATAAAGAGATGCAG
         * * ***** * ******* ******    *** **   ***** ****** **  * *

CB      GTAATACTCAGAACCTAAATGCATTAAGTCGAGGA-AATTGCATATGCGCAACTTG-AAG
CI      GTAAAACCCAAAATCTAAATGCATTNAAGCGAGGA-AACTGCATATCTGCAACTTGGAAG
SC      GCAAAACTCAAAACCTAAAAGCGTTAACTCGTGGGGAATTGCATATCANGCACCTC-AAG
        * ** ** ** ** ***** ** ** * ** **  ** *******     ** *  ***

CB      CATTAGAGGAATAAGTCAATTA-CCGAACCCGCCAATTATCACTGGCATAACAAAGAAAA
CI      TATTAATGGAATTAATCANTTA-CCAAATCCCCCAATTATAACTGGGATAACNNTGAAGA
SC      TATCANCGGCACTAATCAGTTTTCCAAATCCTNCAATTATCACAGGCATTACANAAAAGA
         ** *  ** *  * *** **  ** ** **  ******* ** ** ** **    ** *

CB      AAATCATAACTAATGCATGAC--TAGTTACAACTGCGTTATAAGTAACAGG-ATCTAAAA
CI      AAATCATAACTAATGCNTGGC--TTGGTACTACTGCNTTATATGGNACANGGATCTAAGA
SC      AAATTATGACCAAAGCGTGGTATTGGGTACTACCGGATTATAACACACANG-GTCTAAAA
        **** ** ** ** ** **    * * *** ** * *****    *** *  ***** *

CB      ACTTAGCT-CCTGGGTTATACAA---CCTTCAACG-AATAAGAG--ATCTGAA-TCTTGT
CI      ACTTAANC-CCCGGGNTTTACAN---CCTTTAACGGAATNAGANG-AACTGAA-TNTAGG
SC      ACTTAACTTNCTGGGATTAAAAAGGACTTTCATCGGAATTTAACCGACCTAAAATCTTTG
        *****    * *** *  * *   * ** * ** ***   *  * ** ** * *

CB      TCCA----GCAAGAACAGCTCAAAATCC---AAATACTATG--TAAAACC---TTCCAAA
CI      NCCCC---GNAAGGACANNCCAAAATCC---ANCCCCCCANCTTGGAACC----NCCCAA
SC      NCCCCAGCCCAAAACTGGATCAAAAAACCCAAAAAACTTNTGTTAAAAAACT-TTTCCAA
         **       **        *****  *   *    *      *  **        * **
```

Figure 3.16 Alignment of *Crassostrea belcheri*, *C. iredalei* and *Saccostrea cucullata* COI sequences. Asterisks indicate identical bares among different sequences.

Table 3.8 Sequences of reverse oligonucleotide primers designed from a recombinant clone pNKCi1

Primer	Primer sequence
Forward primer	**HCO2198** : 5′ TAAACTTCAGGGTGACCAAAAAATCA 3′
Reverse primer (pNKCi1)	**R301** : 5′ CTTACTCTTATCATGGCGTTT 3′
	R353 : 5′ TGTAGAAAAATGGGGTTGGGG 3′

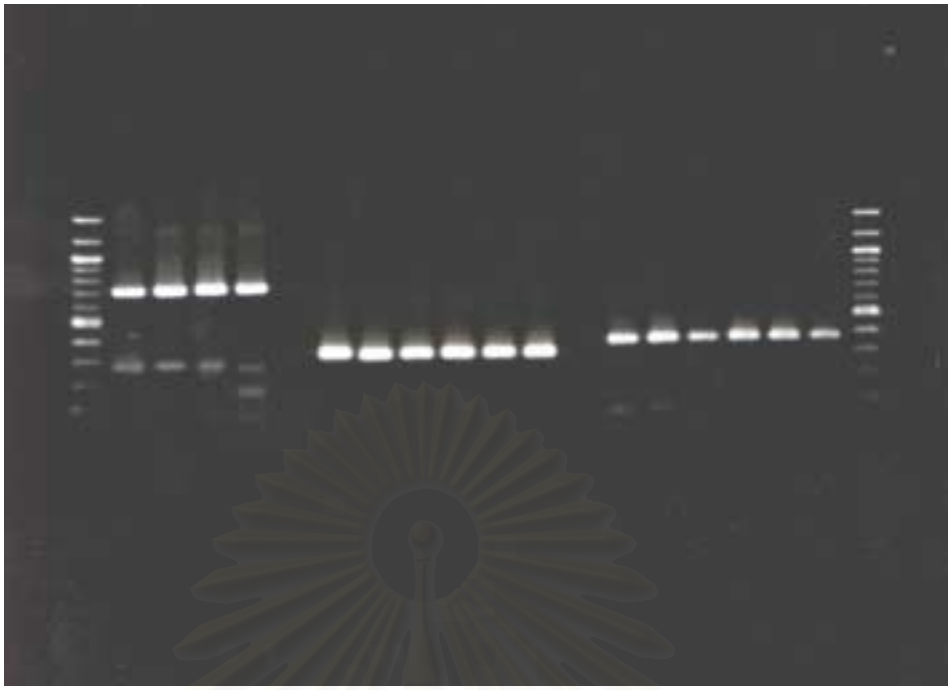

Figure 3.17 Results from amplification of *C. belcheri* and *C. iredalei* DNA using the original primer (LCO1490 and HCO2198) and newly designed primers (HCO2198+R301 and HCO2198+R353) using PCR cycles described in Chapter 2 (Table 2.3). PCR products were electrophoretically analyzed through a 1.0 % agarose gel.

Lane M = A 100 bp DNA ladder (Promega)

Lane 1 = negative control

Lanes 2 - 3 = amplification of *C. belcheri* DNA using LCO1490+HCO2198

Lanes 4 = amplification of *C. iredalei* DNA using LCO1490+HCO2198

Lanes 6 - 8 = amplification of *C. belcheri* DNA using HCO2198+R301

Lanes 9 - 11 = amplification of *C. iredalei* DNA using HCO2198+R301

Lanes 13 - 15 = amplification of *C. belcheri* DNA using HCO2198+R353

Lanes 16 - 18 = amplification of *C. iredalei* DNA using HCO2198+R353

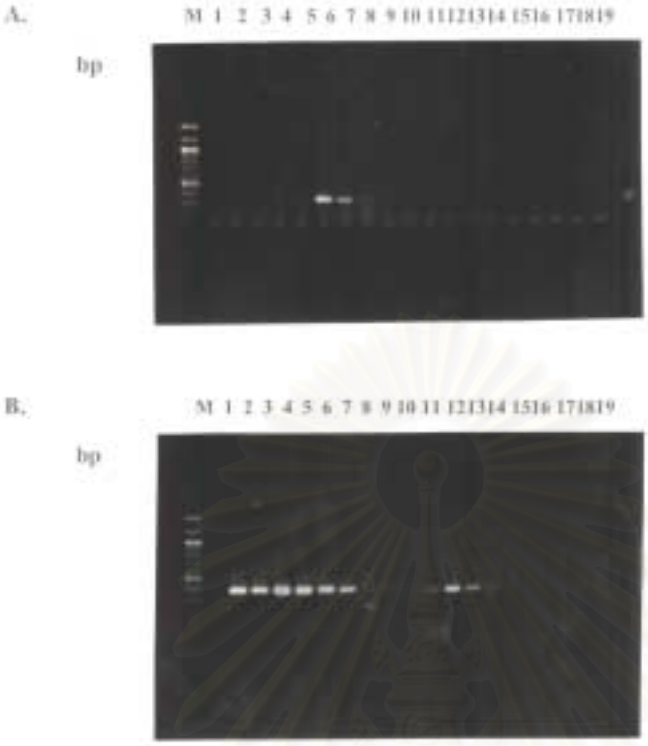

Figure 3.18 Results from amplification of oyster DNA using newly designed primers; HCO2198+R301 (A) and HCO2198+R353 (B), and annealing temperature at 55^{O}C (30 cycles without amplification at lower annealing temperature, 42^{O}C, for 10 cycles). PCR products were electrophoretically analyzed through a 1.0 % agarose gel.

Lane M = A 100 bp DNA ladder (Promega)
Lane 1 = negative control
Lanes 2 – 4 = *C. belcheri*
Lanes 5 – 7 = *C. iredalei*
Lanes 8 – 10 = *Crassostrea* sp.
Lanes 11 – 13 = *S. cucullata*
Lanes 14 – 16 = *S. forskali*
Lanes 17 – 19 = *S. mytiloides*

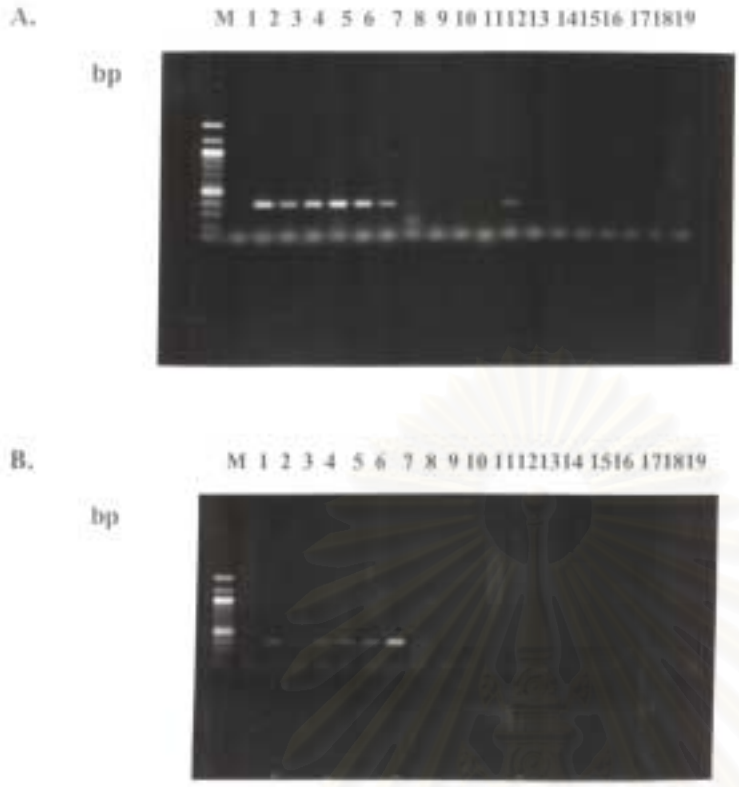

Figure 3.19 Results from amplification of oyster DNA using HCO2198+R353 for 30 cycles at the annealing temperature of 60^{O}C (A) and 62^{O}C (B). PCR products were electrophoretically analyzed through a 1.0 % agarose gel.

Lane M = A 100 bp DNA ladder (Promega)
Lane 1 = negative control
Lanes 2 – 4 = *C. belcheri*
Lanes 5 – 7 = *C. iredalei*
Lanes 8 – 10 = *Crassostrea* sp.
Lanes 11 – 13 = *S. cucullata*
Lanes 14 – 16 = *S. forskali*
Lanes 17 – 19 = *S. mytiloides*

Figure 3.20 Restriction patterns of PCR products amplified from *C. belcheri* and *C. iredalei* using HCO2198+R353 digested with *Mbo* I.

Lane M	=	A 100 bp DNA ladder (Promega)
Lane 1	=	An undigested PCR product (353 bp in length)
Lanes 2 – 7	=	*C. belcheri* (150, 80, 75 bp)
Lanes 8 – 13	=	*C. iredalei* (155, 80, 80 bp)

CHAPTER IV
DISCUSSIONS

Oysters are highly variable in forms and can display xenomorphism (ecomorphological variation) (Littlewood, 1994). The 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 to reach the conclusions on taxonomic identification.

Although *Crassostrea* oysters showed less morphological complexes than do the *Saccostrea* oysters. They still show difficulties for classical taxonomy. For example, the *C. iredalei* in this study had been previously recognized as *C. lugubris*. In the People Republic of China, the most commercially important species is *C. ariakensis* which is extremely difficult to differentiate from *C. iredalei* morphologically (S. Allen, personal communication).

All *Saccostrea* oysters in Thailand had been previously assumed to be only *S. cucullata* (Amornjaruchit, 1988). Subsequently, the existence of *S. commercialis* (Brohmanonda et al., 1988 and Tookwina, 1991) and *S. mordax* in Thailand had been reported (Yoosukh, 2000). More recently, *S. echinata* was newly recognized as *Striostrea (Parastriostrea) mytiloides*.

Visootiviseth et al. (1998) collected samples from Trat, Chonburi, Chumphon, Surratthani, Trang and Satun. Specimens were examined using morphometrics and allozyme electrophoresis (Pgi, Lap, Pgm, Mpi-2, Ap, Est-2, Aat-2, Mdh-2 and Idh-1). These oysters could be differentiated to three groupings; A, B and C, but the scientific name was recognized under *S. cucullata*.

Day et al. (2000) used allozymes and shell morphology to distinguish sympatric species of the rock oyster, *Saccostrea* in Thailand. They collected samples from 12 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, Chumphon; Ko Prab, Suratthani; Ko Patra, Satun and Ban Kantang, Trang). These oysters were identified as *S. commercialis*, *S. cucullata* and *S.*

manilai compared to type specimens. The results create more confused information for the availability of *S. manilai* in Thailand.

