CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
- Automatic micropipettes P10, P20, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)
- A –20 °C Freezer
- A –30 °C Freezer
- A –80 °C Freezer (Bara Laboratory Co., Ltd.)
- Gene pulser (Bio-Rad Laboratories, USA)
- Heating block Bd 1761G-26 (Sybron Thermermolyne Co., USA)
- Incubator BM-600 (Memmert GambH, Germany)
- Microcentrifuge tube 0.5 and 1.5 ml (Bio-RAD Laboratories, USA)
- PCR Thermal cycler : model 2400 (Perkin Elmer)
- PCR Thin wall microcentrifuge tube 0.2 ml (Perkin Elmer)
- PCR Workstation Model # P-036 (Scientific Co., USA)
- Pipette tips 10, 20, 100 and 1000 µl (Bio-RAD Laboratories, USA)
- Power supply : Power PAC 300 (Bio-RAD Laboratories, USA)
- Refrigerated microcentrifuge : Kubota 1300 (Kubota, Japan)
- Spectrophotometer DU 650 (Beckman, USA)
- White/UV Transilluminator : UVP Image Store 7500 (Mitshubichi Electric Corporation, Japan)

2.1.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Agarose (FMC BioProducts, USA)

- Ammonium sulfate (Merck, Germany)
- Bacto-agar (Difco, USA)
- Bacto-yeast extract (Difco, USA)
- Bacto-tryptone (Difco, USA)
- Boric acid (Merck, Germany)
- Bromphenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- 100 mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)
  : 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)
  : 25 mM $MgCl_2$
- D-Glucose (Merck, Germany)
- Hydrochloric acid (Merck, Germany)
- Isoamyl alcohol (Merck, Germany)
- Oligonucleotide primers : 10-mer (Operon Technologies Co. Ltd., and University of British Columbia)
- Phenol, crystal (Fluka, Germany)
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)

2.1.3 Enzymes

- Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA)
- Proteinase K (Gibco BRL life technologies, Inc., USA)
- RNase A (Sigma Chemical Co., USA)

- T4 DNA ligase (Pharmacia, USA)
- *Bam*HI (Promega Corporation Medison, Wisconsin)
- *Eco*RI (New England BioLabs)

2.1.4 Bacterial strain

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

2.1.5 Cloning vectors

- pUC18
- pGEM^{R}-T easy vector

2.2 Sampling

Five oysters species including *C. belcheri* (*N*=20), *C. iredalei* (*N*=20), *S. cucullata* (*N*=26), *S. forskali* (*N*=58) and *S. mytiloides* (*N*=23) and those which are taxonomic uncertainly, *Crassostrea sp.* (*N*=9), *Saccostrea sp.* group 1 (*N*=9), *Saccostrea sp.* group 2 (*N*=9) and *Saccostrea sp.* group 3 (*N*=5), were collected during April 1998 to December 1999. Geographic locations and abbreviations of sampling sites are shown by Fig. 2.1 and Table 2.1, respectively. Oysters were maintained on ice and transported back to the laboratory at Chulalongkorn University. Twelve individuals of the Australian oystersl *S. commercialis* and the mussel, *P. viridis*, collected from Brisbane (Australia) and Chonburi (eastern Thailand) were included as an ingroup and an outgroup references, respectively. The whole specimens were transferred to a –30 °C freezer until required. Alternatively, the adductor muscle was dissected out individually and kept at –80 °C until further used for DNA extraction.

Table 2.1 Sample collection sites 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)	10
Songkhla (east of PT)	CbSK(E)	4
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
Local oyster : *S. cucullata*		
Trad (Gulf of Thailand, east)	ScTD(E)	5
Chantraburi (Gulf of Thailand, east)	ScCT(E)	8
Ranong (west of PT)	ScRN(W)	7
Phuket (west of PT)	ScPK(W)	6
Local oyster : *S. forskali*		
Chantraburi (Gulf of Thailand, east)	SfCT(E)	20
Chonburi, Angsila (Gulf of Thailand, east)	SfCBA(E)	4
Chonburi, Sichang Island (Gulf of Thailand, east)	SfCBS(E)	11
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)	6
Satun (west of PT)	SfST(W)	6
Local oyster : *S. mytiloides*		
Phuket (west of PT)	SmPK(W)	6
Ranong (west of PT)	SmRN(W)	9
Samut Sakhon (Gulf of Thailand, east)	SmSS(E)	8
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)	9
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 reference : *S. commercialis* Brisbane, Australia	Scom	12
An outgroup reference : *P. viridis* Chonburi, Thailand	Pevi	12
Total (*N*)		203

