Chapter II

Materials and Methods

2.1 Equipment

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
-Automatic micropipette P20, P100, P200 and P1000 (Gilson Medical Electronic S.A., France)
-Autoradiography cassette (Research Products International corp., USA)
- A -20 °C Freezer
- A -80 ° C Freezer
- Gel dryer Model 583 (Bio-RAD Laboratories, USA)
- Heating block BD 1761G-26 (Sybron Thermermolyne Co., USA)
- Hyperfilm MP (Amersham International, England)
- Incubator BM-600 (Memmert GambH, Germany)
- Microcentrifuge tube 0.5, 1.5 ml (Bio-RAD Laboratories, USA)
- PCR Thermal cycler: Omnigene-E (Hybaid Limited, England)
- PCR Workstation Model#P-036 (Scientific Co., USA)
- Pipette tips 100, 1000 µl (Bio-RAD Laboratories, USA)
- Power supply: Power PAC 3000 (Bio-RAD Laboratories, USA)
- Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)
- Spectrophotometer DU 650 (Beckman, USA)

- Vertical sequencing gel electrophoresis apparatus (Hoefer, USA)
- Power supplies: Power Pac300 Power Supply (Bio-RAD Laboratories, USA)
- UV transilluminator (UVP Inc.)
-Whatman®3 MM Chromatography paper (Whatman International Ltd., England)

2.2 Chemicals and Reagents

- Absolute ethanol (Merck, Germany)
- Acrylamide (Merck, Germany)
- Ammonium persulfate (Promega, USA)
- Boric acid (Merck, Germany)
- Bromophenolblue (Merck, Germany)
- Chloroform (Merck, Germany)
- Developer (Eastman Kodak Company, USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
-100 mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, USA)
- Fixer (Eastman Kodak Co., USA)
- Formamide (Gibco BRL Technologies, Co., USA)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)
  : 10X PCR buffer (100 mM Tris-HCL pH 8.3, 500 mM KCl)
  : 25 mM $MgCl_2$

- Hydrochloric acid (Merck, Germany)
- Isoamyl alcohol (Merck, Germany)
- N,N-methylene-bis-acrylamide (Amersham, England)
- Oligonucleotide primers (Bio synthesis)
- Phenol crystal (Fluka, Germany)
- Proteinase K (Gibco BRL Technologies, Inc., USA)
- RNase A (Sigma Chemical Co., USA)
- T7 Sequencing kit (Pharmacia Biotech, USA)
  : Mix-Short for each dATP, dCTP, dGTP, dTTP
  : Enzyme dilution buffer (20 mM Tris-HCl pH 7.5, 5 mM DTT, 100 μM BSA/ml, 5% glycerol)
  : Universal Primers
  :Annealing buffer (1M Tris-HCl, 100 mM $MgCl_2$, 160 mM DTT)
  :Labeling Mix-dATP (1.375 μM each dCTP, dGTP and dTTP, 333.5 mM NaCl)
  :Control DNA template
  :T7 DNA Polymerase
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- N,N.N',N'-tetramethylethylenediamine (Gibco BRL Technologies, Inc., USA)

- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Urea (Fluka, Switzerland)
- Xylene cyanol (Sigma, USA)
- [γ-^{32}P]ATP specific activity 3000 Ci/mmol (Amersham International, England)
- [α-^{32}P]dATP specific activity 800 Ci/mmol (Amersham International, England)
- Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, USA)
- T4 Polynucleotide kinase (Pharmacia Biotech, USA)
- Formaldehyde solution, 37 to 41% HCOOH (BDH Chemical Ltd., England)
- Silver nitrate (BDH Chemical Ltd., England)

2.3 Prawn samples

The black tiger prawn (*P. monodon*) broodstock was wild-caught alive from Trad (N=51) in February 1998. Pleopods were excised from freshly killed prawn individuals and immediately placed on dry ice. Specimens were stored at -80°C until required. Genomic DNA of these specimens was prepared by Supungul (1998) and used to construct genomic libraries and to determine polymorphism of microsatellite loci developed in this study.

For genetic linkage mapping analysis, the reference pedigreed family was produced at the Australian Institute of Marine Science (AIMS), Townsville Facility, between 1995-1997. The first generation was wild broodstock (G0) captured from

inshore waters off the North Queensland coast in 1995. The offspring were raised over 17 months to produce the first generation of domesticated broodstock (G1). The (G1) were spawned in March 1997 and their offspring, G2 were raised in nursery tanks to 3 months. Due to a disease outbreak in the facilities, all animals were harvested at that point. Offspring from several families were snap-frozen in liquid nitrogen for future DNA preparation. Pleopods were also preserved from the parental generations (Wilson et al., 2002).

2.4 DNA extraction

2.4.1 The black tiger prawn broodstock (Supungul, 1998)

Genomic DNA was extracted from a pleopod of each prawn using a phenol-chloroform method modified from that of Davis et al. (1986). As soon as possible after removing from a -80°C freezer, a pleopod was transferred into a 1.5 ml microcentrifuge tube containing 400 µl of pre-chilled 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 pre-chilled glass homogenizer. A 40% SDS solution was added to a final concentration 1.0% (W/V). The resulting mixture was then incubated at 65°C for 1 hour following by an addition of 10 µl of proteinase-K solution (30 mg/ml) and 5 µl of a RNase solution (10 mg/ml). The mixture was further incubated at the same temperature for 3 hour. To remove proteins, 91 µl of 5 M potassium acetate, pH 6.5 was added, thoroughly mixed and incubated at 4°C for 10 min prior to centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was decanted to a sterile microcentrifuge. An equal volume of buffer-equilibrated phenol-chloroform-isoamyl

alcohol (25:24:1 v/v) was added and gently mixed. The mixture was then centrifuged at 12,000 rpm for 10 min. The upper aqueous phase was carefully transferred to a new microcentrifuge tube. One-tenth volume of 3 M sodium acetate, pH 5.5 was added. DNA was precipitated by an addition of 2 volume of ice-cold absolute ethanol and kept at -20°C overnight. The precipitated DNA pellet was recovered using a cut tip and briefly wash twice with 70% ethanol. The pellet was air-dried and resuspended in 300 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 use.

2.4.2 The reference family (Wilson et al., 2002)

DNA was prepared from parents and 42 offspring at the Australian Institute of Marine Science (AIMS) as follows. Frozen pleopod or tail muscle samples were weighed and placed in a glass homogenizer with 1 ml homogenisation buffer (100 mM Tris-HCl pH 9.0, 100 mM EDTA, 1% SDS, 100 mM NaCl) per 100 mg tissue. After grinding on ice, the homogenate was placed at 70°C for 60 min prior to addition of 250 µl of 5 M potassium acetate, pH 6.5 per 1 ml grinding buffer used, mixed thoroughly, incubated on ice for 30 min to precipitate proteins and centrifuged at 25,000 g for 15 min at 4°C. The supernatant was decanted. A 0.8 volume of isopropanol was added. The mixture was left at room temperature for 15 min to precipitate DNA. Genomic DNA was either spooled out or precipitated by centrifugation at 25,000 g for 15 min at 20°C. The precipitate was washed with 70% ethanol, air dried and resuspended overnight at room temperature in 1 ml of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 µg/ml RNase. The genomic DNA was then

further purified on Qiagen Genomic-tips according to manufacturer's instructions (Qiagen) and resuspended in 10 mM Tris pH 7.5, 1 mM EDTA. The reference DNA samples were sent to the laboratory at the Department of Biochemistry, Faculty of Science, Chulalongkorn University in the dried form. The reference DNA samples were redissolved with distilled water and kept at 4°C until further used.