Interspecific hybridization between oysters of the genus *Crassostrea* were reported over a century ago (Bouchon-Brandely cited in Gaffney and Allen, 1993). Hybridization between *C. gigas* and *C. rivularis* resulting in viable and fertile progeny (Allen and Gaffney, 1993). Recently, Charoensit (1995) examined interspecific and intergeneric hybridization between *C. belcheri*, *C. iredalei* 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 surprisingly successful. This information indicated further complication on taxonomic identification of oysters through hybridization. Species-diagnostic markers of oysters are thus useful for examination of the correct seed and broodstock species and to estimate degree of hybridization in natural populations of oysters in Thai waters.

Several molecular genetic studies addressing population genetics (Hedgecock and Okazaki, 1984; Karl and Avise, 1992 and 1993; Foltz and Hu, 1996; Small and Chapman, 1997), intraspecific phylogeography (Reeb and Avise, 1990; Hare and Avise, 1998), interspecific phylogenetic relationships and genetic differentiation (Banks et al., 1993; Anderson and Adlard, 1994; Littlewood, 1994; Boudry et al., 1998) have been reported in oysters. Nevertheless, there have been no publications on genetic diversity and species-diagnostic markers of commercial oysters in Thailand based on RFLP analysis. This information is necessary to elevate culture and management efficiency of these taxa.

MtDNA-RFLP analysis has been successfully used for stock structure analysis in commercially important species including molluscs (Reeb and Avise, 1990; Small and Chapman, 1997; Boudry et al., 1998). In this study, restriction analysis of nuclear 18S rDNA and mitochondrial 16S rDNA and COI of indigenous oysters in Thailand with various restriction endonucleases were carried out and indicated high levels of genetic polymorphism in these species.

Molecular taxonomic keys of three commercially important species (*C. belcheri*, *C. iredalei* and *S. cucullata*) could be constructed based on both single digestion patterns and composite haplotypes of PCR-amplified products. Results from RAPD analysis using the same sample set also indicated the failure to identify species-specific markers in *S. mytiloides* and *S. forskali* (Klinbunga et al., 2000 and 2001).

Digestion of the 16S rDNA with *Acs* I revealed mixed patterns (e.g. D+F, D+I, L+I and D+L) in a single digestion. This circumstance was only observed in *S. forskali* and *S. mytiloides*. Therefore, it is regarded as the consequence of interspecific hybridization rather than contribution of paternal mtDNA. It is difficult to prove this circumstance at present because breeding and culturing a large number of *S. forskali* and *S. mytiloides* families in laboratories are not possible.

Oysters (particularly members of the genus *Saccostrea*) are variable in form. 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. Species-specific markers are thus required to unambiguously identify the correct species of commercial oysters in Thailand at various stages (larvae, seed and broodstock) of life cycle.

Based primarily on morphology, four groups of oysters could only be classified at the genus levels. These included *Crassostrea* sp. (from Krabi), *Saccostrea* sp. group 1 (from Suratthani), *Saccostrea* sp. group 2 (from Ranong) and *Saccostrea* sp. group 3 (from Samut Sakhon). Moreover, several oyster individuals could not be unambiguously identified morphologically (Appendix A). These specimens were genetically examined using RFLP and RAPD (Amparyup, 1999) analyses. Species-specific RFLP patterns and RAPD fragments could identify the species status in most of these sample. In addition, results from PCR-RFLP can indicate the maternal species when interspecific hybridization is existent.

Classical taxonomy indicated that *Crassostrea* sp. should have been the juvenile stages of either *C. belcheri* or *C. iredalei*. Analysis of 18S rDNA of nine individuals of *Crassostrea* sp. confirmed that they are not *Saccostrea* oysters. These oysters possessed haplotype XLII

(DIHABCAAFK) and XLIII (CIHABCAAFK) which were not available in any other oysters species in Thailand. Species-specific RAPD markers for *C. belcheri* and *C. iredalei* were not found in these specimens. As a result, the *Crassostrea* sp. should be regarded as a new *Crassostrea* species in Thailand.

Saccostrea sp. group 1 showed composite haplotypes XLVII (DFGCBBBAHQ), XLVIII (DKGCBBBAHP), XLIX (DFFCBBBAIG), L (DKFCBBBAIG) and LI (DFFCBBB AIO) which were not found in any oyster. Nevertheless, it is not regarded as a newly unidentified species because frequencies of these haplotypes were very low. This oyster group was indicated as *S. forskali*-like oyster based on morphological characters. Five composite haplotypes were observed from only 8 investigated individuals suggesting a high genetic diversity of these oysters. A phylogenetic tree based on the percent sequence divergence between pairs of mtDNA composite haplotypes revealed close relationships between these haplotypes with those of *S. forskali*. Therefore, this group of oysters should be regarded as *S. forskali*.

On the basis of morphology, the species status of *Saccostrea* sp. group 2 could not be unambiguously concluded. Three composite haplotypes including XLIV (JJFCBBBBGM, n = 7), XLV (JJFCBBBBGS, n = 1) and XLVI (JJFCBBBBGV, n = 1) were observed from RFLP analysis of 9 individuals. This indicated low polymorphism within this oyster group. The 750 (OPB08) and 1800 bp (UBC220) RAPD fragments were not observed in these specimens (Amparyup, 1999) indicating that they are not *S. cucullata*. In contrast, several fixed RAPD fragments were observed in all individuals of this group. Based on both RFLP and RAPD analyses, *Saccostrea* sp. group 2 should be a new unidentified species in Thailand.

The *Saccostrea* sp. group 3 exhibited both *S. forskali* and *S. mytiloides* characters. Lack of species-specific RFLP patterns (and RAPD markers) prohibited an unambiguous conclusion for the species origin of these oysters. Moreover, a phylogenetic tree based on nucleotide divergence between species placed this group of oysters to be a sister taxon of *S. forskali* whereas that from RAPD analysis allocated *Saccostrea* sp. group 3 to be closely related with *S. mytiloides*. Therefore, the species status of this unidentified oyster still cannot be concluded.

Several oyster individuals constituting of Oys030, Oys039, Oys095, Oys104, Oys121 and Oys122 could not be clearly classified. Using RFLP analysis, the species status of these specimens could be clarified. Oys030, Oys039 and Oys104 were identified as *C. iredalei*-like oysters. The composite haplotype of Oys030 was DEDCBABCDF (XIII) which was commonly found in *S. forskali*. This specimen was then regarded as the introgressive hybrid between *C. iredalei* and *S. forskali* where *S. forskali* served as the maternal species. The Oys039 and Oys104 exhibited the composite haplotype V (BBBBAAAABC) which was restricted to *C. iredalei*. RAPD analysis revealed all *C. irdalei*-specific markers reported by Amparyup (1999). As a result, these specimens are *C. iredalei* showing ecomorphological variation.

The external characteristics of Oys095 showed that it should be a hybrid between *C. belcheri* and *C. iredalei*. Nevertheless, this oyster showed the composite haplotype BBBBB AAABC which has only one mutation step from the most common haplotype V (BBBBA AAABC) in *C. iredalei*. Analysis of Oys095 using RAPD approach revealed all *C. iredalei*-specific RAPD markers whereas those specifically found in *C. belcheri* were not observed suggesting that Oys095 is not a hybrid but it is a pure *C. iredalei* oyster.

The Oys121 and Oys122 could be classified to *Saccostrea* but the exact species name could not be concluded. Species-specific patterns (RFLP) or fragments (RAPD) of *C. belcheri*, *C. iredalei* and *S. cucullata* were not observed in Oys121 and Oys122. These oysters did not show composite haplotypes found in any species of *Saccostrea* but they possessed new composite haplotypes (LVI; DFGABBBAHP and LVII; DFGABBBAIO, respectively) closely related to those of *S. forskali* phylogentically.

Patterns of composite haplotypes distribution in economically important species (*C. belcheri*, *C. iredalei* and *S. cucullata*) were different from those of *S. forskali* and *S. mytiloides*. A common composite haplotype with a high frequency was observed in each economically important species whereas the deficiency of fixed species-specific haplotypes and the presence of many unique haplotypes (observed with low frequencies in a particular species) were observed in *S. forskali* and *S. mytiloides*.

Restriction analysis of the entire mtDNA of the American oyster (*C. virginica*) was carried out in continuously distributed populations from the Gulf of St. Lowrence (Canada) to Brownsville (Texas, USA). A total of 82 composite haplotypes was found in 212 individuals using 13 restriction endonucleases. Two major composite haplotypes; CCCCCCCCCCCCC and XXCXXXXXXCCCC were found. The former was most common in all Atlantic coast locales, with the exception of southeast Florida. The latter was the most common haplotype in all Gulf coast locales. The distribution patterns of composite haplotype in *C. virginica* is similar to those of *S. mytiloides* and *S. forskali* in Thailand.

The haplotype and nucleotide diversity within *Saccostrea* and *Striostrea* oysters was greater than that within *Crassostrea* oysters indicating high genetic differences within *Saccostrea* and *Striostrea* oysters. The highest haplotype and nucleotide diversity within *Saccostrea* species was observed in *S. mytiloides* (0.9044 and 3.2388%, respectively) followed by *S. forskali* (0.8545 and 2.8711%) and *S. cucullata* (0.6285 and 0.3501%). The haplotype and nucleotide diversity for *C. belcheri* and *C. iredalei* were much lower. The results were according to those base on morphological studies (Brock, 1990), allozyme electrophoresis (Buroker et al., 1979; Hedgecock, 1955; Visootiviseth et al., 1998) and RAPD analysis (Klinbunga, 2001). High genetic diversity within each oyster may have reflected correlation between genetic adaptation to climatic or environmental variants (Buroker, 1985).

The haplotype diversity of *Saccostrea* oysters of the present study (using restriction analysis of 16S rDNA and COI gene regions) was as high as that previously reported in other molluscs (using restriction analysis of the entire mtDNA); for example, 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).

Small and Chapman (1997) examined genetic variation of *C. virginica* using RFLP analysis of the 16S rDNA with 10 restriction endonucleases (*Rsa* I, *Msp* I, *Hha* I, *Taq* I, *Mse* I, *Hae* III, *Mbo* I, *Hin*f I, *Hin*d III and *Hinc* II). The average haplotype and nucleotide diversity within species was 0.1079 and 0.1309% which is comparable to those of *C. belcheri* (0.2279 and 0.0945) and *C. iredalei* (0.1857 and 0.0912) in this study.

The percentage nucleotide divergence between *Crassostrea* oysters was quite low while greater divergence was observed between paired of small oyster species (*Saccostrea* and *Striostrea*). Unidentified *Saccostrea* sp. group 1, 2 and 3 showed relatively high nucleotide divergence with *C. belcheri*, *C. iredalei* and *Crassostrea* sp. but lower nucleotide divergence was found when compared with *S. cucullata*, *S. forskali*, *S. mytiloides* and *S. commercialis*.

Neighbor-joining trees constructed from genetic distances between pairs of composite haplotypes (haplotype tree) and nucleotide divergence between oyster species (species tree) indicated clear differentiation between *Crassostrea* and *Saccostrea* oysters. Nevertheless, *S. mytiloides*, a representative of *Striostrea* oyster showed close relationship with *Saccostrea* oysters.

The NJ haplotype tree revealed large genetic differences between each of the *Crassostrea* oyster reflecting less complexity of these oysters based on morphological characters. Clear phylogenetic differences between *C. belcheri*, *C. iredalei* and *Crassostrea* sp. were observed. The *Crassostrea* sp. oyster showed the absence of chromata indicating that they are belonging to the genus *Crassostrea*. The absence of species-specific patterns for *C. belcheri* and *C. iredalei* in all investigated individuals of *Crassostrea* sp. indicated that this group of oysters might be a new unidentified *Crassostrea* species in Thailand.

Conversely, more complex status were observed in the *Saccostrea* and *Striostrea* oysters. While *S. cucullata* and *Saccostrea* sp. group 2 (Ranong) showed monophyletic status, *S. forskali* and *S. mytiloides* did not show that status and could be differentiated into several subgroups. The complexity of genetic relationships within these two species reflect taxonomic difficulties of these oysters. The NJ tree constructed from the percentage of nucleotide divergence between pairs of species provided similar results with that of the haplotype tree.

Formerly, *Striostrea (Parastriostrea)* was regarded as *Saccostrea echinata* (Yoosukh and Duangdee, 1999). On the basis of RFLP analysis, shared composite haplotypes were not observed in other identified oyster species except between *S. forskali* and *S. mytiloides* (XIII; DEDCBABCDF and XIV; DEDABABCDF). The placement of *S. mytiloides* samples as a sister taxon with *S. forskali* suggested that these species are closely related phylogenetically.

Our results based on RAPD analysis of the same sample set support this conclusion (Amparyup, 1999). Accordingly, *S. mytiloides* should be recognized as a member of *Saccostrea* rather than that of *Striostrea*.

It has been reported that *Saccostrea* oysters display ecomorphological variation (Tack et al., 1992). All *Saccostrea* oysters in Thailand were previously assumed to be only *S. cucullata* (Visootiviseth et al., 1998). Results from RFLP analysis indicated that more than one *Saccostrea* species is present in Thai waters implying the previous problems on the use of a part of the species complexes for aquaculture.

The *Saccostrea* oyster originating from Chonburi (Angsila) had been misidentified as *S. commercialis* (Department of Fisheries, 1993). Based on RFLP results, it may not be *S. commercialis* due to lack of species-diagnostic patterns for RFLP analysis typically found in that species and phylogenetic relationships of oysters in this study. Nevertheless, replicate sampling of the extensive non-Thai *S. commercialis* over its geographic range are required to draw an unambiguous conclusion.

