Chapter 2

Materials and Methods

1. Preparation of Mitochondria (Tapper et al., 1983)

Materials

1. The giant freshwater prawn (Macrobrachium rosenbergii de Man)
2. Chemicals and Solutions
   - -MS buffer: 0.21 M manital, 0.07 M sucrose, 0.005 M Tris and 0.005 M EDTA, pH 7.5
   - -Sucrose-TE buffer: 20% sucrose, 50 mM Tris-HCl, pH 7.5 and 10 mM EDTA
   - -1 M Sucrose-TE buffer: 1 M sucrose, 10 mM Tris-HCl, pH 7.5 and 5 mM EDTA
   - -1.5 M sucrose-TE buffer: 1.5 M sucrose, 10 mM Tris-HCl, pH 7.5 and 5 mM EDTA

Methods

1.1 Preparation of crude mitochondria

The Giant freshwater prawn (Macrobrachium rosenbergii de Man) was stunned by cooling below 4°C about 10 minutes. The

hepatopancreas was removed. The tissue was scissor minced and homogenized in 1:5 w/v of MS buffer on ice by passing the glass homogenizer tube up and down past the motor-driver teflon for 6-8 times until the solution was homogeneous.

The homogenate was centrifuged at 3,000 rpm 4°C for 15 minutes in Sorvall SS-34 rotor, to removed nuclei and cell debris. The supernatant was decanted into another centrifuge tube and mitochondria were pelleted from this supernate in a Sorvall SS-34 rotor at 10,000 rpm 4°C for 20 minutes. The crude mitochondria pellets were washed twice with MS buffer and were resuspended in a minimal volume of MS buffer.

1.2 Preparation of purified mitochondria

The crude mitochondria pellets were resuspened in Sucrose-TE buffer 5 ml/10g starting tissue and layered over a discontinuous sucrose gradient consisting 10 ml of 1.5 M Sucrose-TE buffer (bottom layer) and 10 ml of 1.0 M Sucrose-TE buffer (upper layer). The gradient was centrifuged at 40,000 rpm 4°C for 20 minutes in 50.2 Ti rotor of Beckman L9-70 M Ultracentrifuge. The mitochondria at the interface of the two sucrose layers with brown color was removed with a pasteus pipette. The mitochondria solution was diluted with an equal volume of MS buffer and mitochondria was pelleted by centrifugation at 10,000 rpm 4°C for 15 minutes in Sorvall SS-34 rotor. The washing step was repeated again. After the supernatant was discarded and purified mitochondria pellets were resuspended in a minimal volume of MS buffer.

2. Analysis of mitochondria (Ackrell et al., 1978)

Materials

1. Mitochondria pellets were isolated from hepatopancreas of M. rosenbergii
2. Chemicals and solutions
   - -200 mM KH_2PO_4
   - -100 mM KCN
   - -200 mM sodium succinate
   - -0.05% (w/v) 2,6-dichlorophenolindophenol
   - -0.33% (w/v) phenazine methosulfate

Method

The enzyme succinate dehydrogenase was the enzyme-marker for mitochondria which was assayed as follow, Phenazine Methosulfate Method. The reaction mixture consisted of 750 ul of 200 mM KH_2PO_4, 300 ul of 200 mM sodium succinate, 100 ul of 0.05% (w/v) 2,6-dichlorophenolindophenol, 300 ul of 0.33% (w/v) phenazine methosulfate, 30 ul of 100 mM KCN and 1500 ul deionized water, giving a final volume of approximately 3 ml and a pH of 7.5. The solution was protected from light until immediately before use. Enzyme activation was carried out by incubating the stock mitochondria pellet suspension in the presence of succinate at 37°C for 6 minutes. The assay was started by the final addition of 5 ul activated mitochondria pellet, from 1 ml of crude mitochondria which extracted from 5 g hepatopancreas, to the reaction mixture. Enzyme activity was measured against blank, which was treated as the sample except sodium succinate

was not added, with following the change in absorbance at 600 nm 37°C by Shimadzu 240 spectrophotometer.