Abbreviation ; PT = peninsular Thailand

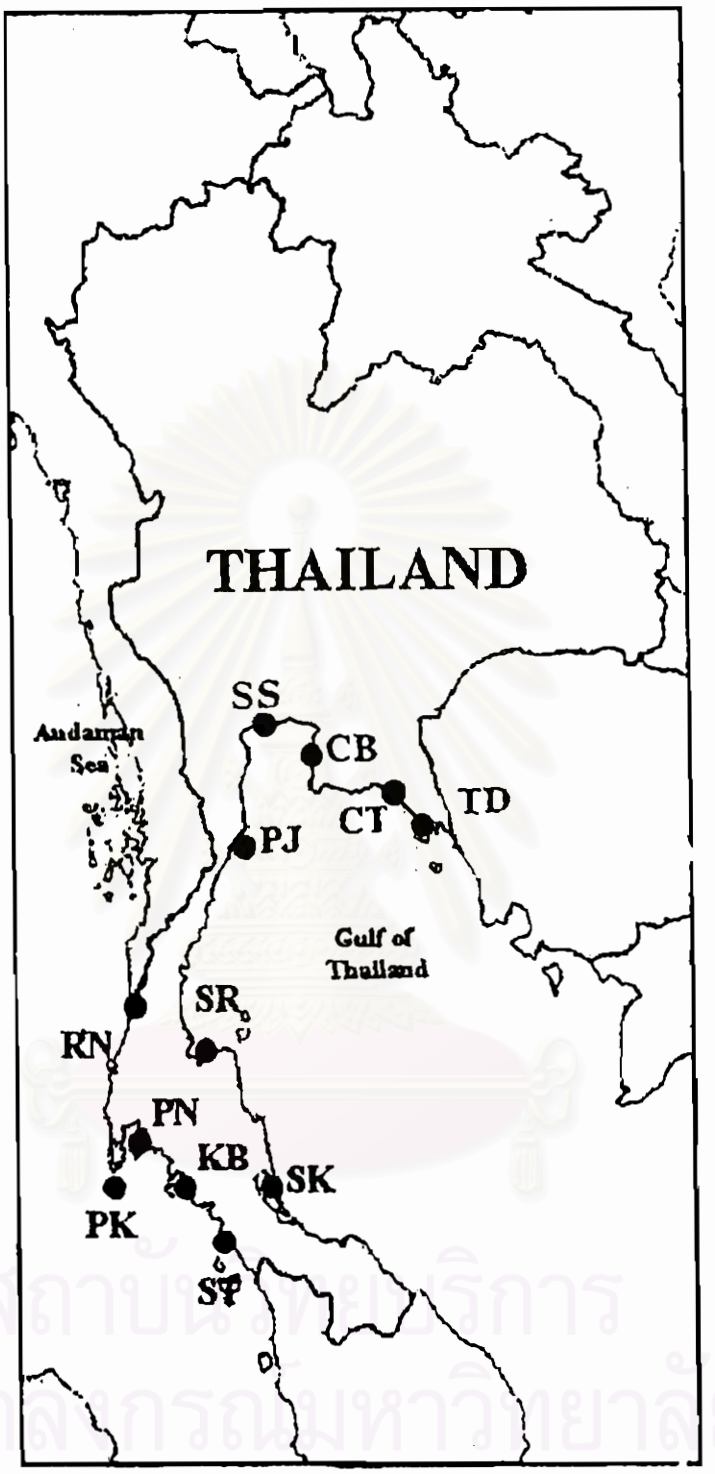

Figure 2.1 Map of Thailand indicating sample collection sites of oysters (*C. belcheri*, *C. iredalei*, *S. cucullata*, *S. mytiloides*, *S. forskali*, *Crassostrea sp.*, *Saccostrea sp.* group 1, *Saccostrea sp.* group 2 and *Saccostrea sp.* group 3) used in this study. Dots represent geographic areas from which at least one oyster species was sampling. Detailed information and abbreviations of sample sites are shown by Table 2.1.