Day et al. (2000) assigned *Saccostrea* oysters from Thailand to 3 species; *S. commercialis*, *S. cucullata* and *S. manilai* using allozymes and shell morphology. On the basis of PCR-RFLP of this study, two unique composite haplotypes (LII; LFCCBBBELN and LIII; LFCEBBBELN) were found in the Australian *S. commercialis* but these composite haplotypes were not found in any Thai oyster indicating that *S. commercialis* was not existent in Thailand.

The amplified COI products revealed non-specific bands. More specific primers for amplification of this gene region are required. Additionally, selective amplification of COI gene from different oyster species may be developed if nucleotide sequences is known. Accordingly, the 710 bp COI product amplified from an individual of *C. belcheri* representing haplotype I (AAAAAAAAAA), that of *C. iredalei* representing haplotype V (BBBBAAAA BC) and that of *S. cucullata* representing haplotype VII (CDCCBBBBCD) were cloned and sequenced.

Comparisons of these sequences with those previously deposited in GenBank revealed significantly matching with COI in other organisms including oysters of genera *Crassostrea*,

Saccostrea and *Ostrea*. This further confirmed that the eluted fragment used for genetic analysis were actually COI gene segment.

The COI sequences obtained were aligned. Nevertheless, high genetic differences among COI of three oysters were observed. Conserved regions which are longer than 20 bases were not found. This prohibited the ability to developed selective amplification of COI for simplification of species diagnosis of *C. belcheri*, *C. iredalei* and *S. cucullata*. Two reverse primers (R301 and R353) were designed and further analyzed against all individuals of *C. belcheri* and *C. iredalei*. The primers HCO2198+R353 PCR successfully amplified from all investigated individuals of both species. The patterns of *Mbo* I-digested 353 bp PCR product of *C. belcheri* and *C. iredalei* were clearly different. The specific and simple diagnostic markers were successfully developed in *Crassostrea* oysters.

The basic information on numbers of species and/or populations of exploited species in a particular area is importance for broodstock selection and breeding programmes (Carvalho and Hauser, 1994). Moreover, knowledge on genetic diversity of oyster is essential for the construction of an appropriate management scheme in these taxa.

The ability to identified broodstock and seed species is crucial for both management and conservation programs in oysters. It is also an essential prerequisite to population studies in the species having taxonomic difficulties like oysters. Basically, individual stocks often differ considerably in their biological characteristics, including recruitment and mortality. They therefore respond independently to exploitation thereby requiring independent management. As a result, population genetic structure of each oyster species in Thailand, which is not available at present, should be investigated extensively.

Identification of species followed by evaluation of heritability for the growth rate of each commercially important species are necessary for broodstock selection and breeding programmes in these taxa. Results of this study illustrated useful genetic markers in *C. belcheri*, *C. iredalei* and *S. cucullata*. Species-diagnostic RFLP patterns can be utilized for identification of seed species and for comparison of growth performance of three commercially important oyster species in the commercial setting conditions.

PCR-RFLP analysis is a promising approach to be utilized for population genetics and systematic studies in various taxa. This simply genetic approach has been successfully used to identify the existence of at least three populations of the honey bee, *Apis cerana* in Thailand (Sihanatavong et al., 1999), to investigate genetic differentiation between populations of the Portuguese oyster (*C. angulata*) and the Pacific oyster (*C. gigas*)(Boudry et al., 1998) and to determine population genetics and phylogeny of the black tiger prawn, *P. monodon* in Thailand (Siludjai, 2000).

This study illustrates the use of this approach to evaluate levels of genetic variation of Thai oysters and to identify species-specific markers to assist taxonomic identification of oysters from genera *Crassostrea*, *Saccostrea* and *Striostrea*. The genetic markers found in this study 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.

CHAPTER V
CONCLUSIONS

1. High genetic diversity of five oysters in Thailand; *C. belcheri*, *C. iredalei*, *S. cucullata*, *S. forskali* and *S. mytiloides* was observed based on restriction analysis of the 16S rDNA, 18S rDNA and COI genes.
2. A total of 54 composite haplotypes was found across investigated oyster species. Species-specific markers were found in *C. belcheri*, *C. iredalei* and *S. cucullata* but not in *S. forskali* and *S. mytiloides*.
3. A neighbor-joining tree indicated distant relationships between *Crassostrea* and *Saccostrea* (including *S. mytiloides*) oysters but closer relationships were observed within each genus.
4. The COI fragment amplified from a representative of *C. belcheri*, *C. iredalei* and *S. cucullata* were cloned and sequenced. Simple species diagnostic of these oysters was successfully developed based on digestion of the HCO2198+R353 product with *Mbo* I.

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APPENDICES

Appendix A

Sampling sites, date of collection and species of oysters (preliminary investigated based on morphology characters)

Code of samples	Location	Date of collection	Species
*Cb 001	Suratthani	16/03/1999	*C. belcheri*
*Cb 002	Suratthani	16/03/1999	*C. belcheri*
Cb 003	Suratthani	16/03/1999	*C. belcheri*
Cb 004	Suratthani	16/03/1999	*C. belcheri*
Cb 005	Suratthani	16/03/1999	*C. belcheri*
Cb 006	Suratthani	16/03/1999	*C. belcheri*
Cb 007	Suratthani	16/03/1999	*C. belcheri*
Cb 008	Suratthani	16/03/1999	*C. belcheri*
Cb 009	Suratthani	16/03/1999	*C. belcheri*
Cb 010	Suratthani	16/03/1999	*C. belcheri*
Cb 011	Ban Na Thab, Songkhla	04/06/1998	*C. belcheri*
*Cb 012	Ban Na Thab, Songkhla	04/06/1998	*C. belcheri*
Cb 013	Ban Na Thab, Songkhla	04/06/1998	*C. belcheri*
Cb 014	Ban Na Thab, Songkhla	04/06/1998	*C. belcheri*
Cb 015	Ao Khao Yau, Ranong	01/06/1998	*C. belcheri*
Cb 016	Ao Khao Yau, Ranong	01/06/1998	*C. belcheri*
Cb 017	Ao Khao Yau, Ranong	01/06/1998	*C. belcheri*
Cb 018	Bo Tho restaurant, Krabi	07/04/1998	*C. belcheri*
Cb 019	Bo Tho restaurant, Krabi	07/04/1998	*C. belcheri*
Cb 020	Bo Tho restaurant, Krabi	07/04/1998	*C. belcheri*
Ci 021	Sriracha, Chonburi	06/10/1998	*C. iredalei*
Ci 022	Sriracha, Chonburi	06/10/1998	*C. iredalei*
Ci 023	Sriracha, Chonburi	06/10/1998	*C. iredalei*

Appendix A (continued)

Code of samples	Location	Date of collection	Species
Ci 024	Sriracha, Chonburi	06/10/1998	*C. iredalei*
Ci 025	Sriracha, Chonburi	06/10/1998	*C. iredalei*
Ci 026	Sriracha, Chonburi	06/10/1998	*C. iredalei*
Ci 027	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	*C. iredalei*
Ci 028	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	*C. iredalei*
Ci 029	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	*C. iredalei*
Oys 030	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	*S. forskali*
Ci 031	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	*C. iredalei*
Ci 032	Ban Na Thab, Songkhla	04/06/1998	*C. iredalei*
Ci 033	Ban Na Thab, Songkhla	04/06/1998	*C. iredalei*
Ci 034	Ban Na Thab, Songkhla	04/06/1998	*C. iredalei*
Ci 035	Ban Na Thab, Songkhla	04/06/1998	*C. iredalei*
Ci 036	Ban Na Thab, Songkhla	04/06/1998	*C. iredalei*
Ci 037	Ban Na Thab, Songkhla	04/06/1998	*C. iredalei*
Ci 038	Ao Khao Yau, Ranong	01/06/1998	*C. iredalei*
Oys 039	Ao Khao Yau, Ranong	01/06/1998	*C. iredalei*
Ci 040	Kog Krai, Phangnga	07/04/1998	*C. iredalei*
Ci 041	Kog Krai, Phangnga	07/04/1998	*C. iredalei*
Ci 042	Kog Krai, Phangnga	07/04/1998	*C. iredalei*
Ci 043	Kog Krai, Phangnga	07/04/1998	*C. iredalei*
Sc 044	Kho Phi, Trad	01/08/1998	*S. cucullata*
Sc 045	Kho Phi, Trad	01/08/1998	*S. cucullata*
Sc 046	Kho Phi, Trad	01/08/1998	*S. cucullata*
Sc 047	Kho Phi, Trad	01/08/1998	*S. cucullata*
Sc 048	Kho Phi, Trad	01/08/1998	*S. cucullata*
*Sc 049	Koh Nui, Ranong	09/05/1998	*S. cucullata*

Appendix A (continued)

Code of samples	Location	Date of collection	Species
Sc 050	Koh Nui, Ranong	09/05/1998	*S. cucullata*
Sc 051	Koh Nui, Ranong	09/05/1998	*S. cucullata*
Sc 052	Koh Nui, Ranong	09/05/1998	*S. cucullata*
Sc 053	Koh Nui, Ranong	09/05/1998	*S. cucullata*
Sc 054	Koh Nui, Ranong	09/05/1998	*S. cucullata*
Sc 055	Koh Nui, Ranong	09/05/1998	*S. cucullata*
Sc 056	Koh Al, Phuket	08/04/1998	*S. cucullata*
Sc 057	Koh Al, Phuket	08/04/1998	*S. cucullata*
Sc 058	Koh Al, Phuket	08/04/1998	*S. cucullata*
Sc 059	Koh Al, Phuket	08/04/1998	*S. cucullata*
Sc 060	Koh Al, Phuket	08/04/1998	*S. cucullata*
Sc 061	Koh Al, Phuket	08/04/1998	*S. cucullata*
Sc 062	Chantraburi	21/05/1998	*S. cucullata*
*Sc 063	Chantraburi	21/05/1998	*S. cucullata*
*Sf 064	Chantraburi	21/05/1998	*S.forskali*
*Sc 065	Chantraburi	21/05/1998	*S. cucullata*
Sc 066	Chantraburi	21/05/1998	*S. cucullata*
*Sc 067	Chantraburi	21/05/1998	*S. cucullata*
Sf 068	Angsila, Chonburi	17/02/1998	*S. forskali*
Sf 069	Angsila, Chonburi	17/02/1998	*S. forskali*
Sf 070	Angsila, Chonburi	17/02/1998	*S. forskali*
Sf 071	Angsila, Chonburi	17/02/1998	*S. forskali*
Sc 072	Chantraburi	21/05/1998	*S. cucullata*
Sc 073	Chantraburi	21/05/1998	*S. cucullata*
Sc 074	Chantraburi	21/05/1998	*S. cucullata*
Sf 075	Chantraburi	21/05/1998	*S. forskali*

Appendix A (continued)

Code of samples	Location	Date of collection	Species
Sf 076	Chantraburi	21/05/1998	*S. forskali*
Sf 077	Chantraburi	21/05/1998	*S. forskali*
*Sf 078	Chantraburi	21/05/1998	*S. forskali*
Sf 079	Chantraburi	21/05/1998	*S. forskali*
Sf 080	Ban Pak Bara, Satun	04/08/1998	*S. forskali*
Sf 081	Ban Pak Bara, Satun	04/08/1998	*S. forskali*
Sf 082	Ban Pak Bara, Satun	04/08/1998	*S. forskali*
*Sf 083	Ban Pak Bara, Satun	04/08/1998	*S. forskali*
*Sf 084	Ban Pak Bara, Satun	04/08/1998	*S. forskali*
Sf 085	Ban Pak Bara, Satun	04/08/1998	*S. forskali*
Sf 086	Ao Khao Yau, Ranong	01/06/1998	*S. forskali*
Sf 087	Ao Khao Yau, Ranong	01/06/1998	*S. forskali*
Sf 088	Ao Khao Yau, Ranong	01/06/1998	*S. forskali*
Sf 089	Ao Khao Yau, Ranong	01/06/1998	*S. forskali*
Sf 090	Ao Khao Yau, Ranong	01/06/1998	*S. forskali*
*Sf 091	Ao Khao Yau, Ranong	01/06/1998	*S. forskali*
Sf 092	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	*S. forskali*
Sf 093	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	*S. forskali*
Sf 094	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	*S. forskali*
Oys 095	Klong Bang Nang Rom, Prachuapkririkhan	02/06/1998	A hybrid between *C. iredalei* and *C. belcheri*
Sf 096	Ban Na Thab, Songkhla	04/06/1998	*S. forskali*
Sf 097	Ban Na Thab, Songkhla	04/06/1998	*S. forskali*
Sf 098	Ban Na Thab, Songkhla	04/06/1998	*S. forskali*
Sf 099	Ban Na Thab, Songkhla	04/06/1998	*S. forskali*

Appendix A (continued)

Code of samples	**Location**	**Date of collection**	**Species**
Sf 100	Ban Na Thab, Songkhla	04/06/1998	*S. forskali*
Sf 101	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Sf 102	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Sf 103	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Oys 104	Koh Sichang, Chonburi	07/05/1999	*C. iredalei*-like oyster
Sf 105	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Sf 106	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Sf 107	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
*Sf 108	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Sf 109	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Sf 110	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Sf 111	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
Sf 112	Koh Sichang, Chonburi	07/05/1999	*S. forskali*
*Sf 113	Chantraburi	21/05/1998	*S. forskali*
*D 114	Chantraburi	21/05/1998	*Dendostrea* sp.
