CHAPTER II

MATERIALS AND METHODS

Materials and chemicals

-Absolute ethanol (Merck, Germany.)

-Agarose gel (Sigma Chemical Co.,USA.)

-Ammonium acetate (Merck, Germany.)

-Bolic acid (Merck, Germany.)

-Bactotryptone (Difco, USA.)

-Bacto yeast extract (Difco, USA.)

-Chloroform (Merck, Germany.)

-Calcium chloride (Merck, Germany.)

-100mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin.)

-[$\gamma^{32}P$] dATP (Dupont, NYR)

-[α-^{35}S] dATP (Dupont, NYR)

-Ethylene diaminetetraacetic acid Disodiam salt Dihydrate (Fluka Chemika BioChemika, Switzerland.)

-ethidium bromide

-Isoamyl alcohol (Merck, Germany.)

-25mM Mg_2CI_2 (Perkin-elmer Cetus, Norwalk, Connecticus.)

-Glycerol (Merck, Germany.)

-Glycogen (Merck, Germany.)

-Glucose (Merck, Germany.)

-Low melting point agarose (Sigma Chemical Co.,USA)

-Oligonucleotide primers (Bio synthesis)

-Kodak XAR film (Kodak, Japan.)

-10 X PCR buffer (Perkin-elmer Cetus, Norwalk, Connecticus.)

-Phenol crystal (Fluka Chemika-BioChemika, Switzerland.)

-Potassium acetate (Merck, Germany.)

-pUC18/ Sma I /Bap (Pharmasia, U.S.A.)

-Sodium chloride (Merck, Germany.)

-Sodium dodecyl sulfate (Sigma Chemical Co., U.S.A.)

-Sucrose (Sigma Chemical Co., U.S.A.)

-Tris-(hydroxy methyl)-amino methane (Fluka Chemika-Bio Chemika, Switzerland.)

-Triton X-100 (Sigma Chemical Co., U.S.A.)

-T_7-sequencing^{TM} kit (Pharmasia, U.S.A.)

-Whatman 3 MM paper (Whatman International Ltd, England)

-Whatman # 42 filter paper (Whatman International Ltd, England)

-Yeast tRNA (Sigma Chemical Co., U.S.A.)

Enzyme

-AmpliTaq DNA polymerase (Perkin-elmer Cetus, Norwalk, Connecticut.)

-*Alu* I (Bethesda Research Laboratories)

-*Hae* III (Bethesda Research Laboratories)

-*Hinc* II (Bethesda Research Laboratories)

-Lysozyme (Sigma Chemical Co., U.S.A.)

-*Rsa* I (Bethesda Research Laboratories)

-Proteinase K (Gibco BRL life Technologies, Inc., USA.)

-T_4 DNA ligase (Bethesda Research Laboratories)

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Host cell

-E. coli strain DH5-α and XL-1Blue

Instrument

-Glass homogenizer

-Refrigerated microcentrifuge (Kubota 1300, Japan)

-Spectrophotometry (Beckman, U.S.A.)

-Gene pulser (Bio-Rad, U.S.A.)

-Hybridization oven (Hybaid, U.S.A.)

-Gel dryer Model 583 (Bio-Rad, U.S.A.)

-Thermal cycle (Perkin Elmer, U.S.A.)

1. Samples

The black tiger shrimp broodstocks were wild-caught alive from four geographically different sites of Thailand. Pleopods were dissected from freshly killed *P. monodon* individuals and kept on ice during transportation back to the laboratory at Department of Biochemistry, Faculty of Science, Chulalongkorn University. The specimens were transferred to a -70°C deep freezer until further use.