2.5 Agarose gel electrophoresis (Sambrook, Fritsch and Maniatis, 1989)

Agarose was mixed with 1X Tris-Borate-EDTA (TBE) buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) to make the final gel concentration of 0.7% (W/V) and heating until complete solubilization. The solution was allowed to cool to approximately 5°C and poured into a chamber set with comb. After the gel hardened, the comb was carefully withdrawn and sufficient 1X TBE buffer was added to cover the gel for approximately 0.5 cm. One microlitre of extracted genomic DNA was mixed with 1/4 volume of the gel-loading dye (0.25% bromphenol blue, 0.25 xylene cyanol FF and 15% ficoll) and loaded into the well. Lambda *Hin*d III fragments were used as a standard DNA marker. The elecrtophoresis was carried out in 1X TBE buffer from cathode to anode at 100 volts until the bromphenol blue dye marker migrated about 3/4 of the gel length. After finishing, the gel was stained in 2.5 μg/ml ethidium bromide (EtBr) solution for 5 min and destained (to remove excessive EtBr) by submerged in an excessive amount of distilled water for 15 min. The nucleic acid bands were visualized as fluorescent bands under UV transilluminator (UVP Inc.) and photographed through a red filter using Kodak Tri-X-Pan 400 film. The exposure time was usually about 10-15 sec.

2.6 Spectrophotometric measuring of DNA concentration

DNA concentration is estimated by spectrophotometry at the OD_{260}. An absorbance or optical density (O.D.) of 1.0 corresponds to 50 μg/ml of double-stranded DNA. DNA concentration of each specimen is estimated in μg/ml by OD_{260} x dilution factor x 50. An estimation of the purity of a sample can be obtained by calculating the ratio of the O.D. at 260 and 280 nm. For a pure preparation of DNA, $OD_{260/280}$ should be7 1.8. Ratios significantly less than this value indicated contamination of sample with protein or phenol (Kirby, 1992).

2.7 Genomic library construction and microsatellite isolation

Before a specific microsatellite locus can be examined, sequence information for the flanking DNA is allowing designation of specific primers. In this study, three different protocols were given for the isolation of microsatellites from genomic DNA of *P. monodon*, including a conventional genomic library and 2 different enriched library establishing protocols. Outlines of library construction and microsatellite isolation of each protocol are illustrated in Figure 2.1.

2.7.1 Construction of conventional genomic library

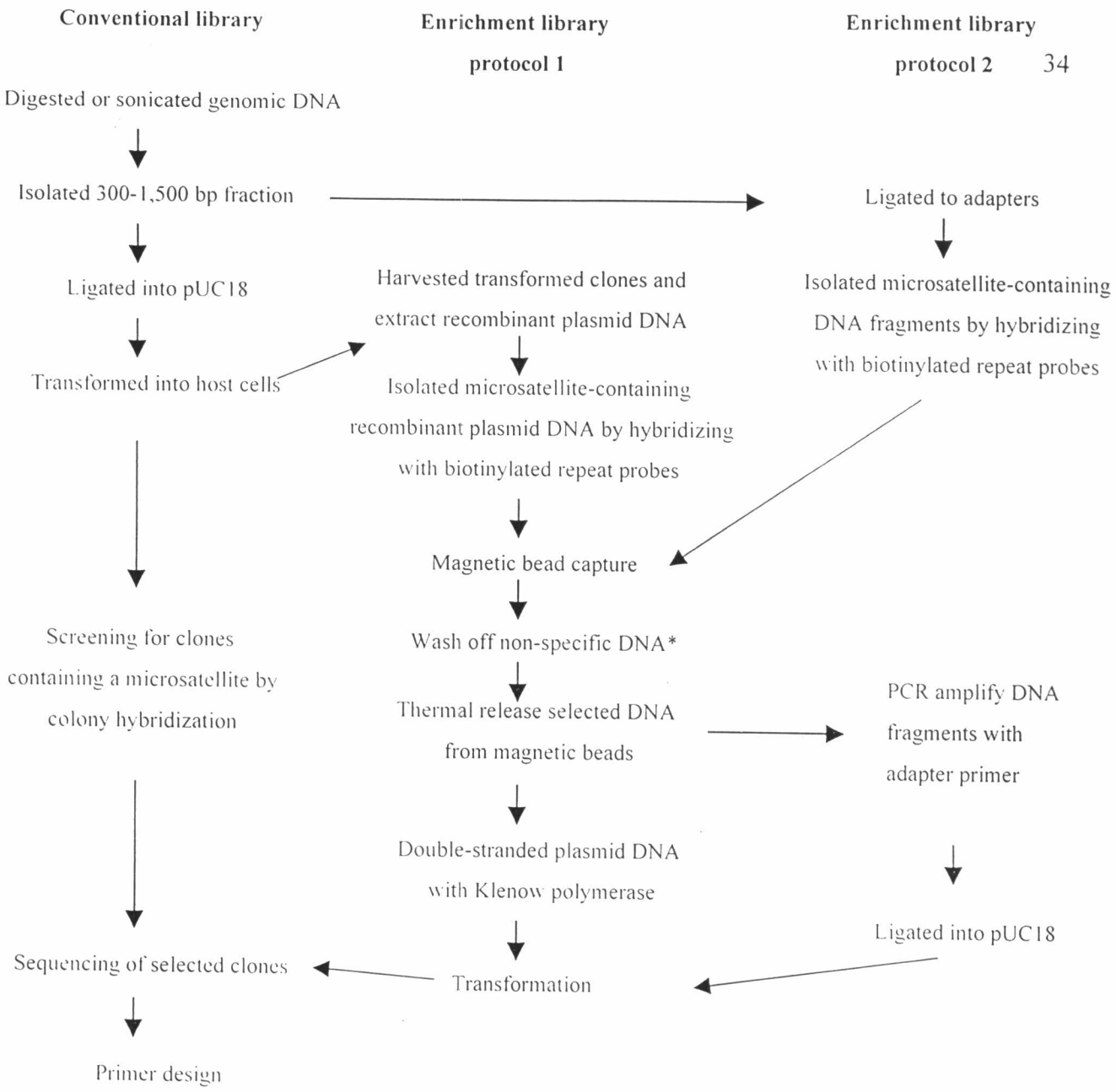
Conventional genomic libraries of *P. monodon* were constructed from 3 different types of DNA fragments. Libraries A and C were constructed from sonicated genomic DNA while libraries B and D were constructed from Dnase I- and *Hinc* II/*Alu* I- digested fragments, respectively. The recognition sequences and cleavage sites of *Hinc* II and *Alu* I were $\overset{5'\ldots GTPy\downarrow PuAC\ldots 3'}{3'\ldots CAPu\uparrow PyTG\ldots 5'}$ and $\overset{5'\ldots AG\downarrow CT\ldots 3'}{3'\ldots TC\uparrow GA\ldots 5'}$, respectively.

2.7.1.1 Preparation of P. monodon DNA

Five microgram of high molecular weight *P. monodon* DNA were fragmented with each of the following methods, sonication, DNase I and *Hin*c II/*Alu* I restriction digestions.