3. Mitochondria DNA extraction

3.1 Cesium chloride-Ethidium bromide gradient (Radloff et al., 1967)

Materials

1. The hepatopancreas of M. rosenbergii about 30 g
2. Chemicals and solutions
   - -cesium chloride (Sigma)
   - -ethidium bromide (Sigma)
   - -isoamyl alcohol (Merck)
   - -absolute ethanal (Merck)
   - -70% ethanal (Merck)
   - -2x pronase buffer: 20 mM Tris, 20 mM EDTA and 300 mM NaCl
   - -2x lysis buffer: 4 mg/ml protenase K in 4% SDS
   - -TE buffer: 10 mM Tris-HCl pH 7.4 and 1 mM EDTA

Method

The crude mitochondria was extracted from 30 g of hepatopancreas as described in Materials and Methods (see section 1.1). The crude mitochondria pellet was resuspended in 2 ml of 2x pronase buffer and was added 2 ml of 2x lysis buffer. The suspension was incubated at 37°C for overnight. The solution was transfered to centrifuge tube and added TE buffer to make a final volume 8 ml. Then 8 g of cesium chloride was dissolved in the

solution and 0.8 ml of 10 mg/ml ethidium bromide was added. Closed circular mtDNA in the DNA solution was separated under cesium chloride bouyant density gradient in an ultraclear centrifuge tube in a 70.1 Ti rotor of Beckman L9-70 M ultracentrifuge at 45,000 rpm for 24 hour at 20°C.

The band position was detected under a UV lamp. The upper band and lower band was removed through a no.25 hypodermic needle (Terumo). Each solution was removed ethidium bromide by adding an equal volume of isoamyl alcohol. The solution was centrifuged at 800 g for 5 minutes at room temperature. Extraction step was repeated about 3-4 time until solution was clear. Two volumes of sterile distilled water were added to dissolve salt, followed by two volumes of absolute ethanal. The solution was mixed and left at -20°C for overnight. The DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes and washed with 70% ethanal twice. The DNA pellet was dried by heat at 65°C for 15 minutes and dissolved in TE buffer.

3.2 Rapid alkaline extraction (Palva and E.T. Palva, 1985)

Materials

1. The hepatopancreas of M. rosenbergii
2. Chemicals and solutions
   - -lysozyme solution: 50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA and 2 mg/ml lysozyme
   - -lysis solution: 0.2 N NaOH and 1% SDS
   - -neutralize solution: 3 M sodium acetate pH 4.8
   - -absolute ethanal
   - -70% ethanal
   - -TE buffer

Method

The crude mitochondria pellet was obtained from 4-5 g hepatopancreas and resuspended in lysozyme solution 100 ul/2.5 g tissue or the purified mitochondria pellet was obtained from 50-60 g hepatopancreas and resuspened in lysozyme solution 100 ul/10 g tissue as described in Materials and Methods section 1

These suspension 100 ul was added 200 ul of lysis solution and throughly mixed. After 5 minutes incubating on ice, 150 ul of neutralize solution was added and gently mixed. The tube was maintained on ice for 1 hour and centrifuged at 10,000 rpm for 10 minutes. The mtDNA in the supernatant was precipitated with 2 volumes of absolute ethanal and kept at -20°C for overnight. The mtDNA was pelleted by centrifigation at 10,000 rpm for 10 minutes and washed with 70% ethanal twice. The mtDNA pellet was dried by heat at 65°C for 15 minutes and dissolved in TE buffer.