2. DNA extraction

DNA was extracted from a pleopod of a *P. monodon* individual using the proteinase-K/phenol-chloroform method as described by Tassanakajon et al. (1996). The pleopod was placed in a microcentrifuge tube containing 400 μl of a prechilled extraction buffer (100 mM Tris pH 9.0, 100 mM NaCl, 200 mM Sucrose, 50 mM EDTA pH 8.0) and thoroughly homogenized with a prechilled glass homogenizer. Forty microliters of a 10% SDS solution (w/v) was added, gently mixed and incubated in a 65°C for at least 1 hour. At the end of the

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incubation period. the mixture was allowed to cool to room temperature before 20 μl of 10 mg/ml proteinase K was added. The reaction mixture was further incubated at the same temperature for approximately 3 hours. An appropriate amount of 5 M of potassium acetate was added to a final concentration of 0.05%. The solution was incubated on ice for 45 minutes and was centrifuged at 12,000 rpm in a refrigerated microcentrifuge (Kubota 1300, Japan) for 10 min at 4°C. The supernatant was transferred to a new 1.5 ml microcentrifuge tube. An equal volume of buffer-equilibrated phenol was added and thoroughly mixed by inversion of the tube for 10 minutes. The mixture was then centrifuged at 12,000 rpm for 10 minutes at room temperature. The aqueous phase was transferred to a new microcentrifuge tube using a wide-bore pipette tip. The resulting aqueous solution was further extracted once with phenol: chloroform: isoamyl alcohol (25:24:1 v/v) and once with chloroform:isoamyl alcohol (24:1). After centrifugation at 12,000 rpm for 10 min, the final extracted solution was transferred to a new 1.5 ml microcentrifuge tube. One-half volume of 7.5 M ammonium acetate was added followed by 2 volumes of ice-cold absolute ethanol. The mixture was mixed and placed in a -20°C freezer overnight to ensure complete precipitation of DNA. The precipitated DNA pellet was removed from a microcentrifuge tube using a wide-bore microtip and rinsed with 70% cold ethanol and air-dried. The DNA pellet was redissolved with an appropriate volume of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at 4°C until further needed.

To roughly estimate the amount of extracted DNA, a few microliters of individually extracted DNA was electrophoresed through a 0.7% agarose gel at 100 volts for 2-3 hours along with a DNA standard (λ/*Hind* III). Since the amount of fluorescence is proportional to DNA length, the quantity of DNA in samples can be estimated by comparing their fluorescence to that of a series of standard DNA (Sambrook et al., 1989). When 500 ng of λ/*Hind* III was applied to a gel, the

amounts of 23.1, 9.4, 6.6, 4.3, 2.3, 2.0, and 0.56 kb DNA fragments correspond to 240, 98, 67, 45, 24, 21 and 6 ng DNA, respectively.

3. Agarose gel electrophoresis

An appropriate amount of agarose was weighed out and heated to dissolve in 1x TBE (89 mM Tris-HCl, 89 mM Boric acid, 2.5 mM EDTA, pH 8.3) to make an appropriate gel concentration. When the agarose cooled to about 50°C, the melted agarose was poured into a gel mould. A comb was then inserted. After completely solidified, the comb was gently removed. The gel was placed in a chamber containing 1x TBE covering the gel to a depth about 1-2 mm. The extracted DNA was mixed with 10x loading dye buffer (0.1% bromophenol blue, 40% ficoll and 0.5% SDS) and mixed well. The samples were slowly applied into the wells. An appropriate amount of λ/*Hind* III was also loaded to the gel and served as a DNA marker. Generally, the gel was run at 100 volts until bromophenol blue had migrated to the other edge of the gel (approximately 2 hours). After electrophoresis, the gel was stained with 0.25 μg/ml ethidium bromide for 10 minutes. The gel was destained in deionized H_2O for 10-15 minutes to leach out unbound ethidium bromide. The DNA was placed on a transluminator (UVP) and visualized under an ultraviolet light. The gel was then photographed using Kodak TMX-400 film.