Sonication of genomic DNA (Moore et al., 1999)

High molecular weight genomic DNA was sonicated with a high Intensity Ultrasonic Processor (Sonic and Materials) to provide fragments of approximately 500-1,900 bp. This was achieved by sonication of 1 ml (5 μg) of DNA for 95 sec with a sonicator set at power 3, output 50 % for 10 sec. After sonication, DNA fragments were precipitated by adding 0.1 volume of 3 M sodium acetate, pH 4.8 and 2 volume of cold (-20^{o}C) absolute ethanol. The tube was mixed by inversion and left at -80^{o}C for 30 min, and centrifuged at 12,000 rpm for 15 min. The supernatant was carefully decanted and 500 μl of cold (-20^{o}C) 70% ethanol were added to wash the pellet. The tube was centrifuged at 12,000 rpm for 10 min and the supernatant was decanted. The residual alcohol was removed by placing the tube in a UniVapo Speed-Vac centrifuge (Uniequip) for 5 min. The DNA was resolubilized by adding 100 μl of TE buffer (100 mM Tris-HCl, 40 mM EDTA, pH 8.0), gently mixing. The sonicated fragments were blunt ended in a 15 μl of reaction mixture containing 500 ng of sonicated fragments, 10 U of T_4 polynucleotide kinase (New England Biolabs), 1 U of Klenow polymerase (New England Biolabs), lX EcoPol Buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol : New England Biolabs), 200 μM of dNTP, 600 μM of ATP and an appropriate amount of ddH_2O. This reaction mixture was incubated at 37^{o}C for



* Selected DNA in the second and third wash solutions were recovered by rehybridizing to biotinlylated probes, recapturing to magnetic beads and the selected DNA was eluted from magnetic beads. These extra steps decrease the salt in wash solutions from selected DNA.

Figure 2.1 Schematic representation of three different protocols for library construction and microsatellite isolation in *P. monodon*. An enrichment protocol 1 is the enrichment of microsatellite based on selection of clones from a genomic library. An enrichment protocol 2 is the enrichment of microsatellite based on selection of DNA fragments from genomic DNA.

30 min, then the reaction was stopped by adding 1 µl of 0.5 M EDTA, pH 8.0. The sonicated fragments were purified by using a QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions.

Enzymatic digestion of genomic DNA

a) DNase I

Five micrograms of high molecular weight *P. monodon* DNA were digested with 0.6 U of DNase I (Phamacia). The reaction mixture contained 100 mM acetate buffer, pH 5.0, 5 mM $MgSO_4$, 5 µg of *P. monodon* DNA, 0.6 U of DNase I and ddH_2O to a volume of 50 µl. The reaction was incubated at room temperature for 5 min and at 70°C for 30 min to stop the reaction.

b) Hinc II and Alu I

Five micrograms of high molecular weight *P. monodon* DNA were incubated with 50 U of *Hinc* II and *Alu* I (Phamacia), 1X restriction enzyme buffer M (1.0 mM Tris-HCl, pH 7.5, 1.0 mM $MgCl_2$, 0.1 mM DTT and 5.0 mM NaCl: Phamacia) and ddH_2O to a total volume of 100 µl. The reaction was incubated at 37°C overnight. The reaction was stopped by adding 25 µl of loading dye (0.1% bromphenol blue, 40 % ficoll 400 and 0.5 % SDS).

Recovery of desired size range DNA fragments (300 - 1,500 bp)

Digested DNA was size selected by running on low melting point agarose and recovered using a phenol freeze fracture procedure (Qian and Wilkinson, 1991). The

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digestion reaction was a subjected to electrophoresis with a size standard ladder (100 bp marker: New England Biolabs) in a 1.5 % low melting point agarose (Seakem) at 70 volt for 2.5 hours. After electrophoresis was completed, a lane containing the DNA marker was chopped from the gel and stained with ethidium bromide (2.5 ng/ml) for 10-15 min. The gel was placed on a UV transilluminator (UVP Inc.) and the distance of the DNA fragment size 300-1,500 bp in length was measured. Then the gel containing fragmented genomic DNA was excised at the same distance, chopped into small pieces and transferred to 1.5 ml microcentrifuge tubes and freezed at - 80°C to solidify.

Two volumes of phenol were added and vortexed until the frozen gel was completely dissolved. A drop of chloroform was added, mixed vigorously and centrifuged at 12,000 rpm, 4°C for 20 min. The upper phase was transferred to new Eppendorf tubes. An equal volume of chloroform was added and mixed. After centrifugation as above, the upper phase was transferred to new tubes. DNA was precipitated by adding 4 M NaCl to a final concentration of 0.2 M followed by 2 volume of absolute ethanol. The tubes were mixed and kept at -80°C overnight. DNA was collected by centrifugation and resuspended in TE buffer (100 mM Tris-HCl, 40 mM EDTA, pH 8.0) for a final volume of 30 µl. The DNA concentration was estimated by comparing the intensity of that of the DNA marker (λ/*Hin*d III) after ethidium bromide staining.

2.7.1.2 Ligation

The DNA fragments were ligated into *Sma* I-digested pUC 18 vector (Pharmacia). The ligation reaction was carried out overnight at 16°C in a 15 µl volume containing 150 ng of DNA fragments, 50 ng of pUC 18, 1.5 µl of ligation buffer (50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol : New England Biolabs), 1 mM of ATP, 25 µg/ml of bovine serum albumin and 3 U of T4 ligase (New England Biolabs).

2.7.1.3 Transformation

For library A, ligated DNA fragments were transformed using commercial competent cells (Ultracompetent cells XL10-Gold™: Stragtagene). For libraries B, C and D, ligated DNA fragments were electroporated (Bio Rad Gene Pulser) into *E.coli* stain XL1-Blue according to the manufacturer's instructions (Bio Rad).

Transformation of ligation products to E.coli XL10-Gold

A tube of competent cells was placed on ice and 2 µl of β-mercaptoethanol were added. The tube was swirled gently and incubated on ice for 10 min. One microlitre of the ligated solution was added into the tube and further incubated on ice for 30 min. The tube was heat-shocked in a 42°C water bath for 30 sec and placed immediately on ice for 2 min. The tube was added with preheated (42°C) NZY broth (1% NZ amine, 0.5% yeast extract, 0.1% NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$ and 20 mM glucose, pH 7.5) and incubated at 37°C for 1 h with shaking at 250 rpm. The transformation mixture was plated on LB agar plates (1% NaCl, 1% tryptone,

0.5% yeast extract, 2% agar) containing 0.1 mg/ml amplicillin, 0.1 mg/ml X-gal and 0.2 M IPTG. The transformant colonies were counted. Then, the partial genomic library was screened for the presence of microsatellite sequences.