2.3 DNA extraction

Genomic DNA was extracted from the adductor muscle of each oyster using a proteinase K /phenol-chloroform method. A piece of adductor muscle was dissected out from a specimen as soon as possible after removing from a –80 °C freezer. 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 pestle. A 40 % SDS solution was added to a final concentration of 1.0 % (w/v). The resulting mixture was then incubated at 65 °C for 1 hour following by an addition of 15 µl of a proteinase K solution (20 mg/ml) and 10 µl of a RNase A solution (10 mg/ml). The mixture was further incubated at the same temperature for at least 3 hours. DNA was extracted by a standard phenol-chloroform method. The extraction was carried out twice by the addition of an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), mixed gently for 15 minutes and centrifuged at 12000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube and further extracted once 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 °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 200 µl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The DNA solution was incubated at 37 °C for 1-2 hours for complete redissolved and kept at 4 °C until further needed.

2.4 Measuring concentrations of extracted DNA using spectrophotometry and electrophoresis

The concentration of extracted DNA samples is estimated by measuring the optical density 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 obtained by calculating a ratio of OD_{260} /OD_{280}. The ratio that much lower than 1.8 indicated contamination of residual proteins or organic solvents in the DNA solution (Kirby, 1992).

Electrophoresis is a standard method used for rough estimation of DNA on the basis of its 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 1x TBE buffer (89 mM Tris-HCl, 8.91 mM boric acid and 2.5 mM EDTA, pH 8.0) at 100 V. The mini-gel was prepared by adding 0.40 g of agarose to 50 ml of 1x TBE buffer. Agarose was solubilized by heating in a microwave oven and allowed to cool to 50-60 °C before poured into a gel mould (8.5x12.5 cm) with a preset well-forming comb. After electrophoresis, the gel was stained with ethidium bromide. DNA concentration was estimated from the intensity of the fluorescent band by comparing with that of λ - *Hin*dIII.

2.5 RAPD Analysis

Amplification conditions

One hundred and three arbitrary primers purchased from Operon Technologies Limited and The Biotechnology Laboratory, University of British Columbia (Canada) were screened. The amplification reactions were performed in a 25 µl reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of a primer, 50 ng of genomic DNA and 1.0 unit of Ampli *Taq* DNA polymerase. The reaction mixture was performed in a DNA Thermal Cyclic (Perkin Elmer Model 2400) programmed for 45 cycles consisting of a 92 °C denaturation for 10 seconds, a 36 °C annealing for 30 seconds and a 72 °C extension for 90 seconds. The final extension was carried out at 72 °C for 5 minutes (Okamura et al., 1993).

2.6 Agarose gel electrophoresis

RAPD products were analyzed by electrophoresed through 1.5% agarose gels. An appropriate amount of agarose was weighed out and mixed with calculated 1X TBE. The solution was boiled in a microwave oven to complete solubilization, and allowed to 60 °C before poured into the chamber set with an inserted comb. When the gel had solidified, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of 1x TBE buffer covering the gel for approximately 0.5 cm.

Ten microlitres of PCR products were mixed with 2 μl of the loading dye (0.25% bromophenol blue and 25% Ficoll in water) and loaded into the well. The 100 bp and 200 bp DNA ladders were used as standard DNA markers. Electrophoresis was operated at 80 volts until bromophenol blue moved to approximately 0.5 cm from the bottom of the gel. The electrophoresed gel was stained with a solution containing 2.5 μg/ml ethidium bromide for 5 minutes and destained in an appropriate amount of water with gently shaking for 20 minutes to remove unbound ethidium bromide from agarose gels. The DNA fragments were visualized as fluorescent bands under a UV transilluminator and photographed through a red filter using Kodak Tri-X-Pan 400 film. The exposure time was usually about 10-15 seconds.

2.7 Statistical analysis

Each RAPD fragment is treated as an independent character. Sizes of each RAPD band were estimated by comparing with a 100 bp ladder and recorded in a matrix to represent an absence (0) or presence (1) of a particular band. The similarity index between individuals was calculated by

$$S_{xy}=2n_{xy}/n_x+n_y$$

where n_x and n_y represent the number of RAPD bands in individuals x and y, and n_{xy} represents the number of shared bands between individuals (Nei and Li, 1979).

Within samples similarity ($\bar{S}$) is calculated as the average of $\bar{S}_{xy}$ across all possible comparisons between individuals within a geographic sample. Between sample similarity is also calculated between random pairs of individuals across samples i and j using the same equation. Genetic distances between paired samples ($\bar{D}_{ij}$) were converted from the indices of similarity between samples using the equation; $\bar{D}_{ij}$= 1- $\bar{S}_{ij}$ (Lynch, 1990).

Phylogenetic relationships between investigated samples of Thai oysters were constructed based on a neighbor-joining method (Saitou and Nei, 1987) using NEIGHBOR implemented in Phylip (Felsenstein, 1993).