4. Measurement of DNA concentration

DNA concentration was spectrophotometrically estimated using a DU 650 spectrophotometer (Beckman, USA). The optical density at 260 nm (OD260) of 1 is equivalent to 50 mg/ml double stranded DNA whereas the ratio of OD260/OD280 reflected the purity of extracted DNA. The value much lower than 1.8 indicated

contamination of residual protein in the solution or from organic solvents while RNA contamination can cause greater value of this ratio.

5. Construction of genomic libraries from *P. monodon*

5.1 Library A

5.1.1 Preparation of DNA fragments for cloning

Fifty micrograms of genomic DNA isolated from an individual of *P. monodon* was separately digested with 25 units of *Alu* I, *Hae* III, *Hin* c II and *Rsa* I (Bethesda Research Laboratories) in a total volume of 250 μl for 24 hours at 37°C. Digested DNA was electrophoretically fractionated in a 1.5% low melting point agarose (Sea Kem) at 70 volt for 2.5 hours. The DNA fragments between 300 to 700 bp in length were recovered from the gel by using a phenol/freeze-fracture procedure (Qian and Wilkinson, 1991). To avoid a long expose of digested DNA to the ultraviolet light, a piece of gel containing a 100 bp ladder was excised, stained with the ethidium bromide solution (2.5 μg/ml of ethidium bromide in water) for 5-10 minutes. The marker bands were then placed on a UV transluminator and visualized under the UV light. The position of the 300 - 700 bp DNA fragments was measured relatively compared to that of the DNA marker before cut off and chopped into small pieces. The gel pieces were transferred to a pre-weight 50 ml sterile tube. The volume of the gel pieces was then estimated (assumed that the weight and volume of the gel are identical). The gel was then left in a -80°C freezer until they are completely solidified. Two volumes of buffer-equilibrated phenol was added. The mixture was vortexed until the frozen gel pieces were completely redissolved. A few drops of chloroform were added and vigorously mixed. The resulting mixture was spun in a Beckman J2-21 refrigerated centrifuge at 10,000 x g for 20 minutes at 2°C. The upper aqueous phase was recovered and dispensed to the other tube and further extracted by the addition of an equal volume of chloroform ; isoamyl alcohol (24:1 v/v). After

centrifugation, 2 μl of 100 mg/ml glycogen was added. A 3 M NaCl solution was added to 0.2 M final concentration. DNA was then ethanol-precipitated and kept at -80°C for at least 30 min. DNA was recovered by centrifugation. The resulting pellet was washed in 70% ethanol and transferred to a sterile 1.5 ml microcentrifuge tube before being precipitated as described above. The resulting DNA pellet was resuspended in 21μl of TE buffer. The yield of isolated DNA was estimated by comparing of their band intensity to that of known concentration DNA marker. This could be carried out by applied an aliquot of 1 μl from each sample into a 1% agarose gel prior to electrophoresis at 100 volt for 2-3 hours as described earlier.

5.1.2 Ligation

An appropriate amount of pUC18 bought from Pharmacia. A blunt-end ligation mixture containing 50 ng of dephosphorylated pUC18, 100 ng pooled *P. monodon* digested DNA, 1x ligation buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$), 0.5 mM ATP, 2.5 mM DTT was carried out in a final reaction volume of 20 μl. These constituents were carefully mixed in a 0.5 ml microcentrifuge tube and heated at 65°C for 15 min. The tube was left to cool to room temperature, and 1 - 7 units of T_4 DNA ligase was added. The reaction was incubated at room temperature (20 to 25°C) for 20 hours (Sambrook et al., 1989).

5.1.3 Transformation

The suitable amount of the ligation mixture was transformed into the host cells, *E. coli* strain DH 5-α [genotype; F^-φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rel*A1 *end*A1 hsdR17 (r_K^-, m_K^+) *pho*A *sup*E44 λ^-thi-1 *gyr*A96 *rec*A1] using 2 procedures described below.