3.3 Phenol extraction

Materials

1. The hepatopancreas of M. rosenbergii
2. Chemicals and solutions
   - -2x pronase buffer: 20 mM Tris, 20 mM EDTA and 300 mM NaCl
   - -2x lysis buffer: 4 mg/ml proteinase K in 4% SDS
   - -Water saturated distilled phenol containing 0.1% of 8-hydroxy quinoline

-3% isoamyl alcohol in chloroform

-diethyl ether

-absolute ethanal

-70% ethanal

-TE buffer

Method

The crude mitochondria pellet was obtained from 4-5 g hepatopancreas and resuspended in 2x pronase buffer 0.5 ml/2.5 g tissue or the purified mitochondria pelleted was obtained from 50-60 g hepatopancreas and resuspended in 2x pronase buffer 0.5 ml/20 g tissue

One ml. of the suspension was added 1 ml of 2x lysis buffer and incubated at 37°C for 24 hours. The solution was extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) 3 times. The aqueous phase was extracted with chloroform : isoamyl alcohol (24:1) twice and extracted with equal volume of diethylether twice. The mtDNA was precipitated by adding 1/10 volume of 3 M sodium acetate pH 5.5 and 2 volumes of absolute ethanal. The mixture was kept at -20°C for overnight. The mtDNA was pelleted by centrifugation at 10,000 rpm for 10 minutes, washed with 70% ethanal twice and dried at 65°C for 15 minutes. The mtDNA was dissolved in TE buffer.

4. Preparation of plasmid DNA (Birnboim and Doly, 1979)

Materials

1. Escherichia coli JM 107 (lac pro, end A1, gyr A96, thi, hsd R17, sup E44, rel A1, F' tra D36, pro AB+, lac Z M15)

harbouring plasmid pUC 12 or recombinant plasmid.

2. Chemicals and solutions

-Luria-Bertani (LB) media, 1 liter containing

Bacto-triptone (Difco)	10 g
Bacto-yeast extract (Difco)	5 g
NaCl	5 g

-Ampicilin (Sigma)

-chloramphenical (Sigma)

-lysozyme solution: 50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA and 2 mg/ml lysozyme

-lysis solution: 0.2 N NaOH and 1% SDS

-neutralize solution: 3 M sodium acetate pH 5.5

-cesium chloride

-ethidium bromide

-isoamyl alcohol

-TE buffer

Methods

4.1 Large scale extraction of plasmid or recombinant plasmid

The single colony of E. coli strain JM 107 containing plasmid vector or recombinant plasmid was grown in 5 ml LB media supplemented with 100 ug/ml ampicilin at 37°C for overnight with shaking. The overnight culture was transferred to 500 ml LB media supplemented with 100 ug/ml ampicilin and shaken at 37°C for 6-8 hour. Then, 170 ug/ml chloramphenical was added to the culture and continuously incubated for 6-14 hour. The cells were collected by centrifugation at 4,000 g in a GSA rotor of Sorvall RC 2-B for 10 minutes at 4°C. The supernatant was discarded,

then 10 ml freshly prepared lysozyme solution was added. The mixture was kept on ice for 30 minutes before the addition of 20 ml of lysis solution. The solution was subsequently neutralized by adding 15 ml of neutralized solution. After gently mixing the solution, the another 1 hour of incubation on ice was performed for complete chromosomal DNA precipitation before centrifugation at 4,000 rpm for 10 minutes at 4°C. The plasmid in the supernatant was transfered to 15 ml centrifuge tube and precipitated by adding 2 volumes of absolute ethanal and left at -20°C for overnight. The DNA pellet, obtained from centrifugation mixture at 6,000 g for 10 minutes, was washed with 70% ethanal and dissolved in 1-2 ml TE buffer. The solution was transfered to centrifuge tube and TE buffer was added to make a final volume 8 ml. Then 8 g cesium chloride was dissolved in the solution and 0.8 ml of 10 mg/ml ethidium bromide was added. Close circular plasmid in the DNA solution was separated under cesium chloride bouyant density gradient in an ultraclear centrifuge tube (Beckman) in a 70.1 Ti rotor of Beckman L9-70M Ultracentrifuge at 45,000 rpm for 24 hour at 20°C.