2.8 Preparation of RAPD products for cloning

2.8.1. Amplification of species-specific fragments using the RAPD primer containing a *Bam*HI restriction site

A *Bam*HI adaptor (5′ CGGGATCCCG 3′) was added to the 5′ end of the primer OPB08 previously generated species-specific markers in *C. belcheri*. The resulting 20 base oligonucleotide primer was synthesized by Bioservice Unit (BSU), National Center Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA). RAPD-PCR was carried out in the presence of *C. belcheri* template using conditions described in 2.5.

2.8.2 DNA fragment elution

After electrophoresis, the 835 bp and 600 bp DNA fragments were excised from a 1.5% agarose gel using a scalpel and placed in a preweighed microcentrifuge tube. Three volumes of the buffer QG (supplied by the manufacturer) were added. The mixture was incubated at 50 °C for 10 minutes or until the gel slice has completely dissolved. The gel mixture was vortexed every 2 to 3 minutes during the incubation period. The mixture should be in yellow after the gel is completely dissolved. The mixture was transferred into a QIAquick column inserted in a 2 ml

collection tube and centrifuged at 12000 rpm for 90 seconds. The flow-through solution was discarded. An another 0.5 ml of buffer QG was added to the QIA quick column and recentrifuged for 90 seconds. After this step, a 0.75 ml of buffer PE (supplied by the manufacturer) was added to the QIAquick column and centrifuged. The flow through solution was discarded. The QIAquick column was centrifuged to remove a trace amount of the washing solution. The QIAquick column was placed into a sterile 1.5 ml microfuge tube. DNA was eluted by an addition of thirty microlitres of buffer EB (10mM Tris-HCl. pH 8.5) or H_2O to the center of the QIAquick membrane and let the column standing for 5 minutes, before centrifuged at 12000 rpm for 90 seconds.

2.8.3 Purification of eluted DNA

After *in vitro* amplification of DNA, *Taq* DNA polymerase remains bound to DNA molecules and therefore interfere digestion of the PCR-amplified DNA with restriction endonucleases. Digestion of PCR products with proteinase K prior to the subsequent digestion with corresponding restriction enzymes significantly increase cloning efficiency by several times (Crowe, 1991). As a result, a proteinase K solution was added to eluted DNA to 50 µg/ml final concentration in the presence of 0.5% SDS. The mixture was incubated for 60 minutes at 65 °C. After cooling to room temperature, the mixture was extracted once with phenol-chloroform and once with chloroform. DNA was recovered by ethanol precipitation.

2.8.4 Digestion of PCR products with *Bam*HI

Five microlitres of the 835 bp and 600 bp fragment were separately digested with 20 units of *Bam*HI in the 200 µl reaction volume using the conditions recommended by the manufacturer. The reaction was incubated at 37 °C overnight. The restricted products were purified by a phenol-chloroform extraction described previously.

2.9 Ligation of PCR products to plasmids

Each of two reamplified DNA fragments (835 bp and 600 bp) was ligated to dephosphorylated pUC18 in a 20 µl reaction volume containing 50 ng of pUC18/ *Bam*HI/ BAP (Pharmacia), 100 ng of DNA fragment, 1x T4 DNA ligase buffer (10 mM Tris-acetate, pH 7.5), 10 mM magnesium acetate and 50 mM potassium acetate), 1 mM of ATP and 3 weiss units of T4 DNA ligase (Pharmacia). The reaction mixture was incubated at 12 °C for 4 to 5 hours.

Taq polymerase have a terminal transferase activity which results in the non-templated addition of a single nucleotide 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 650 bp fragment generated from OPB01 was excised, recovered from the gel, and digested with proteinase K as mentioned earlier. The resulting insert was directly ligated to pGEM^{R}-T easy vector in a 10 µl ligation reaction constituting of 5 µl of 2x Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol; MW 8000), 3 weiss units of T4 DNA ligase, 50 ng of pGEM^{R}-T easy vector, 100 ng of DNA insert. The reaction mixture was incubated overnight at 4 °C before electrotransformed to *E.coli* XL1-Blue.

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

2.10 Preparation of competent cells

A single colony of *E. coli* XL-1 BLUE was innoculated in 10 ml of LB-broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37 °C overnight. The starting culture was inoculated into 1 liter of L-broth and continued culture at 37 °C with vigorous shaking to the OD_{600} of 0.5 to 0.8. The cells

were chilled briefly on ice for 15 to 30 minutes, and harvested by centrifugation in a prechilled rotor at 4000 g for 15 minutes at 4 °C. The pellet were resuspended in 1 liter of cold water and centrifuged as above. After resuspended in 0.5 liter of cold water, the pellet was centrifuged and resuspended in 20 ml of 10% glycerol. The cells were recentrifuged, and finally resuspended in 2 to 3 ml of 10% glycerol. This concentrated cell suspension was devided to 45 µl aliquots. These cells could be used immediately or stored at –70 °C for later used.