5.1.3.1 $CaCl_2$ method

Transformation procedure was carried out according to the instruction for max efficiency DH5-α competent cell recommended by the supplier (Bethesda Research Laboratories). The competent cells kept at -80°C was thawed on ice and gently mixed. Thirty microliters of this was aliquoted into the prechilled tube. One microlitre of the ligation mixture was added and incubated on ice for 30 minutes. The cell mixture were heat shocked at 42°C for exactly 45 seconds and immediately chilled on ice. One milliliter of LB medium was added and further incubated at 37°C for 1 hours in a shaking water bath. Two hundred microliters of which was spread onto the LB agar plate containing 50 μg/ml ampicillin. After the transformed cell suspension was completely absorbed, the plate was inverted and further incubated at 37°C overnight.

5.1.3.2 Electroporation (Dower, et al. 1988)

5.1.3.2.1 Preparation of host cells

A single colony of *E.coli* DH5-α was inoculated to a tube containing 3 ml of LB medium. The tube was incubated at overnight in a 37°C shaking water bath. One milliliter of the overnight culture was added into a 500 ml flask containing 100 ml of L-broth (1% Bactotryptone, 0.5% Bacto yeast extract, 0.5% NaCl). The culture was incubated at 37°C with vigorous shaking for 3-4 hours until the $O.D._{600}$ reached ~0.5-0.7. The bacterial cells were then chilled on ice for 15-30 min, and centrifuged in a pre-cooled rotor (Beckman J2-21, USA) at 4,000 x*g* for 15 min at 4°C. After the supernatant was removed, the cells were resuspended in 100 ml of ice-cold sterile water, gently mixed and recentrifuged as described above. The supernatant was discarded. The resulting pellet was further washed with 50 ml of ice-cold water, followed by 2 ml of ice-cold sterile 10% glycerol and resuspended in a final volume of 200-300 μl ice-cold 10%glycerol. This cell suspension was dispensed in 40 μl aliquots into 0.5 ml

microcentrifuge tubes and stored at -80°C until use. The cell was stable for at least 6 months under these conditions.

5.1.3.2.2. Electrotransformation

Forty microliters of the competent cells was thawed on ice prior to the addition of 1 μl of the ligation mixture. The transformation mixture was gently mixed by swirled and left on ice for approximately 1 minute. The mixture was then electroporated in a pre-chilled 0.2 cm cuvette. The Gene pulser (Bio-Red) was set at 25 μF, 200 Ω and 2.5 kV. After electroporation, the transformed cells were immediately resuspended with 2 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) and transferred to a new tube. The cell suspension was incubated in a 37°C shaking water bath for 1 hour. One hundred microliters of transformed cell suspension was spread on the LB agar plate containing 50 μg/ml ampicillin. After completely absorbed, the plate was inverted and incubated at 37°C overnight. The number of transformants was counted and subsequently screened for positive colonies possessing microsatellite DNA using colony hybridization.

5.2 Library B

Fifty μg of genomic DNA from *P. monodon* was singly digested with 25 units of *Alu* I and *Rsa* I (Bethesda Research Laboratories). The restricted DNA was proceeded as described in 3.1.1. Dephophorylated pUC18 and digested total DNA of *P. monodon* were blunt-end ligated using the protocol mentioned in 3.1.2. Transformation of the recombinant plasmids to the competent cells was carried out using the $CaCl_2$ and eletrotransformation techniques (3.1.3.1 and 3.1.3.2) with the exception that the host strain was replaced by *E. coli* XL1-Blue having the

genotypes *rec A1 end A1 gyr 96 thi-1 hsd R17 Sup E 44 re/A1 lac* [F′*pro* AB *lac*^{q} ZΔ*M 15* Tn *10* (tet^{r})].

6. Screening of microsatellite DNA using colony hybridization

The partial genomic DNA libraries of *P. monodon* were screened for microsatellite DNA using the method developed by Grunstein and Hogness (1975).