*D 115	Chantraburi	21/05/1998	*D. folium*
*Sf 116	Chantraburi	21/05/1998	*S. forskali*
*D 117	Chantraburi	21/05/1998	*D. folium*
*Sf 118	Chantraburi	21/05/1998	*S. forskali*
*Sf 119	Chantraburi	21/05/1998	*S. forskali*
*Sf 120	Chantraburi	21/05/1998	*S. forskali*
Oys 121	Chantraburi	21/05/1998	*Saccostrea* sp.-like oyster
Oys 122	Chantraburi	21/05/1998	*Saccostrea* sp.-like oyster

Appendix A (continued)

Code of samples	Location	Date of collection	Species
Sf 123	Chantraburi	21/05/1998	*S. forskali*
Sf 124	Chantraburi	21/05/1998	*S. forskali*
Sf 125	Chantraburi	21/05/1998	*S. forskali*
Sf 126	Chantraburi	21/05/1998	*S. forskali*
*Sf 127	Chantraburi	21/05/1998	*S. forskali*
Sm 128	Chantraburi	21/05/1998	*S. mytiloides*
Sm 129	Chantraburi	21/05/1998	*S. mytiloides*
Sf 130	Chantraburi	21/05/1998	*S. forskali*
Sf 131	Chantraburi	21/05/1998	*S. forskali*
Sf 132	Chantraburi	21/05/1998	*S. forskali*
*D 133	Chantraburi	21/05/1998	*D. folium*
Sf 134	Chantraburi	21/05/1998	*S. forskali*
*Sm 135	Laem Phun Wa, Phuket	08/04/1998	*S. mytiloides*
Sm 136	Laem Phun Wa, Phuket	08/04/1998	*S. mytiloides*
Sm 137	Laem Phun Wa, Phuket	08/04/1998	*S. mytiloides*
Sm 138	Laem Phun Wa, Phuket	08/04/1998	*S. mytiloides*
Sm 139	Ranong	09/01/1999	*S. mytiloides*
Sm 140	Ranong	09/01/1999	*S. mytiloides*
Sm 141	Ranong	09/01/1999	*S. mytiloides*
Sm 142	Ranong	09/01/1999	*S. mytiloides*
Sm 143	Ranong	09/01/1999	*S. mytiloides*
Sm 144	Ranong	09/01/1999	*S. mytiloides*
Sm 145	Samut Sakhon	10/12/1999	*S. mytiloides*
Sm 146	Samut Sakhon	10/12/1999	*S. mytiloides*
Sm 147	Samut Sakhon	10/12/1999	*S. mytiloides*
Sm 148	Samut Sakhon	10/12/1999	*S. mytiloides*

Appendix A (continued)

Code of samples	**Location**	**Date of collection**	**Species**
Sm 149	Samut Sakhon	10/12/1999	*S. mytiloides*
*Sm 150	Ranong	09/01/1999	*S. mytiloides*
*Sm 151	Ranong	09/01/1999	*S. mytiloides*
*Sm 152	Ranong	09/01/1999	*S. mytiloides*
Sm 153	Samut Sakhon	10/12/1999	*S. mytiloides*
*Sm 154	Samut Sakhon	10/12/1999	*S. mytiloides*
*Sm 155	Samut Sakhon	10/12/1999	*S. mytiloides*
S3 156	Samut Sakhon	10/12/1999	*Saccostrea* sp. group 3
S3 157	Samut Sakhon	10/12/1999	*Saccostrea* sp. group 3
S3 158	Samut Sakhon	10/12/1999	*Saccostrea* sp. group 3
S3 159	Samut Sakhon	10/12/1999	*Saccostrea* sp. group 3
S3 160	Samut Sakhon	10/12/1999	*Saccostrea* sp. group 3
Cs 161	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
Cs 162	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
Cs 163	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
Cs 164	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
Cs 165	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
Cs 166	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
Cs 167	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
Cs 168	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
Cs 169	Klong Bo Tho, Krabi	07/04/1998	*Crassostrea* sp.
S1 170	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1
Sf 171	Koh Prab, Suratthani	04/04/1998	*S. forskali*
Sf 172	Koh Prab, Suratthani	04/04/1998	*S. forskali*
*Sf 173	Koh Prab, Suratthani	04/04/1998	*S. forskali*
S1 174	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1

Appendix A (continued)

Code of samples	Location	Date of collection	Species
S1 175	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1
S1 176	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1
S1 177	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1
S1 178	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1
S1 179	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1
S1 180	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1
S1 181	Koh Prab, Suratthani	04/04/1998	*Saccostrea* sp. group 1
S2 182	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
S2 183	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
S2 184	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
S2 185	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
S2 186	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
S2 187	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
S2 188	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
S2 189	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
S2 190	Koh Nui, Ranong	09/05/1998	*Saccostrea* sp. group 2
Scom 01	Brisbane, Australia	25/10/1998	*S. commercialis*
Scom 02	Brisbane, Australia	25/10/1998	*S. commercialis*
Scom 03	Brisbane, Australia	25/10/1998	*S. commercialis*
Scom 04	Brisbane, Australia	25/10/1998	*S. commercialis*
Scom 05	Brisbane, Australia	25/10/1998	*S. commercialis*
Scom 06	Brisbane, Australia	25/10/1998	*S. commercialis*
*Scom 07	Brisbane, Australia	25/10/1998	*S. commercialis*
*Scom 08	Brisbane, Australia	25/10/1998	*S. commercialis*
*Scom 09	Brisbane, Australia	25/10/1998	*S. commercialis*
*Scom 10	Brisbane, Australia	25/10/1998	*S. commercialis*

Appendix A (continued)

Code of samples	**Location**	**Date of collection**	**Species**
*Scom 11	Brisbane, Australia	25/10/1998	*S. commercialis*
*Scom 12	Brisbane, Australia	25/10/1998	*S. commercialis*
*Pv 01	Chonburi	04/06/1999	*P. viridis*
Pv 02	Chonburi	04/06/1999	*P. viridis*
Pv 03	Chonburi	04/06/1999	*P. viridis*
*Pv 04	Chonburi	04/06/1999	*P. viridis*
*Pv 05	Chonburi	04/06/1999	*P. viridis*
*Pv 06	Chonburi	04/06/1999	*P. viridis*
Pv 07	Chonburi	04/06/1999	*P. viridis*
Pv 08	Chonburi	04/06/1999	*P. viridis*
*Pv 09	Chonburi	04/06/1999	*P. viridis*
*Pv 10	Chonburi	04/06/1999	*P. viridis*
*Pv 11	Chonburi	04/06/1999	*P. viridis*
Pv 12	Chonburi	04/06/1999	*P. viridis*

*Specimens were not included in this study.

Appendix B

Summary of restriction patterns of 16S rDNA, 18S rDNA and an intergenic COI-COII of oysters genera *Crassostrea*, *Saccostrea* and *Striostrea* and the mussel (*P. viridis*) digested restriction endonuclease used in this study

Specimens	16SrDNA						18SrDNA	COI		
	***Acs*I**	***Alu*I**	***Dde*I**	***Dra*I**	***Taq*I**	***Rsa*I**	***Hinf*I**	***Acs*I**	***Dde*I**	***Mbo*I**
C.b 003	A	A	A	A	A	A	A	A	A	A
C.b 004	A	A	A	A	A	A	A	A	A	A
C.b 005	A	A	A	A	A	A	A	A	A	A
C.b 006	A	A	A	A	A	A	A	A	A	A
C.b 007	A	A	A	A	A	A	A	A	A	A
C.b 008	A	A	A	A	A	A	A	A	A	A
C.b 009	A	A	A	A	A	A	A	A	A	B
C.b 010	A	A	A	A	A	A	A	A	A	A
C.b 011	A	A	A	A	A	A	A	A	A	A
C.b 013	A	A	A	A	A	A	A	A	A	A
C.b 014	A	A	A	A	A	A	A	A	A	A
Cb 015	A	A	A	A	A	A	A	A	A	A
Cb 016	A	A	A	A	A	A	A	A	A	A
Cb 017	A	A	B	A	A	A	A	A	A	A
Cb 018	A	A	A	A	A	A	A	A	A	A
Cb 019	A	A	A	A	A	A	A	A	A	A
Cb 020	A	A	A	A	A	A	A	A	A	A
Ci 021	B	B	B	B	A	C	A	A	B	C
Ci 022	B	B	B	B	A	A	A	A	B	C
Ci 023	B	B	B	B	A	A	A	A	B	C
Ci 024	B	B	B	B	A	A	A	A	B	C

Appendix B (continued)

Specimens	16SrDNA						18SrDNA	COI		
	*Acs*I	*Alu*I	*Dde*I	*Dra*I	*Taq*I	*Rsa*I	*Hinf*I	*Acs*I	*Dde*I	*Mbo*I
Ci 025	B	B	B	B	A	A	A	A	B	C
Ci 026	B	B	B	B	A	A	A	A	B	C
Ci 027	B	B	B	B	A	A	A	A	B	C
Ci 028	B	B	B	B	A	A	A	A	B	C
Ci 029	B	B	B	B	A	A	A	A	B	C
Ci 031	B	B	B	B	A	A	A	A	B	C
Ci 032	B	B	B	B	A	A	A	A	B	C
Ci 033	B	B	B	B	A	A	A	A	B	C
Ci 034	B	B	B	B	A	A	A	A	B	C
Ci 035	B	B	B	B	A	A	A	A	B	C
Ci 036	B	B	B	B	A	A	A	A	B	C
Ci 037	B	B	B	B	A	A	A	A	B	C
Ci 038	B	B	B	B	A	A	A	A	B	C
Oys 039	B	B	B	B	A	A	A	A	B	C
Ci 040	B	B	B	B	A	A	A	A	B	U
Ci 041	B	B	B	B	A	A	A	A	B	C
Ci 042	B	B	B	B	A	A	A	A	B	C
Ci 043	B	B	B	B	A	A	A	A	B	C
Sc 044	C	D	C	C	B	B	B	B	C	D
Sc 045	C	D	C	C	B	B	B	B	C	D
Sc 046	C	C	C	C	B	B	B	B	C	E
Sc 047	C	D	C	C	B	B	B	B	C	D
Sc 048	C	D	C	C	B	B	B	B	C	D
Sc 050	C	D	C	C	B	B	B	B	C	D
Sc 051	C	C	C	C	B	B	B	B	C	D

Appendix B (continued)

Specimens	16SrDNA						18SrDNA	COI		
	*Acs*I	*Alu*I	*Dde*I	*Dra*I	*Taq*I	*Rsa*I	*Hinf*I	*Acs*I	*Dde*I	*Mbo*I
Sc 052	C	D	C	C	B	B	B	B	C	D
Sc 053	C	C	C	C	B	B	B	B	C	D
Sc 054	C	D	C	C	B	B	B	B	C	D
Sc 055	C	C	C	C	B	B	B	B	C	D
Sc 056	C	C	C	C	B	B	B	B	C	D
Sc 057	C	C	C	C	B	B	B	B	C	D
Sc 058	C	C	C	C	B	B	B	B	C	E
Sc 059	C	D	C	C	B	B	B	B	C	D
Sc 060	C	D	C	C	B	B	B	B	C	E
Sc 061	C	D	C	C	B	B	B	B	C	D
Sc 062	D	D	C	C	B	B	B	B	C	E
Sc 066	C	D	C	C	B	B	B	B	C	D
Sc	C	D	C	C	B	B	B	B	C	D
Sf 068	E	E	D	C	B	A	B	C	D	F
Sf 069	D	E	D	C	B	A	B	C	D	F
Sf 070	D	E	D	C	B	A	B	C	D	F
Sf 071	D	E	D	C	B	A	B	C	D	F
Sc 072	C	D	C	C	B	B	B	B	C	D
Sc 073	C	C	C	C	B	B	B	B	C	D
Sc 074	C	D	C	C	B	B	B	B	C	D
Sf 075	D	E	D	A	B	A	B	C	D	F
Sf 076	D	E	D	A	B	A	B	C	D	F
Sf 077	D	E	D	C	B	A	B	C	D	F
Sf 079	D	E	D	C	B	A	B	C	D	F
Sf 080	G	F	G	C	B	A	B	C	J	F

Appendix B (continued)

Specimens	16SrDNA						18SrDNA	COI		
	*Acs*I	*Alu*I	*Dde*I	*Dra*I	*Taq*I	*Rsa*I	*Hinf*I	*Acs*I	*Dde*I	*Mbo*I
Sf 081	G	F	G	C	B	A	B	A	J	F
Sf 082	F	F	F	C	B	B	B	A	J	F
Sf 085	D	F	F	A	B	B	B	A	I	R
Sf 086	D	E	D	A	B	A	B	C	D	F
Sf 087	F	F	F	A	B	B	B	A	J	F
Sf 088	F	F	F	C	B	B	B	A	J	F
Sf 089	F (D+F)	F	F	C	B	B	B	A	J	F
Sf 090	I	F	F	C	B	B	B	C	E	H
Sf 092	D	E	D	C	B	A	B	C	D	F
Sf 093	D	E	D	C	B	A	B	C	D	F
Sf 094	D	E	D	C	B	A	B	C	D	F
Oys 095	B	B	B	B	B	A	A	A	B	C
Sf 096	D	E	D	C	B	A	B	C	D	F
Sf 097	D	E	D	C	B	A	B	C	D	F
Sf 098	D	E	E	C	B	A	B	C	D	F
Sf 099	D	E	D	C	B	A	B	C	D	F
Sf 100	D	E	D	C	B	A	B	C	D	F
Sf 101	D	F	D	A	B	A	B	A	D	F