Electrotransformation

a) Preparation of competent cells

E.coli XL1-Blue was steaked on an LB agar plate (1% NaCl, 1% tryptone, 0.5% yeast extract, 2% agar) containing 12.5 μg/ml of tetracycline. A single colony of *E.coli* XL1-Blue was inoculated and cultured as the starter in 10 ml of LB medium (1% NaCl, 1% tryptone, 0.5% yeast extract) containing 2.5 μg/ml of tetracycline overnight at 37°C in a shaking incubator at 250 rpm. One liter of L-broth (0.5% NaCl, 1% tryptone, 0.5% yeast extract) was inoculated with 10 ml of the fresh overnight culture. Cells were grown at 37°C with vigorous shaking to an A_{600} of 0.5-0.8. Cells were harvested by chilling the flask on ice for 15 to 30 min and spinning (Beckman J2-21, USA) at 4000 g for 15 min at 4°C. The supernatant was removed as much as possible. The pellets were resuspended in a total of 1 liter of ice cold water and re-centrifuged as above. The pellets were resuspended with 0.5 liter of ice cold water and centrifuged. The cell pellets were resuspended in 20 ml of ice cold 10% glycerol and centrifuged. The pellets were resuspended to a final volume of 2 ml of ice-cold 10% glycerol, dispensed to 40 μl aliquots into 0.5 ml microfuge tubes and stored at -80°C. The cells were good for at least 6 months.

b) Electrotransformation

Forty microliters of the cell suspension were gently thawed at room temperature and immediately placed on ice. Gene Pulser apparatus (Bio Rad) was set at 25 μF and 2.5 kV. Pulse controller was set at 200 Ω. Two microliters of ligation solution were added into competent cells and incubated on ice for 1 min. The mixture of cells and DNA was transferred to the bottom of a prechilled 0.2 cm electroporation cuvette. The cuvette was placed in a chilled chamber slide which was pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber. The mixture of cell and DNA was pulsed once at the above settings. The cuvette was removed and 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose, pH 7.5) was added. The cell suspension was transferred to a 15 ml tube and incubated at 37°C in a shaking incubator at 225 rpm. The cell suspension was plated on LB agar plates containing 0.1 mg/ml amplicillin, 0.1 mg/ml X-gal and 0.2 M IPTG. The transformant colonies were counted. Then, the partial genomic library was screened for the presence of microsatellite sequence.

2.7.1.4 Partial genomic library screening of microsatellite-containing clones

Microsatellite-containing clones were identified by colony hybridization. The colonies were transferred onto a piece of Whatmann paper#42 and hybridized with microsatellite probes.

Colony lifting onto Whatmann paper (Tassanakajon et al., 1998)

Three asymmetry sites at the edge of the filter were cut. A filter was put on each agar plate for 5 to 10 min. Colonies were blotted onto a piece of Whatman paper #42 (Ashless). The markers on the filters were reported precisely onto the plates and the filters were removed from the plates gently. The colonies on the plates were regrown by incubating at 37°C for 3-4 h and kept at 4°C. The filter was processed by placing on pieces of Whatmann 3 MM paper presoaked with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 7 min, followed by neutralization with neutralizing solution (1 M Tris-HCl pH 7.6, 1.5 M NaCl), 2 times for 3 min each and washed with 2X SSC (0.3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.2% SDS for 30 seconds to 1 min. After air-dried for 15-30 min, DNA was fixed on the filter by baking at 80°C for 2 hours. The filter was gentry rubbed in 2X SSC, 0.2% SDS with a gloved-hand to washed out bacterial cell debris from the filter. The filter was ready for hybridization.

Hybridization of radiolabeled probe to partial genomic libraries of P.monodon

a) Probe labeling

Synthetic oligonucleotide probes, $(GATA)_6$, $(GGAT)_6$, $(GGAA)_5$, $(CACC)_5$ and a concatamer probe $(GAA)_n$ were used in library A screening. Libraries B and C were screened with $(GAA)_8$, $(GATA)_6$, $(CAT)_8$, $(ATG)_8$, $(TCAG)_5$, and $(CATA)_6$ while library D was screened with $(GAA)_8$ and $(GATA)_6$, respectively.

The concatamer probe, $(GAA)_n$ was labeled with [α-^{32}P]dCTP by a random priming method using a Mega Prime DNA Labeling System (Amersham) while

oligoprobes were 5'-end labeling with [γ-^{32}P]ATP. The labeling procedure was followed the user's manual included in the kit. Fifty nanograms of the probe were mixed with 5 µl of primer buffer (random hexamer) in a 33 µl total volume. The DNA probe/primer mixture was denatured at 95°C for 5 min and immediately placed on ice. Ten microliters of 10X labeling buffer, 5 µl of [α-^{32}P]dCTP (2500 µCi/mmol) and 2 µl of Klenow polymerase (1 U/µl) were added to the DNA probe/primer mixture. The reaction was incubated at 37°C for 60 min. In addition, oligonucleotide probes were end labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Fifty nanograms of oligonucleotide probe were end labeled with 2.5 µl of 3000 µCi/mmol [γ-^{32}P]ATP, 10 U of T_4 polynucleotide kinase (New England Biolabs) and 1X polynucleotide kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol : New England Biolabs) in a total volume of 10 µl. The reaction was incubated at 37°C for 60 min and stopped by incubating at 70°C for 10 min. One labeling reaction was used for 50 ml of hybridization buffer.

b) Prehybridization and hybridization (Tiptawonnukul, 1996 and Moore et al.,1999)

Prehybridization is performed to prevent non-specific binding of the probe to the filters. After prehybridization, the actual hybridization is performed in the same buffer containing the probe. Prehybridization and hybridization were performed in a roller bottle oven (Hybaid). For prehybridization, 5 colony-lifted filters were incubated in 25 ml of a prehybridization solution {2X Denhardt's solution [50X Denhardt's solution=1% (w/v) ficoll type 400, 1% (W/V) polyvinylpyrolidone and 1% (W/V) BSA (Fraction V)], 5X SSPE (20X SSPE = 3 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 20 mM

EDTA), 0.5% W/V SDS and 100 μg/ml Yeast tRNA} in a glass tube at the hybridization temperature (see below) for 2 h. An appropriate amount of radiolabeled probe (25 ng/25 ml of hybridization buffer) was denatured at 95°C for 5 min and then placed immediately on ice. The denatured radiolabeled probe was added to the prehybridization tubes. Hybridization was performed overnight. For oligonucleotide-probed filters, low and high stringent hybridization conditions were applied to library screening. Library A was screened with high stringent conditions while the remaining libraries were screened with low stringent conditions. Low stringent conditions were hybridized at Tm-10°C but high stringent conditions were hybridized at Tm-5°C. The GAA concatamer was hybridized at 65°C. After hybridization, the unbound probe was washed off the filter. Filters probed with $(GAA)_n$ were washed 2 times with 2X SSC (20X SSC = 0.3 M NaCl, 0.3 M $Na_3C_6H_2O_7.2H_2O$ pH 7.0), 0.1% SDS at the hybridization temperature for 10 min each and 0.2X SSC, 0.1% SDS at the hybridization temperature for 10 min. For oligonucleotide-probed filters, low stringent conditions were used by washing 2 times with 2X SSC, 0.1% SDS at the hybridization temperature for 10 min each but high stringent condition was treated in the same way except an extra washing step in 0.2X SSC, 0.1% SDS was performed at the room temperature for 10 min. Then, the washed filters were transferred onto filter paper sheets to drain off excess liquid. Filters were wrapped and subjected to autoradiography (Kodak X-OMATAR films) at -80°C with an intensifying screen for several hours to overnight (depending on signal strength). The X-ray film was then developed.

The master plates were aligned with the autoradiograph of the colony hybridization. Individual positive clones were picked up from the master plates and

grown in a 3 ml Terrific broth (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) with 0.1 mg/ml amplicilin overnight at 37°C with shaking. The culture was subjected to plasmid DNA extraction.

2.7.2 Construction of enrichment genomic libraries

Two methods were used to enrich the possibility to discover microsatellite-containing clones. The first method was based on selection of microsatellite sequence of clones in a pUC 18 genomic library whereas the second method was based on selection of genomic DNA fragments before library construction. The procedure for both microsatellite enrichment methods are shown in Figure 2.1.