6.1 Colony blotting

A piece of an appropriate size of Whatman filter paper #42 which were previously labeled with a ball point pen and pre-cut to produce 3 asymmetry sites was carefully placed on the agar surface. The filter was left on the agar plate with the labeled side up until it was completely wet. The position and orientation of the filter was marked using a marker pen before the filter was lift and placed (with the colony side up) on a fresh LB plate containing 50 μg/ml of ampicillin. The bacterial colonies were regrown by incubated at 37°C for 3-4 hours and kept in a 4°C refrigerator for a few hours. The filters were then removed from the plate and placed processed on a stack of Whatman 3 MM paper saturated with a denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 7 min. The filter paper containing lysed colonies were transferred to a pad of 3 MM filter paper soaked with neutralizing solution (1 M Tris-HCl pH 7.6, 1.5 M NaCl) for 3 minutes and transferred to the other stack of 3MM paper saturated with neutralizing solution. After 3 minutes, the neutralized paper was transferred to a stack of 3MM filter paper saturated with 2x SSC, 0.2% SDS (w/v) approximately 1 minute. The filter paper was then placed on a dry sheet of 3MM paper and allowed to air-dry. DNA was fixed to the filter by baking at 80°C for 2 hours *in vacuo*. The filter could be used immediately or stored in a cool dry place.

6.2 End-labeling of the oligonucleotide $(CT)_{15}$ and $(GT)_{15}$ probes

One hundred picomoles of the $(CT)_{15}$ and $(GT)_{15}$ oligonucleotides purchased from Bio-Synthesis was end-labeled in a 10 μl reaction volume containing 6 μl of 3,000 Ci/mmol $[\gamma\text{-}^{32}P]$ dATP (Dupont, NYR), 5 U of T4 polynucleotide kinase (New England Biolabs) and 1μl of 10x One Phor All buffer (100 mM Tris-acetate pH7.5, 100 mM magnesium acetate and 500 mM potassium acetate). The reaction was incubated at 37°C for 30 minutes. A small aliquot (1-2 μl) was taken for determination of the specific activity which could be estimated using the equation;

specific activity (dpm/μg) = total activity incorporated (dpm)/amount of oligonucleotide added (μg)

Generally, the synthesized probe from one reaction was sufficient for hybridization of six filters.

6.3 Prehybridization and Hybridization

The residual cell debris on the filter was removed by gently rubbed in 2x SSC, 0.2% (w/v) SDS before prehybridized in 20 ml of a freshly prepared prehybridization solution (2x Denhardt's solution, 5x SSPE, 0.5% SDS and 100 μg/ml yeast tRNA) at 60°C for at least 1 hours. The labeled $(GT)_{15}$ oligonucleotide probe was directly added to the prehybridization solution. Hybridization was carried out at 60°C overnight in a hybridization oven (Hybaid). The hybridized filters were post-washed twice with 2x SSC, 0.2% SDS at room temperature for 30 minutes each, followed by 0.2x SSC, 0.2% SDS at the same temperature for 30 minutes, 0.1x SSC, 0.2% SDS at 45°C for 30 minutes and finally with 0.1x SSC, 0.2% SDS at 60°C for 30 minutes. The hybridized filter was wrapped between two sheets of cling film and subjected to autoradiography at -80°C (Kodak XAR film or Hyperfilm MP, Americium) in the presence of a pair of intensifying screens for approximately 2 hours. Positive clones was identified by aligning of

the master plate on the autoradiograph. Colonies having strong hybridization signal were individually picked up with a sterile toothpick and placed in a screw-capped tube containing 3 ml of Terrific supplemented with 100 mg/ml ampicillin. The inoculum was cultured at 37°C with shaking for 24-36 hours. Five hundred microliters of each culture was mixed with 100 μl of 80% glycerol in a 1.5 ml microcentrifuge tube and kept at -80°C as the preserved stock culture. The remaining of the culture was used for a miniprep of plasmid DNA for DNA sequencing.