Sf 102	D	F	D	A	B	A	B	C	D	F
Sf 103	D	F	F	C	B	B	B	A	H	R
Oys 104	B	B	B	B	A	A	A	A	B	C
Sf 105	D	E	D	A	B	A	B	C	D	F
Sf 106	D	K	F	A	B	B	B	A	H	R
Sf 107	D	K	F	C	B	A	B	A	H	R
Sf 109	D	E	D	C	B	A	B	A	H	F

Appendix B (continued)

Specimens	16SrDNA						18SrDNA	COI		
	*Acs*I	*Alu*I	*Dde*I	*Dra*I	*Taq*I	*Rsa*I	*Hinf*I	*Acs*I	*Dde*I	*Mbo*I
Sf 110	D	F	F	C	B	A	B	C	H	F
Sf 111	D	F	F	C	B	A	B	A	H	F
Sf 112	D	K	F	C	B	B	B	A	H	F
Oys 121	D	F	G	A	B	B	B	A	H	P
Oys 122	D	F	G	A	B	B	B	A	I	O
Sf 123	D	E	D	C	B	A	B	C	D	F
Sf 124	D	E	D	A	B	A	B	D	D	F
Sf 125	D	E	D	A	B	A	B	C	D	F
Sf 126	D	E	D	A	B	A	B	C	D	F
Sm 128	D	E	D	C	B	A	B	C	D	I
Sm 129	D	E	D	C	B	A	B	C	D	F
Sf 130	D	E	D	C	B	A	B	C	D	F
Sf 131	D	E	D	A	B	A	B	C	D	F
Sf 132	D	E	D	C	B	A	B	C	D	F
Sf 134	D	E	D	C	B	A	B	C	D	F
Oys 030	D	E	D	C	B	A	B	C	D	F
Sm 136	D	F	F	D	B	B	B	A	K	J
Sm 137	H	H	F	D	B	B	B	A	K	J
Sm 138	I	H	F	D	B	B	B	A	K	J
Sm 139	D	G	F	D	B	B	B	A	K	J
Sm 140	L (D+L)	H	F	D	B	B	B	A	K	J
Sm 141	L (L+I)	H	F	D	B	B	B	A	K	J
Sm 142	L (L+I)	H	F	D	B	B	B	A	K	J

Appendix B (continued)

Specimens	16SrDNA						18SrDNA	COI		
	*Acs*I	*Alu*I	*Dde*I	*Dra*I	*Taq*I	*Rsa*I	*Hinf*I	*Acs*I	*Dde*I	*Mbo*I
Sm 143	L (L+I)	H	F	D	B	B	B	A	K	J
Sm 144	I (D+I)	H	F	D	B	B	B	A	K	J
Sm 145	D	E	D	C	B	A	B	C	D	F
Sm 146	D	E	D	C	B	A	B	C	D	F
Sm 147	D	E	D	A	B	A	B	C	D	F
Sm 148	D	E	D	C	B	A	B	C	D	F
Sm 149	D	B	D	C	B	A	B	C	D	F
Sm 153	D	B	D	A	B	A	B	C	D	F
S3 156	D	E	D	A	B	A	B	C	D	F
S3 157	D	E	D	C	B	A	B	C	D	F
S3 158	D	E	D	C	B	A	B	C	D	F
S3 159	D	E	D	C	B	A	B	C	D	F
S3 160	D	E	D	C	B	A	B	C	D	F
Cs 161	D	I	H	A	B	C	A	A	F	K
Cs 162	C	I	H	A	B	C	A	A	F	L
Cs 163	C	I	H	A	B	C	A	A	F	L
Cs 164	D	I	H	A	B	C	A	A	F	K
Cs 165	D	I	H	A	B	C	A	A	F	K
Cs 166	D	I	H	A	B	C	A	A	F	K
Cs 167	D	I	H	A	B	C	A	A	F	K
Cs 168	D	I	H	A	B	C	A	A	F	K
Cs 169	D	I	H	A	B	C	A	A	F	K
S1 170	D	F	G	A	B	A	B	A	H	P
Sf 171	D	F	G	A	B	A	B	A	H	P

Appendix B (continued)

Specimens	16SrDNA						18SrDNA	COI		
	*Acs*I	*Alu*I	*Dde*I	*Dra*I	*Taq*I	*Rsa*I	*Hinf*I	*Acs*I	*Dde*I	*Mbo*I
Sf 172	D	F	G	A	B	B	B	A	I	R
S1 174	D	F	G	C	B	B	B	A	H	Q
S1 175	D	K	G	C	B	B	B	A	H	P
S1 176	D	F	F	C	B	B	B	A	I	G
S1 177	D	F	F	C	B	B	B	A	I	G
S1 178	D	F	F	C	B	B	B	A	I	G
S1 179	D	K	F	C	B	B	B	A	I	G
S1 180	D	F	F	C	B	B	B	A	I	O
S1 181	D	F	F	C	B	B	B	A	I	O
S2 182	J	J	F	C	B	B	B	B	G	M
S2 183	J	J	F	C	B	B	B	B	G	S
S2 184	J	J	F	C	B	B	B	B	G	M
S2 185	J	J	F	C	B	B	B	B	G	V
S2 186	J	J	F	C	B	B	B	B	G	M
S2 187	J	J	F	C	B	B	B	B	G	M
S2 188	J	J	F	C	B	B	B	B	G	M
S2 189	J	J	F	C	B	B	B	B	G	M
S2 190	J	J	F	C	B	B	B	B	G	M
Scom 01	L (L+I)	F	C	C	B	B	B	E	L	N
Scom 02	L (L+I)	F	C	C	B	B	B	E	L	N
Scom 03	L (L+I)	F	C	C	B	B	B	E	L	N

Appendix B (continued)

Specimens	16SrDNA						18SrDNA	COI		
	***Acs*I**	***Alu*I**	***Dde*I**	***Dra*I**	***Taq*I**	***Rsa*I**	***Hinf*I**	***Acs*I**	***Dde*I**	***Mbo*I**
Scom 04	L (L+I)	F	C	E	B	B	B	E	L	N
Scom 05	L (L+I)	F	C	C	B	B	B	E	L	N
Scom 06	L (L+I)	F	C	C	B	B	B	E	L	N
Pv 02	K	L	I	F	C	D	C	F	M	T
Pv 03	K	L	I	F	C	D	C	F	M	T
Pv 07	K	L	I	F	C	D	C	F	M	T
Pv 08	K	L	I	F	C	D	C	F	M	T
Pv 12	K	L	I	F	C	D	C	F	M	T

Appendix C

Pairwise genetic distances of 54 composite haplotypes generated from digestion of 16S rDNA with *Acs* I, *Alu* I, *Dde* I, *Dra* I, *Taq* I and *Rsa* I, 18S rDNA with *Hinf* I and COI with *Acs* I, *Dde* I and *Mbo* I

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I

II 0.00407275772

III 0.00394177006 0.00836845962

IV 0.05924648373 0.05167653480 0.04927199018

V 0.05167653480 0.04478630881 0.04283566641 0.00394177006

VI 0.04854313886 0.04695925129 0.03993325417 0.01009182394 0.00560528503

VII 0.11567399756 0.11386946520 0.11746185402 0.09565329933 0.09408019036 0.08269876958

VIII 0.11567399756 0.11386946520 0.11746185402 0.08718672935 0.08569894173 0.08269876958
0.00827704939

IX 0.10556297276 0.10389142492 0.10722204868 0.08866576272 0.08718672935 0.07698391337
0.00389039543 0.00389039543

X 0.12838747010 0.12641586449 0.13033832340 0.09408019036 0.09249637804 0.08929815659
0.00401857789 0.00401857789 0.00827704939

XI 0.12803070312 0.12600564307 0.13003285403 0.09292492100 0.09128506125 0.08796847970
0.00858546649 0.00858546649 0.01287216774 0.00454351294

XII 0.10766599028 0.09742602656 0.10911450333 0.08548801033 0.07815249610 0.08847655917
0.07587404258 0.06840670137 0.07000422817 0.07418632834 0.07331468444

XIII 0.09455210586 0.08434723685 0.09616639338 0.07307686613 0.06571969232 0.07540325218
0.07065741688 0.06336108349 0.06499844980 0.06892807389 0.06388645441 0.00457082428

XIV 0.08564908434 0.07505685013 0.08743894477 0.07156037851 0.06400553149 0.07386953067
0.08434723685 0.07687989564 0.07834706561 0.08279401776 0.07331468444 0.01177770727
0.00457082428

XV 0.11989527924 0.10874835413 0.13322504887 0.08906217885 0.08219110923 0.09206739823
0.05727722185 0.06285165210 0.06428660120 0.05590080729 0.05439809470 0.03113634839
0.03225610541 0.04127528678

XVI 0.10296610637 0.09128506125 0.11686393489 0.07168192170 0.06437824487 0.07391886782
0.05582981677 0.06153388493 0.06302181404 0.05439809470 0.05293539332 0.03962772419
0.04127528678 0.06199690063 0.00440262882

XVII	0.11746185402	0.10389142492	0.13417809767	0.07463897318	0.07329898399	0.08420280067
	0.04892249493	0.05355824862	0.05486856254	0.04766139926	0.04599037039	0.04259430443
	0.04422427692	0.06590897403	0.01637833890	0.01116590242		
XVIII	0.09079217658	0.08875517364	0.10567306417	0.08702992488	0.08519016428	0.07253210705
	0.03953291877	0.04762358458	0.04463144104	0.04221625958	0.03232720033	0.07953109278
	0.06121335831	0.05560976832	0.06655099212	0.05019396097	0.04069934289	
XIX	0.10852109745	0.09449291210	0.12575272174	0.07307686613	0.07168192170	0.08279401776
	0.07224188531	0.07698380129	0.07797140722	0.07131300277	0.06840552043	0.06302075154
	0.06590897403	0.04887378693	0.02552231742	0.01763916184	0.00427021893	0.03109258595
XX	0.09721539982	0.09565329933	0.10886613580	0.09013569683	0.08866576272	0.10389142492
	0.04854555665	0.05369248905	0.05514490778	0.04714086951	0.04593842213	0.04356961574
	0.04083594284	0.04778736198	0.03101562789	0.03820679501	0.02499751784	0.04186483483
	0.03406210097					
XXI	0.09292492100	0.08279401776	0.09455210586	0.07168192170	0.06437824487	0.07391886782
	0.07722165126	0.06892807389	0.07065741688	0.07538695092	0.07042027815	0.00881446137
	0.00407275772	0.00881446137	0.03512016764	0.04435070258	0.04730585802	0.06649535451
	0.06940160307	0.04453285734				
XXII	0.08620926843	0.07587404258	0.08794996257	0.07307686613	0.06571969232	0.07540325218
	0.07676676875	0.06947043535	0.07101593171	0.07513214439	0.06830504571	0.02184072119
	0.01048475859	0.00457082428	0.05189252385	0.06495424810	0.06878532637	0.05618644669
	0.05093714186	0.05476698257	0.01472134065			
XXIII	0.07822015948	0.07625322738	0.09079217658	0.08519016428	0.07432994201	0.06303174761
	0.04637086917	0.05579534504	0.05217767607	0.04957365445	0.03970403643	0.07815520719
	0.05957101524	0.05383184366	0.06097789767	0.04420892198	0.05714720402	0.02288182411
	0.04778741486	0.05347577225	0.06477180319	0.05446169524		
XXIV	0.09079217658	0.08875517364	0.10567306417	0.08702992488	0.08519016428	0.07253210705
	0.03953291877	0.04762358458	0.04463144104	0.04221625958	0.03232720033	0.08669199119
	0.06820100917	0.06277066673	0.06199690063	0.04552788363	0.04463144104	0.00290333118
	0.03511981551	0.04583636878	0.07432994201	0.06317409756	0.01868830392	
XXV	0.09922929607	0.08460089625	0.10140695245	0.06649535451	0.05791397813	0.06888029588
	0.06570198051	0.07232299263	0.07386953067	0.06421756806	0.05455650611	0.03919497451
	0.02127505185	0.01445582700	0.05204880320	0.03476859671	0.03836630670	0.03586891095
	0.02838099862	0.04762358458	0.02701614231	0.01608057652	0.03404762209	0.04169663930
XXVI	0.10442850333	0.09024872842	0.10647070864	0.07540615312	0.06717896964	0.07818025388
	0.06745804148	0.07391886782	0.07540325218	0.06603891885	0.05581855504	0.02127505185
	0.01360215613	0.00910475433	0.03089942998	0.05204880320	0.05619247149	0.04006681271

	0.03836630670	0.03548589920	0.01907408078	0.01360215613	0.03836604273	0.04574511213
	0.00529877203					
XXVII	0.08564908434	0.08384039869	0.09791101916	0.09791101916	0.09602693131	0.08201282656
	0.03138523603	0.03993325417	0.03689398434	0.03407034867	0.02853934959	0.06121335831
	0.05283870403	0.06121335831	0.05057569639	0.04369489660	0.03274953874	0.01076939071
	0.04463144104	0.02661191489	0.05785993786	0.06223865961	0.01944332050	0.01393934702
	0.04006681271	0.04624576292				
XXVIII	0.08016325266	0.07822015948	0.09280421501	0.11062018354	0.10858159794	0.09188602485
	0.04342450550	0.05217767607	0.04887378693	0.04637086917	0.03658567515	0.08669199119
	0.06820100917	0.06277066673	0.07697777878	0.06087714403	0.04887378693	0.00921948941
	0.03946479879	0.04186483483	0.07432994201	0.06317409756	0.01868830392	0.01267402033
	0.04169663930	0.04574511213	0.00777978776			
XXIX	0.07505685013	0.07331468444	0.08564908434	0.10848796444	0.09412257251	0.08016634232