2.7.2.1 Enrichment of microsatellites based on selection of clones from genomic library

The basic protocol (Shepard and Rae, 1997) was followed with modifications. Before the enrichment step was done, the genomic library was constructed as in conventional protocol.

Partial genomic library construction

Genomic library was constructed with randomly sheared genomic DNA as the conventional library A. After the genomic library construction, the protocol was modified from the conventional method in which transformed colonies were eluted from plates and plasmid DNA was extracted.

Preparation of plasmid DNA for construction of an enriched genomic library

Transformed colonies were harvested from a plate by adding 5 ml of LB broth and incubating at 4°C with shaking for 1 h. The cell suspension was combined for plasmid DNA isolation. Plasmid DNA was isolated using a NucleoBond Plasmid Purification Kit (Clontech) as described in the user's manual. The broth was centrifuged at 600 g for 15 min at 4°C. The supernatant was discarded. The pellet of bacterial cells was resuspended in 12 ml of Buffer S1 (50 mM Tris, 10 mM EDTA, pH 8.0, 100 μg/ml RNase), followed by adding 12 ml of buffer S2 (200 mM NaOH, 1% SDS). The suspension was mixed gently by inverting the tube 6-8 times and incubated at room temperature for 2-3 min. After this, 12 ml of buffer S3 (2.8 M potassium acetate, pH 5.1) were added. The suspension was mixed gently by inverting the tube 6-8 times and incubated on ice for 5 min. The suspension was centrifuged at 10,000 g at 4°C for 45 min and followed by filtration with NucleoBond Folded Filter. A NucleoBond column was equilibrated with 5 ml of buffer N2 (100 mM Trisl, 15% ethanol, 900 mM potassium chloride, pH 6.3). The clear suspension was loaded onto the column. After the column was empty by gravity flow, the column was washed twice with 12 ml of buffer N3 (100 mM Tris, 15% ethanol, 1.15 M potassium chloride, pH 6.3) each and the plasmid DNA was eluted with 12 ml of buffer N5 (100 mM Tris, 15 % ethanol, 1 M potassium chloride, pH 8.5). The plasmid DNA was precipitated by adding 8.4 ml of isopropanol and placed at room temperature for 1 h. Then, plasmid DNA was recovered by centrifugation at 10,000 g for 30 min at 4°C. The supernatant was discarded, the pellets were washed with 70% ethanol, centrifuged at 10,000 g at 4°C for 10 min and dried in a vacuum with heat for 10 min. Dried

pellets were then redissolved in 80 µl of sterile ddH_2O. Concentration and purity of plasmid DNA were determined by UV spectrophotometer DU 650 (Beckman) and 1% agarose gel electrophoresis.

Isolation of microsatellite-containing clones using particle separation

Two micrograms of double-stranded plasmid library were mixed with 50 ng of the 5'-biotinylated $(GATA)_6$ probe in a total volume of 9 µl in a 1.5 ml microfuge tube. The plasmid DNA was denatured by adding 1 µl of 1 N NaOH and incubated at room temperature for 5 min. The denatured reaction was neutralized by the addition of 100 µl pre-warmed Tris-hybridization solution [6X SSPE (20X SSPE = 3 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 20 mM EDTA), 0.1% Tween 20, 50 mM Tris-HCl, pH 7.4] and incubated at 50°C for 1 h. During the hybridization reaction, streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal A.S.) were prepared by transferring 30 µl (300 µg) to a 1.5 ml microfuge tube and washing 3 times with an equal volume of 1X phosphate buffer saline pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), 0.1% BSA and finally resuspending in 20 µl of Tris-hybridization solution. The beads were collected with a magnetic particle concentrator (Promega). At the end of the hybridization reaction, the 20 µl of washed magnetic beads were transferred to the hybridization tube. Probe-hybridized plasmids were captured by placing on a rotating wheel for 3 h at hybridization temperature. Following this incubation, the beads were washed with 2X SSPE, 0.1% tween 20; 0.8X SSPE, 0.1% tween 20 and 0.5X SSPE, 0.1% tween 20, respectively. Each wash solution was performed 15 min each at the hybridization temperature with 80 µl of the wash solution. To collect beads, the tube was placed in a magnetic particle

concentrator and the supernatant was removed. Beads were resuspended in 50 µl distilled water and left at room temperature for next step (elution step). The second and the third wash solutions; 0.8X SSPE, 0.1% tween 20 and 0.5X SSPE, 0.1% tween 20 were retained for library constructions. Target plasmid DNA in the wash solutions were recoverd by rehybridizing to 5'-biotinylated $(GATA)_6$ probe and recapturing to magnetic beads. Each wash solution was mixed with 50 ng of 5'-biotinylated probe in a total volume of 110 µl of the Tris-hybridization solution. The reaction was incubated at the hybridization temperature for 1 h. The 10 µl of washed magnetic beads were transferred to the hybridization tube. Probe-hybridized plasmids were captured by incubation for 3 h at the hybridization temperature with beads kept in suspension by placing the tube on a rotating wheel. Beads were washed 3 times at the hybridization temperature with 6X SSPE, 0.1% tween 20 for 5 min each. Beads were collected with the magnetic particle concentrator (Promega) and resuspended in 100 µl ddH_2O.

The captured single-stranded DNA was eluted from beads in distilled water by incubating at 80°C for 5 min to thermal release captured plasmids from the oligos and streptavidin-coated magnetic beads. Beads were collected with a magnet particle concentrator and the supernatant was transferred to a fresh tube. Each supernatant was purified by passage through a QIA quick PCR Purification kit (Qiagen). Each purified supernatant was concentrated in a vacuum with heat to a final volume of 20 µl. The single-stranded DNA from each purified supernatant was double-stranded by primer extension. Twenty microlitre of single-stranded DNA was mixed with 20 ng of $(GATA)_6$ in a 1.5 ml tube. The tube was incubated at 95°C for 3 min and then left at

room temperature to cool back. The tube was added with 250 μM of dNTP, 250 μM of ATP, 1X Klenow buffer (10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothritol, 2 U of Klenow polymerase: New England Biolabs) and 10 U of T_4 polynucleotide kinase (New England Biolabs) then incubated at 37°C for 1 h. The reaction was stopped by incubating at 70°C for 5 min. Double-stranded DNA was precipitated by adding 10% 3 M sodium acetate pH 4.8, 2 μl glycogen and 2 vol absolute ethanol and placed at -80°C overnight. Double-stranded DNA was pelleted after centrifugation for 20 min, vacuum-dried, resuspended in 10 μl ddH_2O and left to dissolve for 30 min. After that, dsDNA solution was evaporated in vacuum with low heat until the solution was decreased from 10 μl to 3 μl. Three microliters of dsDNA were electroporated into *E.coli* XL1-Blue (2.7.1.3). The transformed bacteria were grown on LB plate overnight. Individual transformed colonies were picked and grown in 3 ml Terrific broth with 0.1 mg/ml amplicilin overnight at 37°C with shaking. The cultures were subjected to plasmid DNA extraction.