7. Characterization of microsatellite-containing clones by DNA sequencing

7.1 Preparation of plasmid DNA template for Sequencing

A mini-prep based on the boiling procedure developed by Holmes and Quigley (1981) was used for rapid isolation of plasmid DNA from large numbers of recombinants for DNA sequencing. The overnight culture was poured into a 1.5 ml microcentrifuge tube. The cells were pelleted by centrifugation at 10,000 x *g* in a microcentrifuge for 10-15 seconds and completely resuspended in 180 μl of STET buffer (8% Sucrose, 5% Triton X-100, 50 mM Tris-HCl, 50 mM EDTA) followed by 20 μl of a lysozyme solution (10 mg/ml in STET buffer). The mixture was immediately mixed by vortexed before left at room temperature for 75 seconds. The tube was then placed in a 100°C bath containing for 75 seconds and spun at 10,000 x *g* for 10 minutes at room temperature. The cell debris was removed by a toothpick and discarded. The DNA was precipitated by an addition of an equal volume of ice-cold isopropanol and mixed thoroughly before placed in a -80°C freezer for at least 10 minutes. DNA was recovered by centrifugation for at 12000 x*g* for 10 minutes at room temperature. The supernatant was discarded. The resulting DNA pellet was air dried and resuspended in 80 μl of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA).

7.2 Sequencing method

DNA sequences of the cloned DNA inserts from the positively hybridized clones were determined by double-stranded chain terminated sequencing method using T7-sequencing ™ kit (Pharmacia) as described below (Sanger et al., 1977, Sambrook et al., 1989). Ten microliters of extracted plasmid DNA from each positive clone was denatured by adding 2.25 μl of 2 N NaOH and incubated for 10 min at room temperature. The mixture was then added with 1.25 μl of 3 M sodium acetate (pH 4.8) followed by 100 μl of absolute ethanol and thoroughly mixed before placed at -80°C for 15 min. The precipitated DNA was recovered by centrifugation at 12000 x *g* for 15 minutes at room temperature. The pellet was washed with ice-cold 70% ethanol and recentrifuged for 10 min at the same speed. The supernatant was removed and discarded. The DNA pellet was air-dried and redissolved in 11 μl of distilled water and 2 μl of annealing buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The resuspended template could be either immediately used for annealing with the primer or stored at 4°C for subsequent use. Six picomoles of the appropriate primer mix (5 pmol/μl of the universal or reverse primer) was added to the resuspended template and incubated at 65°C for 5 minutes followed by at 37°C for 10 minutes before placed at room temperature for at least 5 minutes. The tube was briefly centrifuged before immediately proceeded to the labeling\extension reaction. The labeling reaction was set up by mixing of 1.5 μl of the labeling mix-dATP, 1 μl of [α-^{35}S] dATP (>1,000 Ci/mmol, Dupont) and 0.5 μl of the five-fold diluted T7 DNA polymerase. Subsequently, 3.4 μl of this solution was added to each of the template-primer mix (the T, C, G, A tubes) and incubated at room temperature for 5 minutes. At the end of the labeling period, 3.4 μl of the reaction was transferred into each of the four termination mixes pre-warmed at 37°C. The mixture was mixed by brief centrifugation and incubated at 37°C for 5 minutes. Five microliters of stop solution (10 mM NaOH, 99% formamide, 0.1%

bromophenol blue and 0.1% xylene cyanol) was added to each tube and mix gently. After denaturation, the sequenced DNA sample was ready for electrophoretically analyzed. Practically, the sequencing reaction can be performed using either the "Read Short" or "Read Long" conditions. The four nucleotide mixes of these are shown in Table 2.1. The former condition allows DNA sequences to be read up to approximately 500 nucleotides from the primer while approximately 1000 nucleotides can be examined by the latter. The sequencing products were electrophoretically fractionated in a 8% denaturing polyacrylamide gel prepared with 1X TBE (89 mM Tris-HCI, 89 mM boric acid, 2 mM EDTA pH 8.3) in the presence of 7.8 M urea at 1200 volts for approximately 2.5 hours. The gel was then immersed in a fixing solution (10% methanol and 10% acetic acid) for at least 30 min before blot-transferred onto a piece of Whatman 3MM paper and dry *in vacuo* using a gel dryer for 2 hours (Model 583, Bio-Rad). The gel was autoradiograped with Kodak KAR5 or Hyperfilm MP overnight at room temperature without the intensifying screens.