The band position was detected under a UV lamp. The upper band consisted of bacterial chromosomal DNA. The middle band consisted of nicked circular plasmid DNA, and the lower band consisted of circular plasmid DNA. The lower band was removed through a no. 25 hypodermic needle (Teruma), 1-2 ml of close circular plasmid was collected and an equal volume of isoamyl alcohol was added to extract ethidium bromide. The solution was centrifuged at 800 g for 5 minutes at room temperature. Extraction step was repeated about 3-4 times until the solution was clear. Two volumes of sterile distilled water were added to dissolve salt, followed by 2 volumes of absolute ethanal. The solution was mixed well and left at -20°C for overnight. The

plasmid was collected by centrifugation at 10,000 rpm (Eppendrof) for 10 minutes. The plasmid DNA pellet was washed twice with 70% ethanal, and dried at 65°C for 15 minutes. The pellet was dissolved in TE buffer.

4.2 Small scale plasmid extraction

E. coli strain JM 107 containing plasmid vector or recombinant plasmid were grown overnight at 37°C on LB agar supplemented with 100 ug/ml ampicilin. A single colony was inoculated into 3-5 ml LB media was added 100 ug/ml ampicilin. The culture was incubated at 37° C in a shaking incubator for overnight. The cells were collected by centrifugation at 10,000 g for 5 minutes in microcentrifuge tube. The suspended cells in 100 ul lysozyme solution were added 200 ul of lysis solution. The mixture was incubated on ice for 5 minutes before the addition of 150 ul of neutralized solution and left on ice for 1 hour then the precipitate was discarded by centrifugation at 10,000 rpm for 10 minutes. Plasmid DNA in the supernatant was precipitated with absolute ethanal at -20°C for overnight. The plasmid DNA pellet was washed with 70% ethanal twice and dried at 65°C for 15 minutes. The plasmid DNA was dissolved in TE buffer.

5. Agarose gel electrophoresis (Maniatis, 1982)

Materials

1. DNA solution
   agrarose gel (Sigma)
   Tri-x film (Kodak)
2. Chemicals and solutions

-TBE buffer: 89 mM Tris-HCl pH 8.3, 89 mM boric acid and 2.5 mM EDTA

-loading dye: 0.1% bromphenol, 40% ficall and 5 mM EDTA

Method

This electrophoresis is a standard method used to separate, identify and purify DNA fragment on the basis of different mobilities due to different sizes. Agarose gel can be prepared in various concentration (0.5-1.5%) by dissolved the agarose gel in TBE buffer and heated to completely dissolved, cooled to 60°C and poured into chamber set with a comb of a desired thickness.

DNA sample was mixed with 1/3 volume of loading dye and was loaded into the well. Electrophoresis was performed at 80-100 volts by TBE buffer. After electrophoresis, the gel was stained in 2.5 ug/ml ethidium bromide for 5 minutes then destained in distilled water by shaking at room temperature until the background was clear under ultraviolet light. The gel was photographed using Tri-x film in a olympus camera with a red filter and UV-illuminator (Chromato-VUE Transilluminator, C-61).

6. Restriction endonuclease digestion (Maniatis, 1982)

Materials

1. Buffer: Restriction endonuclease can be divied into three classes, depending on the requirement of ionic strength in buffer system. By following this scheme (table 2), three stock buffers need to prepare as 10x stock solution which can be stored

at -20°C.

2. Restriction endonuclease enzymes: By fllowing the table 3, the optimum condition for each enzyme.

All restriction enzymes were stored in buffer containing 50% glycerol at -20°C. In the reaction mixture, enzyme was not more than 10% of total volume except Eco R1* because a high glycerol concentration inhibited enzyme activity. One unit of enzyme was definded as the amount required to complete digest 1 ug of lamda DNA for 1 hour in the recommended buffer at optimal temperature.

Methods

6.1 Restriction enzyme digestion for characterization of mtDNA

10 ug of extracted mtDNA from various method (see section 3) was digested with some restriction enzymes for characterization of mtDNA using 2 ul of suitable 10x salt buffer, 15-20 units of restriction enzyme in a total volume of 20 ul. The mixture was incubated at optimum temperature for overnight and analyzed on 0.7% agarose gel electrophoresis.