	0.03846941996	0.04854313886	0.04478630881	0.04174477654	0.03631263106	0.05957101524
	0.05110178273	0.05957101524	0.04915171488	0.04212430092	0.04478630881	0.01944332050
	0.05714720402	0.03689398434	0.05603801110	0.06065213759	0.01797654985	0.02324877726
	0.03836604273	0.04469296082	0.00733437786	0.00941238846		
XXX	0.08018423943	0.07069905031	0.08186031401	0.06692123205	0.05915598986	0.06906124786
	0.06597656867	0.05924648373	0.06084058562	0.06430390809	0.05966129228	0.01689858432
	0.00979448683	0.01689858432	0.04390814818	0.03708564726	0.03968618365	0.05259125494
	0.05136413423	0.04569899871	0.01446497685	0.01890742086	0.03975650886	0.05910704876
	0.02089978231	0.02734313002	0.03569883607	0.04670551445	0.03405785199	
XXXI	0.09292492100	0.08279401776	0.10469046406	0.07834706561	0.07028013477	0.08123087651
	0.04962574627	0.05532981443	0.05691246218	0.04809632230	0.04246939239	0.02587829024
	0.02073665310	0.02587829024	0.01977417380	0.02863932794	0.03178338460	0.03802960806
	0.05351532169	0.02227807574	0.02325274060	0.03138523603	0.02838099862	0.04269568541
	0.02338264888	0.01553067396	0.02198670351	0.03372727294	0.02053478712	0.01770784525
XXXII	0.08384039869	0.07331468444	0.09602693131	0.06906124786	0.06065213759	0.07155610443
	0.04854313886	0.05438301817	0.05601755292	0.04695925129	0.04165639031	0.03372727294
	0.02587829024	0.03372727294	0.02863932794	0.02111161185	0.02404697278	0.03174737580
	0.03591012393	0.02890818132	0.02853934959	0.03542864511	0.02174397753	0.03653108535
	0.01649980961	0.02338264888	0.01776920701	0.02733657845	0.01621070110	0.01352235995
	0.00471946897					
XXXIII	0.09602693131	0.08384039869	0.11048059276	0.07849165013	0.07678251892	0.09219655547
	0.03993325417	0.04478630881	0.04629205167	0.03846941996	0.03304571530	0.04417812247
	0.03620587534	0.04417812247	0.03717462362	0.02997219809	0.02198886934	0.02886927818

	0.03361658260	0.02661191489	0.03960098876	0.04560583092	0.03174737580	0.03332198832
	0.02579136390	0.03232720033	0.01556542275	0.01730822841	0.01776920701	0.02236712224
	0.01180231027	0.00733437786				
XXXIV	0.09455210586	0.08434723685	0.09616639338	0.08742244635	0.07834706561	0.08279401776
	0.07065741688	0.06336108349	0.06499844980	0.06892807389	0.06388645441	0.01011676946
	0.00541796183	0.01011676946	0.04134352854	0.05057569639	0.05341035777	0.06121335831
	0.07530704136	0.04615325028	0.01009182394	0.01590272041	0.05957101524	0.06820100917
	0.02838099862	0.02053478712	0.05283870403	0.06820100917	0.05110178273	0.01578595472
	0.02928467123	0.03463146081	0.04673563458			
XXXV	0.09634762663	0.09449291210	0.11046793675	0.07920601554	0.07754731606	0.08267737183
	0.04730585802	0.05204777400	0.05341035777	0.04599037039	0.03846941996	0.06400553149
	0.05167653480	0.05613559016	0.05623803100	0.04572264175	0.03641860480	0.03009290389
	0.04295133884	0.03759571544	0.05601755292	0.05737685488	0.03292028806	0.03443788718
	0.03601022467	0.04275136049	0.02342944598	0.03009290389	0.03463146081	0.04442689123
	0.03138523603	0.02587829024	0.02342944598	0.05763876419		
XXXVI	0.08240730519	0.08085732049	0.09247120423	0.07651885539	0.07505071627	0.07929608282
	0.05761513936	0.04872792069	0.04988637397	0.05635805458	0.05486856254	0.06465819132
	0.05747574623	0.06465819132	0.06270617840	0.05514490778	0.03860901190	0.04980232272
	0.04215841140	0.04470002630	0.06137627590	0.06644622918	0.05340131358	0.05480215146
	0.05737685488	0.06747124098	0.04089952103	0.04521634493	0.04883864854	0.04813679935
	0.05143815764	0.04399699066	0.03676348869	0.06273087166	0.01334716489	
XXXVII	0.08159471391	0.07999824672	0.09188751136	0.07559086722	0.07407703180	0.07838887476
	0.05617456209	0.04709414301	0.04830075998	0.05486856254	0.05341035777	0.06289594896
	0.05611720840	0.06289594896	0.06137627590	0.05415595599	0.03753452361	0.04842549664
	0.04089952103	0.03888983161	0.06008429002	0.06465819132	0.05208683882	0.05353982527
	0.05613559016	0.06571969232	0.03968618365	0.04373449645	0.04778736198	0.04711596330
	0.04992532469	0.04283566641	0.03545755357	0.06148740957	0.01205559239	0.00380118457
XXXVIII	0.08794996257	0.08620926843	0.09999982603	0.07316406045	0.07159052026	0.07587404258
	0.05341035777	0.04422427692	0.04548477641	0.05204777400	0.04478630881	0.05983476556
	0.04778736198	0.05208683882	0.06220821236	0.05199237176	0.04139073677	0.03594376186
	0.04793749299	0.04260527770	0.05167653480	0.05340131358	0.03915121085	0.04070149216
	0.04267809486	0.04915134363	0.02883422221	0.03594376186	0.04122395894	0.04084616460
	0.03689398434	0.03160389002	0.02883422221	0.05322551732	0.00394177006	0.00918541835
	0.00782260777					
XXXIX	0.08240730519	0.08085732049	0.09247120423	0.07651885539	0.07505071627	0.07929608282
	0.05761513936	0.04872792069	0.04988637397	0.05635805458	0.05486856254	0.06465819132

	0.05747574623	0.06465819132	0.06270617840	0.05514490778	0.04345441280	0.04980232272
	0.04830075998	0.04470002630	0.06137627590	0.06644622918	0.05340131358	0.05480215146
	0.05737685488	0.06747124098	0.04089952103	0.04521634493	0.04883864854	0.04813679935
	0.05143815764	0.04399699066	0.03676348869	0.06273087166	0.01334716489	0.00512834904
	0.00380118457	0.00918541835				
XL	0.09292492100	0.08279401776	0.09455210586	0.06571969232	0.05904889066	0.06745804148
	0.06170918659	0.05532981443	0.05691246218	0.06004421185	0.05470634378	0.00881446137
	0.00407275772	0.00881446137	0.02686202840	0.03589594098	0.03895359602	0.05337886846
	0.06085358494	0.03447812483	0.00867119460	0.01472134065	0.05178210485	0.05957101524
	0.01680382058	0.00911495711	0.04505824747	0.05957101524	0.04336513467	0.01446497685
	0.01543876296	0.02053478712	0.03007736932	0.01009182394	0.04478630881	0.05143815764
	0.04992532469	0.04138977000	0.05143815764			
XLI	0.08384039869	0.07331468444	0.08564908434	0.06400553149	0.05714720402	0.06570198051
	0.07540325218	0.06887199021	0.07028013477	0.07391886782	0.06406280627	0.01624384172
	0.00881446137	0.00427305728	0.03589594098	0.05682328808	0.06085358494	0.04748359234
	0.04342450550	0.04138977000	0.01360215613	0.00881446137	0.04574511213	0.05383184366
	0.00974935970	0.00442724683	0.05337886846	0.05383184366	0.05178210485	0.02179303993
	0.02053478712	0.02838099862	0.03802960806	0.01497798519	0.04915134363	0.05859284069
	0.05666604882	0.04560583092	0.05859284069	0.00471946897		
XLII	0.08016325266	0.07822015948	0.08208280551	0.06284089633	0.07610908518	0.06477180319
	0.10211843291	0.11446656903	0.10389687265	0.11255437771	0.10442377141	0.10410236501
	0.08519016428	0.08018104055	0.11649174895	0.10136276920	0.10389687265	0.07714529380
	0.09581908820	0.10565894590	0.08332947948	0.08016325266	0.07504966202	0.07714529380
	0.08391129193	0.08687733777	0.08018104055	0.08831467148	0.08898986353	0.08408793291
	0.09386449364	0.08898986353	0.10415242711	0.08519016428	0.09079217658	0.09999982603
	0.09977256110	0.09280421501	0.09999982603	0.08332947948	0.07818926078	
XLIII	0.08215168990	0.08027856561	0.08400353847	0.05915598986	0.07069905031	0.06065213759
	0.08050894834	0.09064730654	0.08233601098	0.08870352451	0.10211843291	0.10736561256
	0.09518687151	0.08702992488	0.11966229623	0.10389687265	0.10640046608	0.08214712253
	0.09791101916	0.10809362354	0.09351902567	0.08743894477	0.07087481074	0.08214712253
	0.08898986353	0.09386449364	0.08702992488	0.09313002982	0.09581908820	0.09064746384
	0.10389687265	0.09581908820	0.11062018354	0.09518687151	0.08743894477	0.09188751136
	0.09138084429	0.08921007189	0.09188751136	0.09351902567	0.08519016428	0.00909816981
XLIV	0.09408019036	0.09249637804	0.10556297276	0.09565329933	0.09408019036	0.09090222458
	0.03234132078	0.02814220728	0.02958285467	0.03084282032	0.02986283240	0.06180694050
	0.05691246218	0.07028013477	0.05076260972	0.04962574627	0.03825689615	0.04762358458

	0.05189252385	0.03480222545	0.06170918659	0.06302181404	0.06119665424	0.05217767607
	0.06570198051	0.06745804148	0.03548589920	0.04762358458	0.04327043226	0.05322551732
	0.04447566075	0.04327043226	0.03548589920	0.05691246218	0.04730585802	0.04220472602
	0.04086277489	0.04422427692	0.04220472602	0.04962574627	0.06303211090	0.11446656903
	0.11686393489					
XLV	0.09565329933	0.09408019036	0.10722204868	0.09721539982	0.09565329933	0.09249637804
	0.03383107434	0.02566580329	0.03101562789	0.02814220728	0.02711460802	0.06333908427
	0.05848364749	0.07168192170	0.05223159592	0.05114573007	0.03956210993	0.04887378693
	0.05302213151	0.03617187424	0.06336108349	0.06450112940	0.06260446273	0.05347577225
	0.06717896964	0.06887199021	0.03689398434	0.04887378693	0.04478630881	0.05476241974
	0.04593842213	0.04478630881	0.03689398434	0.05848364749	0.04861865388	0.04345441280
	0.04215841140	0.04548477641	0.04345441280	0.05114573007	0.06437824487	0.11635790578