8. Polymorphism of *P. monodon* microsatellites

8.1 Primer Design

Once microsatellite region of the insert was identified, the unique flanking sequences of were used to design PCR primers. The criteria used for development of a pair of primers to *in vitro* amplify a particular microsatellite locus were: (1) the primers should be in the proximity to the repeat array as much as possible; (2) the GC content of both forward and reverse primers should be approximately in equal; (3) when possible, the primers should possess at least one C or G at their 3'end; (4) there should be no potential annealing between primers; and (5) the primers should be 19 to 23 bp in length (Kamonrat, 1996).

Table 2.1 Components of the sequencing mixes

Type	Components
'A' Mix-short	840 μM each dCTP, dGTP and dTTP; 93.5 μM dATP; 14 μM ddATP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'C' Mix-short	840 μM each dATP, dGTP and dTTP; 93.5 μM dCTP; 14 μM ddCTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'G' Mix-short	840 μM each dATP, dCTP and dTTP; 93.5 μM dGTP; 14 μM ddGTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'T' Mix-short	840 μM each dATP, dCTP and dGTP; 93.5 μM dTTP; 14 μM ddTTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'A' Mix-Long	840 μM each dCTP, dGTP and dTTP; 93.5 μM dATP; 2.1 μM ddATP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'C' Mix-Long	840 μM each dATP, dGTP and dTTP; 93.5 μM dCTP; 2.1 μM ddCTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'G' Mix-Long	840 μM each dATP, dCTP and dTTP; 93.5 μM dGTP; 2.1 μM ddGTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'T' Mix-Long	840 μM each dATP, dCTP and dGTP; 93.5 μM dTTP; 2.1 μM ddTTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl

8.2 Amplification of microsatellite loci through PCR

Determination of the length of amplified alleles and their polymorphism status of each microsatellite locus was carried out through PCR and one of the primers was labeled with [γ-^{32}P] ATP. It was crucial to determine whether the reverse or forward labeled primer gave more readily scorable products. Basically, the primer was labeled in a 10 μl labeling reaction constituting 10 pmol of one of the primers, 10 units of T4 polynucleotide kinase (New England Biolabs), 1 μl of 10X One Phor All buffer (100 mM Tris-acetate pH7.5, 100 mM magnesium acetate and 500 mM potassium acetate) and 3 μl of [γ-^{32}P] ATP (3,300 μCi/mmol, Dupont).

The labeling reaction was incubated at 37°C for 30 minutes. The reaction was then terminated at 65°C for 15 minutes. Eight *P. monodon* individuals