2.7.2.2 Enrichment of microsatellite based on selection from genomic DNA fragments (Shepard and Ray, 1997; Piertney et al., 1998)

Preparation of P. monodon DNA for construction of an enriched genomic library

Ten micrograms of high molecular weight *P. monodon* DNA were digested with 100 U of *Sau*3 AI (New England Biolabs) in a 100 μl reaction volume containing 1X Buffer (10 mM NaCl, 1 mM Bis Tris Propane-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, pH 7.9: New England Biolabs). The reaction was incubated overnight at

37°C and then stopped by adding 25 µl of loading dye (0.1% bromphenol blue, 40% ficoll 400 and 0.5% SDS). Digested DNA was sized-selected as described in section 2.7.1.1. The size-selected DNA was ligated with oligonucleotides, SAU-1(5' GCGGTACCCGGGAAGCTTGG) and SAU-2 (GATCCCAAGCTTCCCGGGTACC-GC) that contained a *Sau*3 AI restriction size. The ligation reaction was carried out at 16°C overnight in a total volume of 80 µl containing 6 µg of DNA fragments, 6 µg of oligonucleotides, 1X ligation buffer (5.0 mM Tris-HCl pH 7.5, 1.0 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.1 mM ATP, 2.5 µg/ml bovine serum albumin : New England Biolabs) and 18 U of T_4 ligase (New England Biolabs). The mixture of fragments was purified by a QIA quick PCR Purification kit (Qiagen) following the manufacturer's instructions.

Isolation of microsatellite-containing DNA fragments using particle separation

Four micrograms of purified fragments were mixed with 2 µg of 5'-biotinylated oligonucleotide in 100 µl of 6X SSPE, 0.1% tween 20, denatured at 95°C for 10 min and then placed on ice. The 5'-biotinylated oligonucleotides, $(GATA)_6$ and $(GAA)_8$ were used as captured molecules. The reaction was carried out for 3 h at 50°C for $(GATA)_6$ and at 54°C for $(GAA)_8$. Three hundred micrograms of streptavidin-coated Magnetic beads (Promega) were prepared, hybridized to biotinylated oligonucleotide-DNA fragment complex, washed, and released captured molecules were released as describe in Section 2.7.2.1. Each supernatant was purified by a QIAquick PCR Purification kit (Qiagen). The purified single-stranded DNA was eluted from the column with 40 µl of ddH_2O. The purified ssDNA was amplified with SAU-1

oligonucleotide as a primer. The reactions were carried out in a 25 µl volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.6 U *Taq* polymerase (Finnzymes), 0.2 µM SAU-1 and 10 µl of ssDNA for $(GATA)_n$ enrichment or 2 µl of ssDNA for $(GAA)_n$ enrichment . Reactions began with a 3 min 94°C denaturing step, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The reactions were terminated with a final extension step of 10 min at 72°C. PCR products were purified by a QIAquick PCR purification kit (Qiagen). The concentration of the products were estimated manually after electrophoresis through a 1.5% agarose gel. PCR fragments were digested with *Sau*3 AI in a 40 µl reaction volume containing 1X buffer (10 mM NaCl, 1 mM Bis Tris Propane-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, pH 7.9: New England Biolabs), 4 U *Sau*3 AI (New England Biolabs) and 1.4 µg of PCR fragments. The digested fragments were purified by a QIAquick PCR Purification kit (Qiagen) to remove salt, denatured enzyme and free cleaved adapter fragments

Cloning of microsatellite-enriched fragments

The enriched fragments were ligated into a pUC 18/*Bam* HI vector (Phamacia). The ligation was done overnight at 16°C in a volume of 15 µl containing 150 ng of DNA fragments, 50 ng of pUC 18 vector, 1.5 µl of 10X ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM dithiothreitol, 1 mM of ATP, 25 µg/ml of bovine serum albumin: New England Biolabs) and 3 U of T_4 ligase (New England Biolabs). The ligation reaction was electroporated (BioRad Gene Pulser) into *E.coli* strain XL1-Blue and selected on LB agar plates overnight as described in Section 2.7.1.3. Transformed colonies were picked up individually and grown in 3 ml of a Terrific broth

supplemented with 0.1 mg/ml amplicillin overnight at 37°C with shaking. The culture was subjected to plasmid DNA extraction.

2.8 Preparation of plasmid DNA for sequencing

Plasmid DNA was isolated from bacterial colonies and digested with restriction enzyme to verify the insert size. Clones with inserts ≥ 300 bp were selected for sequencing.

2.8.1 Plasmid DNA extraction

Plasmid DNA was extracted by boiling procedure (Holmes and Quigley, 1981) for rapid isolation of plasmid DNA from large numbers of colonies. The culture was transferred to a 1.5 ml microfuge tube. The tube was spun at 12,000 rpm for 30 sec to pellet the bacterial cells. The supernatant was decanted and the pellet was resuspended with 180 µl STET buffer (8% sucrose, 5% tritonX-100, 50 mM Tris-HCl, 50 mM EDTA). The tube was added with 20 µl of 10 mg/ml lysozyme in STET buffer, immediately vortexed and left at room temperature for 75 sec and then placed in the boiling water for 75 sec. The tube was spun at 12,000 rpm for 10 min and the cell debris at the bottom of the tube was removed using a toothpick. DNA was precipitated by adding an equal volume of cold isopropanal and placed at -80°C for at least 10 min. The tube was spun at 12,000 rpm for 10 min. The supernatant was discarded and the tube was placed in a speedvac to remove the residual ethanol. The DNA was resolubilized by adding 30 µl TE buffer.

2.8.2 Restriction digestion of plasmid DNA

Approximately 300 ng of extracted plasmid DNA were digested with restriction enzymes in a 10 µl reaction volume containing 1X NEB buffers 3 (10 mM NaCl, 5 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, pH 7.9: New England Biolabs), 1 U *Eco* RI (New England Biolabs), 1 U *Pst* I (New England Biolabs) and 100 µg/ml BSA at 37°C for 1 h. At the end of the incubation period, 10 µl of digested solution was mixed with 5 µl of loading dye/RNase (0.1% bromphenol blue, 40% ficoll 400, 0.5% SDS and 80 ng/µl RNase), loaded onto 1.2% agarose gel and electrophoresed at 100V. A 100 bp ladder (New England Biolabs) was included as a marker. When the electrophoresis was completed, the gel was stained with ethidium bromide and visualized with the UV-transilluminator.

2.9 DNA sequencing

Plasmid DNA was sequenced using the dideoxy nucleotide chain termination with T_7 sequencing kit (USB Corporation).

2.9.1 Sequencing reaction

Ten microlitres of plasmid DNA were mixed with 2.25 µl of 2 N NaOH and incubated for 10 min at room temperature. The denatured mixture was precipitated with 1.2 µl of 3 M sodium acetate pH 4.8, 1 µl of distilled water and 30 µl of absolute ethanol at -80°C for 15 min. The precipitated plasmid DNA was collected by centrifugation at 12,000 rpm for 15 min. The pellet was washed with 70% ethanol and dried in a vacuum centrifuge with heat for 10 min. Dried pellets were then dissolved in

11 µl distilled water and 2 µl annealing buffer. Six and a half microlitres of plasmid DNA were annealed with 1.1 µl of 5 pmole/µl the universal or reverse primer, incubated at 65°C for 5 min, transferred to 37°C for 10 min and allowed to cool at room temperature for 5 min. The annealing mixture was ready to proceed to the next step.