2.11 Electrotransformation

The competent cells were thawed on ice for 5 minutes. One or two microlitres of the ligation mixture was added and gently mixed by pipetting. The mixture was left on ice for approximately 1 minute. The mixture was eletroporated in a prechilled 0.2 cm cuvette using a Gene pulser (Bio-Rad) with the setting parameters of 25 µ F200Ω and 2.5 KV. After electroporation, the mixture were immediately removed from the cuvette and added to a new tube containing 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37 °C for 1 to 2 hours. Approximately 10-30 µl of this were spreaded on a selective LB agar plates containing 50 µg/ml of ampicillin, 25 µg/ml of IPTG and 20 µg/ml of X-Gal and further incubated at 37 °C overnight (Sambrook et al., 1989). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.12 Isolation of recombinant plasmid DNA

Plasmid DNA was isolated using a modification of the alkaline lysis DNA method (Li et al., 1997). A white colony was inoculated into a sterile tube containing LB broth supplemented with 50 µg/ml of ampicillin and incubated with shaking at 37 °C overnight. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged at 10,000 rpm for 30 seconds. The supernatant was carefully decanted. One hundred microlitres of solution 1 (50 mM glucose, 10 mM EDTA, pH

8.0, 25 mM Tris-HCl, pH 8.0) was added to the cell pellet and vortexed. Two hundred microlitres of solution 2 (0.2 N NaOH and 1% SDS) was added and gently mixed by inversion of the tube. One hundred and fifty microlitres of solution 3 (3 M sodium acetate, pH 4.8) was added and mix by trapping of the tube. The tube was centrifuged at 10,000 rpm for 30 seconds to pellet cell debris. The supernatant was transferred into a new microcentrifuge tube. An equal volume of cold absolute ethanol was added and mixed by inversion following by centrifugation at 12,000 rpm for 10 minutes. The supernatant was removed by pipetting. The pellet was dried *in vacuo* for 5 minutes. The pellet was resuspended in 50 µl of TE buffer. DNA solution was incubated at 65 °C for 10 minutes to inactivate any residual DNases. The reaction mixture was centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred to a new tube. RNase A was added to a final concentration of 200 µg/ml to digest contaminating RNA. The reaction volume was made up to 50 µl by TE buffer before incubated at 37 °C for 30 minutes. Plasmid DNA was stored at –20 °C.

2.13 Detection of recombinant plasmids

The recombinant DNA cloned from a *Bam*HI adapter method (835 bp and 600 bp from OPB08) was digested with *Bam*HI in a 20 µl reaction mixture at 37 °C overnight.

The existence of the insert for a 650 bp fragment cloned by a T-A cloning method was examined by digestion of recombinant DNA with *Eco*RI. The reaction was carried out in a 20 µl standard mixture at 37 °C overnight.

At the end of digestion period, the resulting product was electrophoretically analyzed by 1% agarose gel. The size of DNA insert is compare with that of a 100 bp DNA ladder.

2.14 DNA sequencing

Three recombinant clones (pPACB1, pPACB2 and pPACB3) exhibiting 650 bp, 835 bp and 600 bp inserts were sequenced for both directions using an automatic sequencer (ABI-PRISM, Model 377) at the Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus.

2.15 Primer design, sensitivity and species-specific tests

Forward and reverse primers of each insert were designed using the Oligo 4.0 program. PCR conditions were optimized and tested for the sensitivity of each pair of primers against varying concentrations of *C. belcheri* template (16.25 pg to 25 ng). Amplification reactions were performed in a 25 µl reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.4 µM of each primer and 1.0 unit of Ampli *Taq* DNA polymerase. The reaction was carried out for 25 cycles composing of a 94 °C denaturation step for 45 seconds, a 62 °C annealing step for 30 seconds and a 72 °C extension step for 45 seconds. The final extension was carried out at 72 °C for 5 minutes. Ten microliters of the amplification products are electrophoresed through 1.4% agarose gels and visualized by ethidium bromide staining.

For species-specific examination, all individuals of local oysters, *S. commercialis* and *P. viridis* were analyzed by primer pPACB1-F and pPACB1-R using the same PCR conditions as those used for the sensitivity test.