	0.11868436755	0.00389039543				
XLVI	0.10389142492	0.10220644514	0.11746185402	0.09565329933	0.09408019036	0.09090222458
	0.03234132078	0.02814220728	0.02958285467	0.03084282032	0.02986283240	0.06180694050
	0.05691246218	0.07028013477	0.05076260972	0.04962574627	0.03825689615	0.04762358458
	0.05189252385	0.03480222545	0.06170918659	0.06302181404	0.06119665424	0.05217767607
	0.06570198051	0.06745804148	0.03548589920	0.04762358458	0.04327043226	0.05322551732
	0.04447566075	0.04327043226	0.03548589920	0.05691246218	0.04730585802	0.04220472602
	0.04086277489	0.04422427692	0.04220472602	0.04962574627	0.06303211090	0.11446656903
	0.11686393489	0.00259879901	0.00667756791			
XLVII	0.09412257251	0.09219655547	0.10848796444	0.08564908434	0.08384039869	0.07155610443
	0.02625637102	0.03407034867	0.03138523603	0.02863832647	0.02288122768	0.06649535451
	0.05785993786	0.06649535451	0.04435070258	0.03720619075	0.03548589920	0.01944332050
	0.04762358458	0.03274953874	0.06360903925	0.06741029272	0.01115407823	0.01588667818
	0.04398860681	0.05017257836	0.00733437786	0.01588667818	0.01621070110	0.03924534258
	0.02438474001	0.02015690281	0.01776920701	0.05785993786	0.02198670351	0.03955644237
	0.03829419658	0.02736154413	0.03955644237	0.04934745006	0.05791397813	0.07818926078
	0.07610908518	0.03846941996	0.03993325417	0.03846941996		
XLVIII	0.09602693131	0.09412257251	0.11048059276	0.09791101916	0.09602693131	0.08201282656
	0.02758485837	0.03548589920	0.03274953874	0.03001465836	0.02438474001	0.07610908518
	0.06738256334	0.07610908518	0.05057569639	0.04369489660	0.04138977000	0.02474219995
	0.05347577225	0.03829419658	0.07429004383	0.07678251892	0.01255088520	0.02089978231
	0.05203421293	0.05791397813	0.01206076128	0.01393934702	0.01405732705	0.04668082522
	0.03007736932	0.02605882362	0.01556542275	0.06738256334	0.02736154413	0.04089952103

	0.03968618365	0.03311868229	0.04089952103	0.05785993786	0.06649535451	0.09107349152
	0.08702992488	0.03993325417	0.04138977000	0.03993325417	0.00427305728	
XLIX	0.09412257251	0.09219655547	0.10848796444	0.07678251892	0.07505685013	0.06406280627
	0.02275804531	0.03407034867	0.02758485837	0.02863832647	0.02288122768	0.05957101524
	0.05110178273	0.05957101524	0.04435070258	0.03720619075	0.02758485837	0.01255088520
	0.03953291877	0.03274953874	0.05603801110	0.06065213759	0.02579136390	0.01588667818
	0.03836604273	0.04469296082	0.01405732705	0.02324877726	0.02441537763	0.04494245770
	0.02853934959	0.02441537763	0.02175648849	0.05110178273	0.02198670351	0.03955644237
	0.03829419658	0.02736154413	0.03955644237	0.04336513467	0.05178210485	0.07818926078
	0.07610908518	0.03407034867	0.03548589920	0.03407034867	0.01253745911	0.01776920701
L	0.09412257251	0.09219655547	0.10848796444	0.08564908434	0.08384039869	0.07155610443
	0.02275804531	0.03407034867	0.02758485837	0.02863832647	0.02288122768	0.06649535451
	0.05785993786	0.06649535451	0.04915171488	0.04212430092	0.03138523603	0.01588667818
	0.04342450550	0.03689398434	0.06360903925	0.06741029272	0.03015903038	0.01944332050
	0.04398860681	0.05017257836	0.01776920701	0.01944332050	0.02015690281	0.05125013679
	0.03304571530	0.02903440249	0.01776920701	0.05785993786	0.02587829024	0.03955644237
	0.03829419658	0.03160389002	0.03955644237	0.04934745006	0.05791397813	0.08898986353
	0.08519016428	0.03407034867	0.03548589920	0.03407034867	0.01621070110	0.01405732705
	0.00285867891					
LI	0.10647070864	0.10442850333	0.12399413413	0.07678251892	0.07505685013	0.06406280627
	0.02625637102	0.03407034867	0.03138523603	0.02863832647	0.02288122768	0.05957101524
	0.05110178273	0.05957101524	0.04435070258	0.03720619075	0.02758485837	0.01255088520
	0.03953291877	0.03274953874	0.05603801110	0.06065213759	0.02174397753	0.01588667818
	0.03836604273	0.04469296082	0.01405732705	0.02324877726	0.02441537763	0.04494245770
	0.02853934959	0.02441537763	0.02175648849	0.05110178273	0.02198670351	0.03955644237
	0.03829419658	0.02736154413	0.03955644237	0.04336513467	0.05178210485	0.07818926078
	0.07610908518	0.03407034867	0.03548589920	0.03407034867	0.01253745911	0.01405732705
	0.00285867891	0.00588554928				
LII	0.10488116820	0.10324947952	0.10650128862	0.09681021092	0.09528958815	0.09222217228
	0.03843626928	0.04254206915	0.04385028434	0.03717563837	0.03563504976	0.06947043535
	0.06417347128	0.07840260833	0.05194420680	0.05076260972	0.04385028434	0.04974848105
	0.05829464130	0.02849786809	0.06962624808	0.07996299676	0.04855932071	0.04974848105
	0.06168191643	0.06361159596	0.02913089804	0.04564781789	0.03978629990	0.05415595599
	0.04114678713	0.03978629990	0.03685099901	0.06417347128	0.04075722388	0.04773816837
	0.04645631481	0.04588511582	0.03852039822	0.05633703997	0.07060454121	0.10296610637

	0.09565329933	0.04189047820	0.04332691693	0.04189047820	0.02785727535	0.03284424416
	0.03548953259	0.03978629990	0.03548953259			
LIII	0.10488116820	0.10324947952	0.10650128862	0.09681021092	0.09528958815	0.09222217228
	0.04524938371	0.04935518358	0.05056393724	0.04409120564	0.04224988669	0.07840260833
	0.07059085039	0.07840260833	0.05875732122	0.05727722185	0.05056393724	0.04974848105
	0.05829464130	0.03348270498	0.07614086021	0.07996299676	0.04855932071	0.04974848105
	0.06168191643	0.06361159596	0.03524024990	0.04564781789	0.04599037039	0.06008429002
	0.04766139926	0.04599037039	0.04296035087	0.07059085039	0.04075722388	0.04773816837
	0.04645631481	0.04588511582	0.03852039822	0.06285165210	0.07060454121	0.10296610637
	0.09565329933	0.04702301397	0.04838452517	0.04702301397	0.03406134585	0.03895359602
	0.04169360309	0.04599037039	0.04169360309	0.00258498578		
LIV	0.13012144128	0.12855627254	0.14634217772	0.11008404468	0.10874835413	0.10607019591
	0.11889259457	0.11889259457	0.12027361726	0.11750889831	0.11556926276	0.12861415986
	0.13012144128	0.12861415986	0.10950821982	0.10741013730	0.11078423350	0.10474572944
	0.10874835413	0.11205963427	0.14292377721	0.13012144128	0.09466475777	0.10474572944
	0.11177600742	0.11365472182	0.10673218968	0.11492945835	0.11516753526	0.13183576706
	0.10741013730	0.10532907786	0.11667344886	0.13012144128	0.10045477676	0.11526741362
	0.11333363872	0.10170279604	0.11526741362	0.11701619019	0.11516753526	0.12716140621
	0.11817187342	0.10950821982	0.12027361726	0.10950821982	0.09692715217	0.10673218968
	0.09692715217	0.10532907786	0.09692715217	0.11161552033	0.11161552033	

Appendix D

Sequence similarity:

D.1 *C. belcheri*

Sequences producing significant alignments:	Score (bits)	E Value
ref|NC_001276.1| Crassostrea gigas mitochondrion, complete ...	391	e-106
gb|AF177226.1|AF177226 Crassostrea gigas mitochondrial DNA,...	391	e-106
gb|AF152568.1|AF152568 Crassostrea sikamea cytochrome oxida...	363	6e-98
gb|AF152569.1|AF152569 Crassostrea ariakensis cytochrome ox...	359	1e-96
gb|AF152567.1|AF152567 Crassostrea angulata cytochrome oxid...	355	2e-95
gb|AF152565.1|AF152565 Crassostrea gigas cytochrome oxidase...	339	9e-91
dbj|AB033687.1|AB033687 Crassostrea gigas mitochondrial co1...	230	6e-58
gb|AF152566.1|AF152566 Crassostrea virginica cytochrome oxi...	94	9e-17
gb|AF112288.1|AF112288 Ostrea aupouria cytochrome oxidase s...	86	2e-14
gb|AF112285.1|AF112285 Ostrea chilensis from Foveaux Strait...	86	2e-14
gb|AF112289.1|AF112289 Ostrea chilensis from Moturekareka I...	80	1e-12
gb|AF112287.1|AF112287 Ostrea angasi cytochrome oxidase sub...	70	1e-09
gb|AF112286.1|AF112286 Ostrea chilensis from Quempillen cyt...	64	8e-08
gb|AF049518.1|AF049518 Leptodea fragilis USA: Cahaba River,...	54	8e-05
gb|AF049508.1|AF049508 Potamilus purpuratus USA: Cahaba Riv...	54	8e-05
gb|AF049507.1|AF049507 Potamilus purpuratus USA: Cahaba Riv...	54	8e-05
gb|AF125422.1|AF125422 Lebbeus carinatus isolate Shrimp lcP...	52	3e-04
gb|AF125421.1|AF125421 Lebbeus carinatus isolate Shrimp lcP...	52	3e-04
gb|AF156502.1|AF156502 Alasmidonta marginata UMMZ 265695 cy...	50	0.001
gb|AF080672.1|AF080672 Tegula felipensis cytochrome oxidase...	50	0.001
gb|AF080671.1|AF080671 Tegula mariana cytochrome oxidase su...	50	0.001
dbj|AB026512.1|AB026512 Ecnomiosa sp. Ecn1 mitochondrial CO...	50	0.001
gb|AF156501.1|AF156501 Unio caffer UMMZ 265692 cytochrome c...	48	0.005
gb|AF156499.1|AF156499 Unio pictorum cytochrome c oxidase s...	48	0.005
gb|AF080660.1|AF080660 Tegula funebralis cytochrome oxidase...	48	0.005
gb|AF156514.1|AF156514 Ptychobranchus fasciolaris UMMZ 2657...	46	0.019
gb|AF156500.1|AF156500 Unio caffer UMMZ 265692 cytochrome c...	46	0.019
gb|AF080674.1|AF080674 Tegula fasciata cytochrome oxidase s...	46	0.019
gb|AF049519.1|AF049519 Leptodea fragilis USA: Elk River, AL...	46	0.019
gb|AF049511.1|AF049511 Potamilus alatus USA: Clinch River, ...	46	0.019
gb|AF049510.1|AF049510 Potamilus alatus USA: Elk River, AL,...	46	0.019