Labeling mixture including 0.5 µl of distilled water, 1.5 µl Labeling Mix-dATP (1.37 µM dCTP, dGTP and dTTP), 0.8 µl enzyme dilution buffer, 0.2 µl T_7 DNA polymerase and 0.4 µl [α-^{35}S]dATP (12.5 µCi/µl) was prepared. Labeling mixture was added to the annealing mixture and incubated at room temperature for 5 min. The reaction (2.4 µl) was aliquoted to each of 4 tubes containing 1.25 µl pre-warmed appropriate termination mixture (each mixture contains all four dNTPs at suitable concentrations and the appropriate ddNTP at a concentration of 14 µM). The tubes were incubated at 37°C for 5 min, and 2 µl of the stop solution (10 mM NaOH, 99% formamide, 0.1% bromphenol blue and 0.1% xylene cyanol) was added to each tube.

2.9.2 Sequencing product separation and detection

The sequencing products were heated at 95°C for 3 min and immediately placed on ice for 15 min. Three and a half microlitres of denatured mixture were loaded onto a 8% denaturing polyacrylamide gel (76 g acrylamide, 4 g N, N' methylenebisacrylamide, 7.66 M urea/ 1 litre in 1XTBE) and electrophoresed with 1X TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8.3) at 50 W for 2 h. After electrophoresis, the gel was transferred onto a piece Whatmann 3 MM paper,

dried with a gel dryer (Model 583, Biorad) for 2 h and exposed to X-ray film (Kodak X-OMATAR film) at the room temperature for 3-7 days, depending on the intensity of the signals. The X-ray film was then developed using X-ray developer and fixer (Kodak).

2.10 Design and synthesis of PCR primer pairs

PCR primers were designed from non-repetitive flanking regions of the microsatellite loci using OLIGO 4.0 (National BioScience). The criteria for primer designing were; 1) primers placed as closed to the repeat array as possible, 2) primers were 18-25 base in length with at least one G or C at the 3' end, and melting temperature was 55-70°C, Tm = 2 (A+T) + 4 (G + C), 3) base distribution of the primers were random, avoiding polypurine and polypyrimidine tracts, 4) the difference of the melting temperature of any primer pair was not exceed 5°C 5) ΔG of annealing between pairs of primers and secondary structure of each primer was not lower than -5. The primers were synthesized from commercial company (Biosynthesis, USA).

2.11 Microsatellite analysis

Primers were used for amplification of microsatellites by the polymerase chain reaction (PCR). PCR products were separated by denaturing polyacrylamide gel electrophoresis and detected by autoradiography.

2.11.1 PCR amplification of microsatellites

Microsatellite amplification was assessed using radioactive end-labeled PCR primers. Typically, the forward primer for each microsatellite locus was end-labeled

using T_4 polynucleotide kinase. The labeling reaction was carried out in a 10 µl volume containing 10 pmole of forward primer, 1X of T_4 polynucleotide kinase buffer (0.5 M Tris-HCl pH 7.6, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine: New England Biolabs) , 30 µCi of [γ-^{32}P]ATP (3,000 µCi/mmole: Amersham) and 10 U of T_4 polynucleotide kinase (New England Biolabs). The labeling reaction was incubated at 37°C for 30 min and terminated by heat-inactivation of T_4 polynucleotide kinase at 65°C for 15 min.

Amplification was performed in a Hybaid Omn-E Thermal cycle (Hybaid limited United Kingdom) in a 5 µl reaction. This consisted of approximately 15 ng of genomic DNA, 0.425 µM of the unlabeled forward primer, 0.45 µM of the reverse primer, 0.025 µM of the labeled forward primer, 1X PCR buffer (10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Tritonx-100: Finnzymes), 200 µM each of dNTPs and 0.15 U of *Taq* polymerase. A drop of mineral oil was added to the surface of the reaction mixture. The PCR cycling program began with a 3 min of a 95°C denaturing step, followed by 30 cycles of 1 min at 95°C, 2 min at the primer-specific annealing temperature (see Table 2.1) and 1 min at 72°C. The reaction was terminated with a final extension step of 10 min at 72°C. After the amplification was completed, each reaction was added with 4 µl of the stopping/loading dye (10 mM NaOH, 99% formamide, 0.1% bromphenol blue and 0.1% xylene cyanol).

Table 2.1 Annealing temperature for each microsatellite locus.

Annealing temperature (°C)	Microsatellite locus
50	CUPmo 20, 22, 24
52	CUPmo 15, 16, 17
54	CUPmo 4, 9, 12, 19
56	CUPmo 1, 2, 3, 5, 6, 7, 11
58	CUPmo 13, 14, 28
60	CUPmo 21, 23
62	CUPmo 25, 26, 29
66	CUPmo 27

2.11.2 DNA standard for estimation of microsatellite alleles

A M13 sequencing ladder was used as a DNA standard for estimation of sizes of microsatellite alleles. M 13 sequencing reaction was prepared using a T_7 DNA Sequencing kit. M13 control template was annealed with the universal primer in a 14 µl reaction mixture containing 5 µl of control template, 5 µl of distilled water, 2 µl of annealing buffer and 10 pmole of the universal primer. The annealing mixture was incubated at 65°C for 15 min and placed at room temperature for 10 min. The annealing mixture was ready to proceed to next step.

Labeling mixture including 1µl of distilled water, 3 µl of labeling Mix-dATP (1.37 µM dCTP, dGTP and dTTP), 1.7 µl of the enzyme dilution buffer, 0.3 µl T_7 DNA Polymerase and 1µl of 800 Ci/mmole [γ-^{32}P]dATP was prepared. Six microlitres

of this mixture were then added to the annealing mixture. The labeling/extension mixture was incubated at the room temperature for 5 min. Five microlitres of this mixture were aliquoted to each of 4 tubes containing appropriate termination mixtures (each mixture contains all four dNTPs at suitable concentrations and appropriate ddNTP at a concentration of 14 μM). The tubes were incubated at 37°C for 5 min, and 30 μl of the stopping/loading dye (10 mM NaOH, 99% formamide, 0.1% bromphenol blue, and 0.1% xylene cyanol) were added. A few drops of mineral oil were added to the surface of each tube.

2.11.3 PCR product separation and detection

PCR products and the M13 sequencing marker were denatured at 95°C for 15 min and immediately placed on ice. Three and a half microlitres of denatured samples were loaded onto a 6% denaturing acrylamide gel (57 g acrylamide, 3 g N, N' methylenebisacrylamide, 7.66 M urea/ 1 litre in 1X TBE) and electrophoresed with 1X TBE buffer at 50 W for 2-4 h, depending on PCR product size. The gel was transferred onto a piece of Whatmann 3 MM paper, dried and exposed to X-ray film (Kodak X-OMATAR films) at -80°C overnight. The X-ray film was developed and then allowed to dry. The microsatellite alleles were size-estimated by comparing with the M13 mp 18 sequencing ladder.

2.12 Polymorphism analysis of microsatellite loci

Microsatellite polymorphism was characterized by genotyping about 40-50 wild-caught *P. monodon* broodstock from the Trad located in the Gulf of Thailand. A genotype of each *P. monodon* individual was scored from an electrophoretically

observed patterns of each locus. Allele sizes were scored in terms of numbers of base pairs, which were determined by comparison with the M13 sequencing ladder. When shadow bands were present, the largest dark band was considered to be the actual allele (Stoner, Quattre and Weissman, 1997).

2.12.1 Allele number and frequency

Allele number was determined by direct count of a particular allele.

For diploid taxa, the frequency of a particular allele can be calculated as

$$P = (2N_{AA}+N_{Aa})/2N$$

Where p is the frequency of the A allele, N is the total number of individuals and N_{AA} and N_{Aa} are the number of homo- and heterozygotes for such a locus (Nei, 1978).