6.2 MtDNA and pUC12 digestion for cloning

20 ug of purified-mitochondria DNA (see section 3.3) was digested with Sau 3A1 for cloning using 3 ul of 10x medium salt buffer, 24 units of Sau 3A1 in a total volume of 30 ul. The mixture was incubated at 37°C for overnight to obtain complete digestion. The enzyme was removed by phenol-chloroform-ether

Table 2 10x buffers for restriction enzyme digestion

Buffer	NaCl (mM)	Tris-HCl pH 7.5 (mM)	$MgCl_2$ (mM)	Dithiothreitol (DTT) (mM)
low	0	10	10	1
médium	50	10	10	1
high	100	50	10	1

Table 3 Restriction endonuclease enzymes used in this study

Enzyme	Salt	Incubation temperature (oC)	Recognition sequence
Acc I	med	37	5'...GT¦CTAC...3'
Ava 1	med	37	5'...G¦PyCGPuG..3'
Bst U1	med	37	5'....CG¦CG....3'
Bam H1	med	37	5'...G¦GATCC...3'
Dra 1	med	37	5'...TTT¦AAA...3'
Eco R1	high	37	5'...G¦AATTC...3'
Eco R1*	low	37	5'....¦AATT....3'
	(15% glyceral)		
Hae 111	med	37	5'....GG¦CC....3'
Hinc 11	med	37	5'...GTPy¦PuAC..3'
Hha 1	med	37	5'....GCG¦C....3'
Hind 111	med	37	5'...A¦AGCTT...3'
Pst 1	med	37	5'...CTCGA¦G...3'
Rsa 1	med	37	5'....GT¦AC....3'
Sau 3A1	med	37	5'....¦GATC....3'
Sst 1	med	37	5'...GAGCT¦C...3'
Sma 1	med	37	5'...CCC¦GGG...3'
Xba 1	high	37	5'...T¦CTAGA...3'

extraction and DNA precipitated by ethanal precipitation. The DNA pellet was washed once with 70% ethanal and dissolved in 20 ul TE buffer and analyzed on 1.2% agarose gel electrophoresis.

The plasmid vector pUC12 (Fig. 1) which extracted by CsCl-EtBr Gradient method (see section 4.1) was digested with Bam H1. The reaction mixture, 20 ul, contained 10 ug plasmid DNA, 2 ul of 10x medium salt buffer and 20 units of Bam H1. The mixture was incubated at 37°C for 2-3 hour. After, the digested plasmid DNA was phenolized and precipitated. The DNA pellet was washed once in 70% ethanal, then dissolved in 20 ul TE buffer and analyzed on 0.7% agarose gel electrophoresis to check the complete digestion.

6.3 Recombinant plasmid digestion for southern blot hybridization

Southern blot hybridization was used to select highly repetitive inserted fragment from recombinant clones previously selected by colony hybridization. The recombinant plasmid extracted by small scale plasmid extraction (see section 4.2) was single digested with Eco R1 for opening the closed circular form to linear form. The reaction mixture, 20 ul, contained 500 ng of recombinant plasmid DNA, 2 ul of 10x high salt and 5 units of Eco R1. The mixture was incubated at 37°C for 3 hour prior to loading onto 0.7% agarose gel electrophoresis.

After selected the different size and highly repetitive of inserted fragment from single enzyme digestion recombinant plasmid. The two enzymes digestion were performed to separate inserted fragment from plasmid vector pUC12. The reaction mixture, 20 ul, contained 1 ug of recombinant plasmid DNA, 2 ul of 10x high salt, 5 units of Eco R1 and 5 units of Xba 1. The