gb|AF049509.1|AF049509 Potamilus purpuratus coloradoensis U...	46	0.019
gb|AF049502.1|AF049502 Potamilus inflatus USA: Black Warrio...	46	0.019
gb|AF049501.1|AF049501 Potamilus inflatus USA: Black Warrio...	46	0.019
gb|AF049500.1|AF049500 Potamilus inflatus USA: Black Warrio...	46	0.019
gb|AF049499.1|AF049499 Potamilus inflatus USA: Black Warrio...	46	0.019
dbj|AB026501.1|AB026501 Laqueus rubellus mitochondrial COI ...	46	0.019
gb|AF236070.1|AF236070 Clithon spinosus cytochrome oxidase ...	44	0.075
gb|AF242839.1|AF242839 Neocalanus plumchrus cytochrome oxid...	44	0.075
gb|AF093839.1|AF093839 Strophitus undulatus cytochrome c ox...	44	0.075
gb|AF156511.1|AF156511 Quadrula quadrula UMMZ 265699 cytoch...	44	0.075
gb|AF000063.1|AF000063 Sepia opipara cytochrome c oxidase s...	44	0.075
gb|AF080663.1|AF080663 Tegula atra cytochrome oxidase subun...	44	0.075
gb|U32210.1|CPU32210 Cucumaria pallida cytochrome oxidase 1...	44	0.075

D.2 *C. iredalei*

Sequences producing significant alignments:	Score (bits)	E Value
ref|NC_001276.1| Crassostrea gigas mitochondrion, complete ...	303	5e-80
gb|AF177226.1|AF177226 Crassostrea gigas mitochondrial DNA,...	303	5e-80
dbj|AB033687.1|AB033687 Crassostrea gigas mitochondrial co1...	299	8e-79
gb|AF152569.1|AF152569 Crassostrea ariakensis cytochrome ox...	262	2e-67
gb|AF152567.1|AF152567 Crassostrea angulata cytochrome oxid...	258	3e-66
gb|AF152565.1|AF152565 Crassostrea gigas cytochrome oxidase...	252	2e-64
gb|AF152568.1|AF152568 Crassostrea sikamea cytochrome oxida...	222	2e-55
gb|AF112288.1|AF112288 Ostrea aupouria cytochrome oxidase s...	117	7e-24
gb|AF244292.1|AF244292 Dysdera arabisenen cytochrome c oxid...	115	3e-23
gb|AF244287.1|AF244287 Dysdera bandamae cytochrome c oxidas...	101	4e-19
gb|AF244291.1|AF244291 Dysdera arabisenen cytochrome c oxid...	100	2e-18
gb|AF244320.1|AF244320 Dysdera propinqua cytochrome c oxida...	96	2e-17
gb|AF244272.1|AF244272 Dysdera enghoffi cytochrome c oxidas...	96	2e-17
gb|AF244271.1|AF244271 Dysdera enghoffi cytochrome c oxidas...	96	2e-17
gb|AF244321.1|AF244321 Dysdera propinqua cytochrome c oxida...	94	1e-16
gb|AF244285.1|AF244285 Dysdera unguimmanis cytochrome c oxi...	94	1e-16
gb|AF244274.1|AF244274 Dysdera rugichelis cytochrome c oxid...	94	1e-16
dbj|D89277.2|D89277 Turbo cornutus mitochondrial COI gene f...	92	4e-16
ref|NC_001878.1| Florometra serratissima mitochondrion, com...	90	1e-15
gb|AF049132.1|AF049132 Florometra serratissima mitochondrio...	90	1e-15
gb|AF244319.1|AF244319 Dysdera propinqua cytochrome c oxida...	88	6e-15
gb|AF170860.1|AF170860 Coenonympha tullia cytochrome c oxid...	86	2e-14
gb|AF244317.1|AF244317 Dysdera liostethus cytochrome c oxid...	86	2e-14
gb|AF037993.1|AF037993 Undaria pinnatifida cytochrome C oxi...	86	2e-14
gb|AF244283.1|AF244283 Dysdera guayota cytochrome c oxidase...	84	9e-14
gb|AF244235.1|AF244235 Harpactocrates radulifer cytochrome ...	84	9e-14
gb|U08902.1|TPU08902 Trichoniscus pusillus pusillus clone V...	84	9e-14
gb|AF244288.1|AF244288 Dysdera tilosensis cytochrome c oxid...	82	4e-13
gb|AF244282.1|AF244282 Dysdera chioensis cytochrome c oxida...	82	4e-13
gb|AF244281.1|AF244281 Dysdera chioensis cytochrome c oxida...	82	4e-13
gb|AF244290.1|AF244290 Dysdera yguanirae cytochrome c oxida...	80	1e-12
gi|9799562 Unknown	78	6e-12
gi|9799487 Unknown	78	6e-12
gb|AF170875.1|AF170875 Battus philenor cytochrome c oxidase...	78	6e-12
ref|NC_001322.1| Drosophila yakuba mitochondrion, complete ...	78	6e-12
gb|AF244277.1|AF244277 Dysdera gibbifera cytochrome c oxida...	78	6e-12
gb|AF077918.1|AF077918 Bombus sichelii cytochrome oxidase I...	78	6e-12
gb|AF103954.1|AF103954 Lasioglossum erythrurum cytochrome o...	78	6e-12
gb|AF150927.1|AF150927 Paraclemensia acerifoliella cytochro...	78	6e-12
gb|AF013736.1|AF013736 Montastrea cavernosa cytochrome oxid...	78	6e-12
emb|AJ236958.1|CRE236958 Calathus rectus mitochondrial tRNA...	78	6e-12
emb|AJ236965.1|CPI236965 Calathus pilosipennis mitochondria...	78	6e-12
emb|AJ236964.1|CPI236964 Calathus pilosipennis mitochondria...	78	6e-12
emb|X03240.1|MIDYRRN Drosophila yakuba mitochondrial DNA mo...	78	6e-12
gb|AF066990.1|AF066990 Bombus nevadensis cytochrome oxidase...	74	9e-11
gb|AF112287.1|AF112287 Ostrea angasi cytochrome oxidase sub...	74	9e-11
gb|AF137511.1|AF137511 Diastylis bispinosa cytochrome oxida...	74	9e-11
gb|AF170873.1|AF170873 Iphiclides podalirius cytochrome c o...	72	3e-10

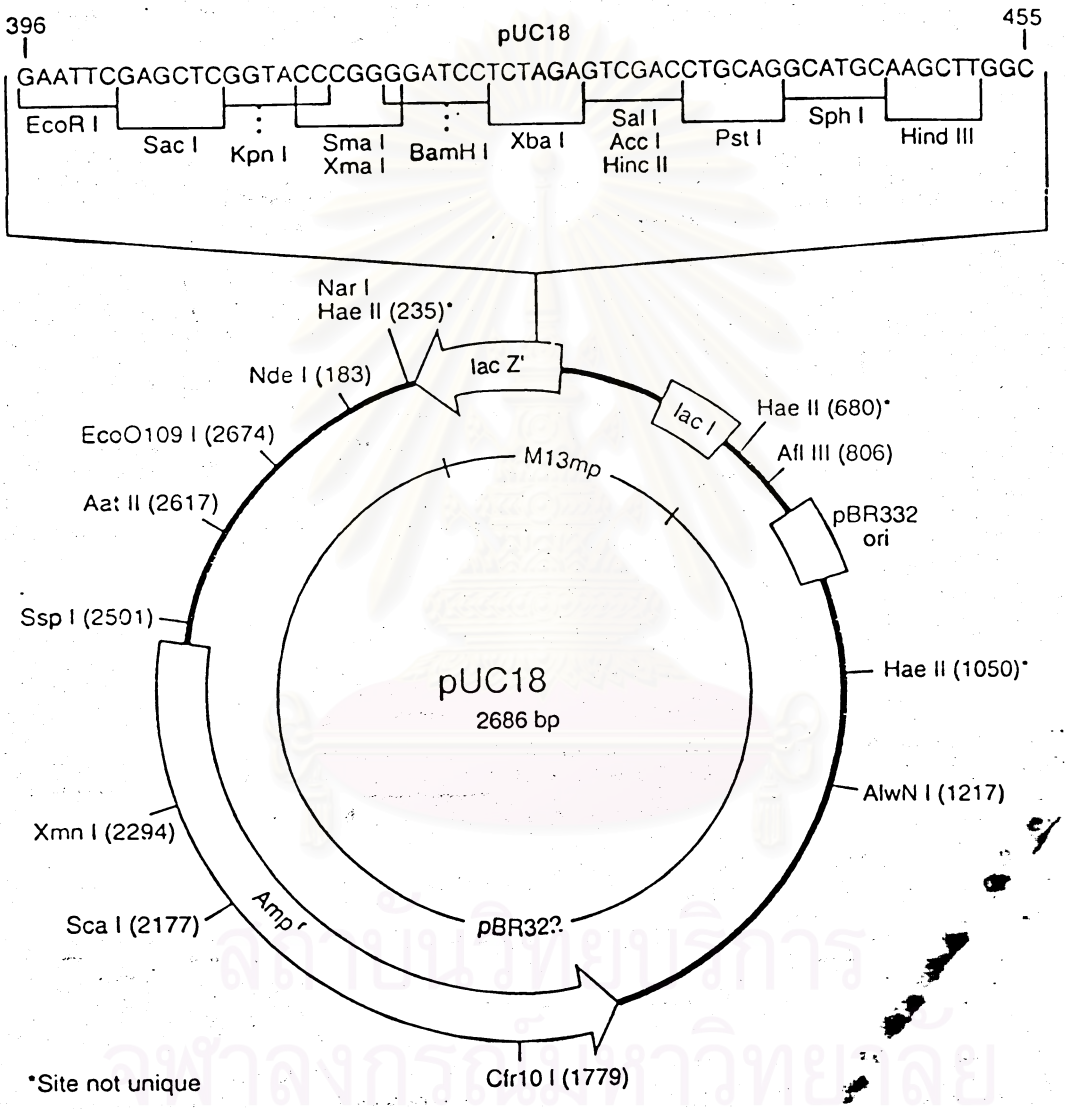
D.3 *S. cucullata*

Sequences producing significant alignments:	Score (bits)	E Value
gb|AF112287.1|AF112287 Ostrea angasi cytochrome oxidase sub...	121	4e-25
ref|NC_001276.1| Crassostrea gigas mitochondrion, complete ...	109	2e-21
gb|AF177226.1|AF177226 Crassostrea gigas mitochondrial DNA,...	109	2e-21
gb|AF112288.1|AF112288 Ostrea aupouria cytochrome oxidase s...	109	2e-21
dbj|AB033687.1|AB033687 Crassostrea gigas mitochondrial co1...	109	2e-21
gb|AF276827.1|AF276827 Phytomyza flavicornis cytochrome oxi...	101	4e-19
gb|AF250945.1|AF250945 Apis cerana cytochrome oxidase subun...	101	4e-19
gb|AF143845.1|AF143845 Bombus sp. 'western Pennsylvania iso...	101	4e-19
dbj|AB020223.1|AB020223 Ditylum brightwelli mitochondrial c...	98	6e-18
gb|AF200828.1| Drosophila melanogaster isolate Oregon R mit...	96	3e-17
gb|U37541.1|DMU37541 Drosophila melanogaster complete mitoc...	96	3e-17
gb|J01404.1|DROMTM1 Drosophila melanogaster tRNA-Trp, tRNA-...	96	3e-17
ref|NC_001709.1| Drosophila melanogaster mitochondrion, com...	96	3e-17
gb|AF129322.1|AF129322 Helebops docimus cytochrome c oxidas...	96	3e-17
gb|M57910.1|DROMTTGNC Drosophila melanogaster mitochondrial...	96	3e-17
gb|AF170859.1|AF170859 Hylephila phyleus cytochrome c oxida...	94	1e-16
gb|AF244274.1|AF244274 Dysdera rugichelis cytochrome c oxid...	94	1e-16
gb|AF044022.1|AF044022 Eurytides marcellus cytochrome oxida...	94	1e-16
gb|AF077771.1|AF077771 Macrosiphum sp. 'Burnaby, B.C. Canad...	94	1e-16
dbj|AB020228.1|AB020228 Thalassionema nitzschioides mitocho...	94	1e-16
gb|AF180083.1|AF180083 Panorpa similis cytochrome oxidase I...	92	4e-16
gb|AF170873.1|AF170873 Iphiclides podalirius cytochrome c o...	90	2e-15
gb|AF222718.1|AF222718 Chrysodidymus synuroideus mitochondr...	90	2e-15
ref|NC_001878.1| Florometra serratissima mitochondrion, com...	90	2e-15
gb|AF049132.1|AF049132 Florometra serratissima mitochondrio...	90	2e-15
gb|AF132831.1|AF132831 Dermacentor variabilis cytochrome c ...	88	6e-15
gb|AF244264.1|AF244264 Dysdera nesiotes cytochrome c oxidas...	88	6e-15
gb|AF244243.1|AF244243 Dysdera lancerotensis cytochrome c o...	88	6e-15
gb|AF077770.1|AF077770 Macrosiphum creeli cytochrome oxidas...	88	6e-15
gb|AF276866.1|AF276866 Phytomyza opacae isolate Pop cytochr...	86	2e-14
gb|AF276851.1|AF276851 Phytomyza nemopanthi cytochrome oxid...	86	2e-14
gb|AF276846.1|AF276846 Phytomyza n. sp. 4 isolate Pam cytoc...	86	2e-14
gb|AF276838.1|AF276838 Phytomyza glabricola isolate PcoE cy...	86	2e-14
gb|AF276836.1|AF276836 Phytomyza glabricola isolate Pgl cyt...	86	2e-14
gb|AF132827.1|AF132827 Boophilus microplus cytochrome c oxi...	86	2e-14
gb|AF132825.1|AF132825 Boophilus annulatus cytochrome c oxi...	86	2e-14
gb|AF244321.1|AF244321 Dysdera propinqua cytochrome c oxida...	86	2e-14
gb|AF104625.1|AF104625 Scathophaga stercoraria cytochrome o...	86	2e-14
gb|AF178679.1|AF178679 Galathealinum brachiosum mitochondri...	86	2e-14
gb|AF103980.1|AF103980 Lasioglossum calceatum cytochrome ox...	86	2e-14
gb|AF150907.1|AF150907 Coptodisca kalmiella cytochrome oxid...	86	2e-14
gb|AF044004.1|AF044004 Papilio cresphontes cytochrome oxida...	86	2e-14
gb|AF013737.1|AF013737 Montastrea annularis cytochrome oxid...	86	2e-14
gb|AF013736.1|AF013736 Montastrea cavernosa cytochrome oxid...	86	2e-14
gb|U49023.1|TMU49023 Tegeticula maculata extranea cytochrom...	86	2e-14
gb|AF244305.1|AF244305 Dysdera ratonensis cytochrome c oxid...	84	1e-13
gb|AF244304.1|AF244304 Dysdera ratonensis cytochrome c oxid...	84	1e-13
gb|AF244292.1|AF244292 Dysdera arabisenen cytochrome c oxid...	84	1e-13

Appendix E

Restriction mapping of plasmid pUC18 (E.1) and pGEM^{R}-T easy vector (E.2)

E.1 pUC18

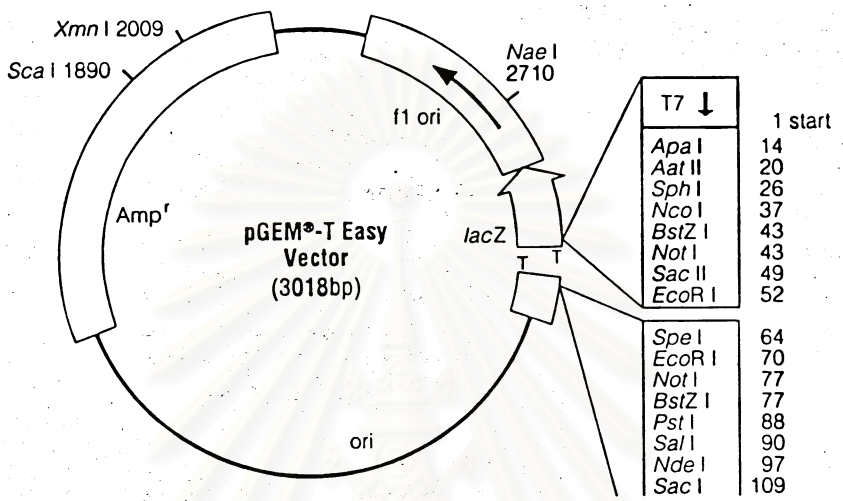


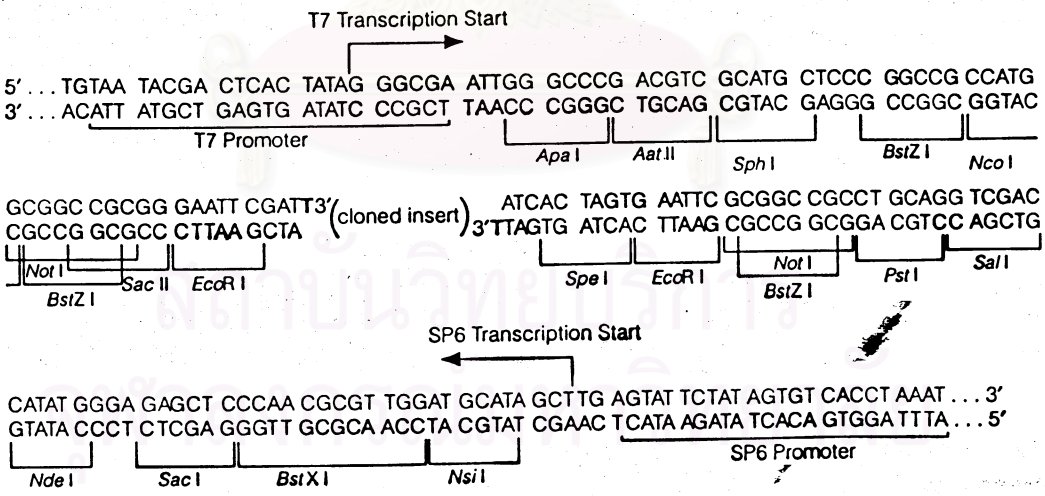
Polycloning Sites
pUC18

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser		Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC

EcoRI SacI KpnI SmaI XmaI BamHI XbaI SalI AccI HincII PstI SphI HindIII

E.2 pGEM^{R}-T easy vector





BIOGRAPHY

Miss Neerawan Khamnamtong was born on July 23, 1975 in Ubonratchathani, Thailand. She graduated with the degree of Bachelor of Science in Biochemistry from Chulalongkorn University in 1997. She has studied for a degree of Master of Science at the program of Biotechnology, Chulalongkorn University since 1998.