2.12.2 Observed heterozygosity

The observed heterozygosity (h_{obs}) was determined as number of observed heterozygote individuals divided by the number of all individuals (Weinmayr, Vautrin and Solignac, 2000).

2.12.3 Expected heterozygosity

When the population is in Hardy-Weinberg equilibrium, the expected heterozygosity (h_{exp}) was estimated using the equation;

$$h_{exp} = 1 - \Sigma p_i^2$$

where p_i is the frequency of i^{th} allele (Nei, 1978).

2.12.4 Hardy-Weinberg equilibrium

Each microsatellite locus was tested for departure from Hardy-Weinberg equilibrium using GENEPOP version 2 (dememorization number was 25000, and 500 batches for each test with 10000 iterations per batch). The probability to reject null hypothesis (Ho: genotype frequencies of an investigated sample at a given locus conform Hardy-Weinberg expectations) were further adjusted using the sequential Bonferroni technique (Rice, 1989). This was carried out by dividing the initial significant level ($P<0.05$) by the number of population and tested loci.

2.12.5 Polymorphic information content

Polymorphism information content (PIC) of each microsatellite locus was calculated as described by Botstein et al. 1980.

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2\right) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2$$

Where p_i is the frequency of the i-th allele, p_j is the frequency of the j-th alleles and n is the number of different alleles in the sample.

2.13 Multiplex analysis of microsatellite loci

Two or more microsatellite loci were simultaneously amplified in the same PCR reaction. The labeled products were concurrently electrophoresed in a single lane of a denaturing polyacrylamide gel.

Multiplex PCR of microsatellites was amplified by radioactive-labeled approach in a Hybaid Omn-E Thermo-Cycle (Hybaid limited, United Kingdom). Amplification of multiplex sets A, B, C and D was carried out using touchdown PCR. PCR reaction components and profiles for multiplex PCR were given in Tables 2.2 and 2.3, respectively. After amplification, reactions were stopped by adding 4 μl of a stop dye (10 mM NaOH, 99% formamide, 0.1% bromphenol blue, and 0.1% xylene cyanol). PCR products were denatured for 15 min at 95°C before loaded to a 6% denaturing polyacrylamide gel and electrophoresed as described in section 2.11.3. The gel was dried in vacuums and exposed to X-ray film overnight. Alleles were size-estimated by comparing with the M13mp18 sequencing ladder.

Table 2.2 PCR reaction components of 4 multiplex systems.

Reagents	Multiplex PCR			
	Set A CUPmo 2+11+15+16	Set B CUPmo 4+13+19	Set C CUPmo 14+21+23	Set D CUPmo 22+24
DNA (ng)	10-20	10-20	10-20	10-20
dNTP (mM)	0.3	0.3	0.3	0.2
$MgCl_2$ (mM)	3	3	3	2
KCl (mM)	100	100	100	100
Tris-HCl, pH8.0 (mM)	10	10	10	10
Tritonx-100 (%)	0.1	0.1	0.1	0.1
Taq (U)	0.36	0.36	0.36	0.36
Forward primer (nM)	380, 380, 380, 100	380, 200, 380	380, 100, 760	760, 130
Reverse primer (nM)	440, 440, 440, 110	440, 210, 440	440, 110, 900	900, 150
Labeled forward primer (nM)	25, 25, 25, 12	25, 12, 25	25, 6, 50	50, 6

Table 2.3 PCR thermal profile for 4 multiplex PCR of microsatellites of *P. monodon*.

Name	Multiplex PCR			
	Set A CUPmo 2+11+15+16	Set B CUPmo 4+13+19	Set C CUPmo 14+21+23	Set D CUPmo 22+24
First denaturing	95°C, 3 min, 1 cycle	95°C, 3 min, 1 cycle	95°C, 3 min, 1 cycle	95°C, 3 min, 1 cycle
Denaturing	95°C, 30 s	95°C, 30 s	95°C, 30 s	95°C, 30 s
Annealing	56 °C, 30 s (-1 per cycle)	56 °C, 30 s (-1 per cycle)	65 °C, 30 s (-1 per cycle)	56 °C, 30 s (-1 per cycle)
Extension	65°C, 4 min	65°C, 4 min	65°C, 4 min	65°C, 4 min
	6 cycles	6 cycles	7 cycles	6 cycles
Denaturing	95°C, 30 s	95°C, 30 s	95°C, 30 s	95°C, 30 s
Annealing	50°C, 30 s	50°C, 30 s	58°C, 30 s	50°C, 30 s
Extension	64°C, 4 min	64°C, 4 min	64°C, 4 min	64°C, 4 min
	20 cycles	20 cycles	20 cycles	20 cycles
Final extension	65°C, 10 min	65°C, 10 min	65°C, 10 min	65°C, 10 min
	1 cycle	1 cycle	1 cycle	1 cycle

2.14 Silver staining detection of microsatellite amplification

Amplification of each microsatellite locus was performed using the same profile as was radioactive-labeled PCR with the exception that non-radioactive labeling primer was added. After PCR amplification, 4 µl of a stop dye were added and the products were denatured at 95°C for 15 min, loaded onto 8% denaturing polyacrylamide gel and electrophoresed as described in section 2.11.3. A silver staining protocol used to stain PCR products in polyacrylamide gel was that described

by Soot-anan (1999). After electrophoresis, gel was submerged for 30 min in 10% acetic acid. The gel was then briefly rinsed 3 times with ultrapure water for 2 min each and incubated in a staining solution (0.1% silver nitrate containing 1.5 ml/liter of 37% formaldehyde) for 30 min. The excess silver ions were eliminated by brief rinse with the ultrapure water. Gel was developed in a cold (8-10°C) developing solution, (3% sodium carbonate containing 1.5 ml/liter of 37% formaldehyde and 200 µl/liter of 10 mg/ml sodium thiosulfate) and agitated until the first bands were visible. The developing solution was poured off and the fresh developing solution was added. The gel was continued development until optimal image intensity was obtained. The developing reaction was stopped using 10% acetic acid with agitating for 5 min. The gel was rinsed 2 times with the ultrapure water for 2 min each, transferred onto a piece of Whatmann 3 MM paper and dried with a gel dryer.

2.15 Application in prawn genome mapping

Parents and 42 offspring of the reference family were genotyped using 10 microsatellite loci. They were CUPmo 1, 2, 4, 9, 12, 13, 15, 19, 22 and 24. Seven microsatellite loci (CUPmo 1, 2, 9, 12, 15, 22 and 24) were analyzed as the single locus PCR whereas CUPmo 4, 13 and 19 were analyzed using multiplex loci (set B).

The amplification, separation and visualization of alleles, using denaturing gel electrophoresis and radiolabeling technique were carried out as described in section 2.11. The genotyping data derived from the amplification of 10 microsatellite loci was sent to molecular Animal Genetic Centre, University of Queensland, Australia for linkage mapping analyses.

The two-way pseudo-testcross strategy with the software program MAPMAKER/exp (F2 backcross model) was applied in the analyses. A LOD score of 3.5 and maximum θ=0.30 were set as linkage threshold for grouping markers. Groups were then analysed using multipoint mapping functions to define the most likely map orders. Map distance in centiMorgans was calculated using Haldane's mapping function (Wilson et al., 2001). The 10 microsatellite markers were placed on the preliminary genetic map of *P. monodon* along with AFLP markers.