originating from different geographic locations was used to test polymorphism of microsatellites loci. Generally, amplification reaction was carried out in the 5 µl standard reaction constituting 15 ng DNA template, 0.6 µM of primer 1, 0.575 µM of primer 2, 0.025 µM [γ-^{32}P] ATP labeled primer 2, 1 µl of 10x PCR buffer (100 mM Tris-HCl H 8.3, 500 mM KCl, 10 mM $MgCl_2$, 0.1% gelatin), 200 µM dNTPs, 0.25 U of *Taq* Polymerase (Perkin Elmer) and 0.05% of tween20 (Kamonrat & Cook, 1998). The amplification reaction was overlaid with a drop of mineral oil to prevent evaporation. The thermal cycling was composed of 7 cycles of a denaturation step for 1 minute at 94°C, an annealing step for 30 seconds at the most optimal annealing temperature (T_A) of each primer, and an elongation step for 1 minute at 72°C followed by 38 cycles of a 30 second denaturation at 90°C, a 30 second annealing at T_A, and a 1 minute extension at 72°C. And the touch down PCR were utilized. Initially, the reaction mixture was pre-denaturated at 94°C for 1 minute, an annealing step at the most suitable Ta for 1 minute and an extension step at 72°C for 1 minute. These were carried out for 3 cycles. Subsequently, the amplification was performed as described above with the exception that a pre-denaturation step was omitted and the annealing temperature were gradually decreased for 2°C below the most optimal annealing temperature every 3, 3 and 4 cycles. At the end of these steps, the SCR reaction was performed for 20 cycles constituting of the typical denaturation temperature for 30 seconds, Ta-7°C below the most optimal annealing temperature of each locus for 30 seconds and the typical extension temperature for 30 seconds. Initially, the annealing temperature (T_A) for each primer was estimated to be five degree Celsius below the melting temperature calculated using the Wallace's rules. As a result,

$$T_A\ (^{\circ}C) = [\ 4\ (\text{total number of G and C}) + 2\ (\text{total number of A and T})] - 5$$

If the Tm values of the forward and reverse primers were, however, not identical, the annealing temperature used in the PCR was chosen from the primer having lower T_A. The actual optimal annealing temperature was further adjusted from autoradiography results. For example, when more than one or two stutter bands appeared, the annealing temperature was raised with a 1°C increment. In contrast, if no products were visualized after autoradiography, the annealing temperature was sequentially lowered for 1°C until the optimal temperature for each primer pair was obtained.

8.3 Preparation of the M13 sequencing marker

To prepare the sequencing marker, the primer/template annealing reaction was set up by mixing of 5 μl of M13 mp template, 5 μl of ddH_2O, 2 μl of annealing buffer and 2 μl of 4 ng/μl of FSP (Forward Sequencing Primers). The reaction was incubated at 65°C for 15 min and allowed to cool to room temperature for 10 min. While the primer/template mix was incubated, labeling mixture was made by the addition of 1μl of ddH_2O, 3 μl of labeling dNTPs mix (0.2 M each of dATP, dCTP, dGTP and dTTP), 2.0 μl of diluted T7 polymerase (0.3 μl of T7 polymerase and 1.7 μl of the dilution buffer) and 1.0 μl of 800 Ci/mmol [α-^{32}P] dATP. Six microliters of this was added to the annealing reaction. The reaction was then incubated at room temperature for 5 min. The reaction was terminated by adding 4.5 μl of labeling reaction to each of the 4 tubes containing 2.5 μl of short mix A, C, G and T, and incubated at 37°C for 5 minutes. Forty microliters of stop solution and 1 drop of mineral oil were added into each tube. The M13 sequencing marker can be stored at -20°C until required. The M13 sequencing marker was heated at 94°C for 2 min before snapped chilled on ice. Approximately, 3-3.5 μl of the marker was loaded onto the gel (Yanish-Perron et al., 1985).

8.4 Electrophoretic analysis of amplified microsatellite DNA

After the amplification was complete, 5 μl of a stop dye solution (10 mM NaOH, 99% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol) was added to each PCR amplified reaction. The resulting mixture was denatured at 94°C for 15 min. Subsequently, 3.5 μl of the denatured solution was loaded onto a 8% denaturing polyacrylamide sequencing gel and electrophoresed at 1200 volts for approximately 2-3 hours depending on the expected sizes of the products. After electrophoresis, the gel was fixed with an enough amount of the fixing solution (10% methanol and 10% acetic acid) for 30 minutes before blot-transferred to Whatmann 3 MM paper and vacuum dried at 80°C for 2 hours before subjected to autoradiography detection with the intensifying screens at -80°C overnight. Microsatellite alleles were relatively sized by compared with a sequencing ladder (generated from single-stranded M13 DNA and loaded along-side of the investigated PCR products).

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