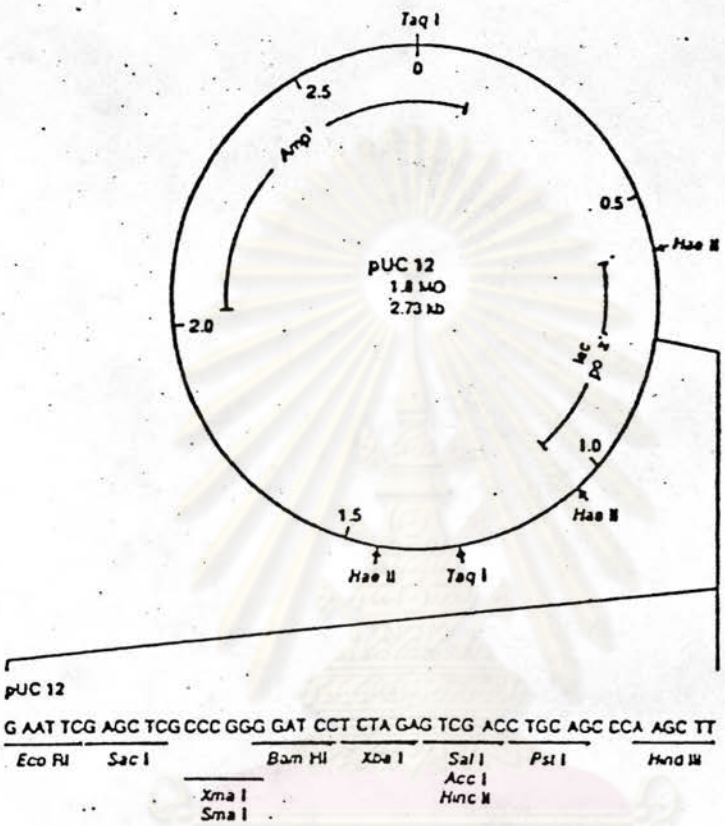

Figure 1 Physical map of vector pUC12

mixture was incubated at 37°C for 3 hour. Then, the reaction was analyzed on 1.2% agarose gel electrophoresis.

6.4 MtDNA from individual digestion

The mitochondria DNA extracted from individual of prawn was digested with Sau 3A1 to analyze RFLP of mtDNA in prawn from various resources (see section 10).

The reaction mixture, 30 ul, contained 20 ug of mtDNA, 3 ul of medium salt and 24 units of Sau 3A1. The mixture was incubated at 37°C for overnight. The mixture was stopped reaction and decrease volume by heating at 65°C for 20 minutes prior to loading onto 1.2 % agarose gel electrophoresis.

6.5 Restriction enzyme digestion for mapping

500 ng of recombinant DNA no. 1, or vector pUC12, was digested with various restriction enzymes for restriction mapping using 2 ul of 10x salt buffer, 2-3 units of restriction enzyme in a total volume of 20 ul. The mixture was incubated at optimum temperature for 2-3 hour and analyzed on 0.7-1.2% agarose gel electrophoresis.

7. Ligation

Materials

1. DNA: mtDNA digested with Sau 3A1
   pUC12 digested with Bam H1
2. Chemicals and solutions
   -10x ligation buffer contained:

6 mM potassium chloride

10 mM Tris-HCl pH 7.5 (Sigma)

10 mM $MgCl_2$ (H & W ; JT-Baker)

11 mM DTT (Sigma)

1 mM ATP (Sigma)

100 g/ml nuclease-free bovine serum albumin (BRL)

1 mM spermidine which was already neutralized to pH 7.5 (Sigma)

-T_4 DNA ligase (BRL)

-double distilled water

Method

The 1 ug of mtDNA was digested with Sau 3A1 and 200 ng of vector pUC12 was digested with Bam H1 (see section 6.2). Both DNA were mixed and warmed at 65°C for 10 minutes. After cooling on ice, the mixture was added 1/10 of final volume of 10x ligation buffer, 2 units of T_4 DNA ligase and make final volume to 6 ul with double distilled water. The ligation was done at 14°C for 16-18 hours. The mixture was heated at 65°C for 10 minutes to stop the reaction and quick cool on ice. Then the ligation product was transformed into E. coli strain JM 107 (Fig. 2).

8. Transformation (Hanahan, 1983)

Materials

1. Bacteria: E. coli strain JM 107

   Ligation product DNA

2. Chemicals and solutions

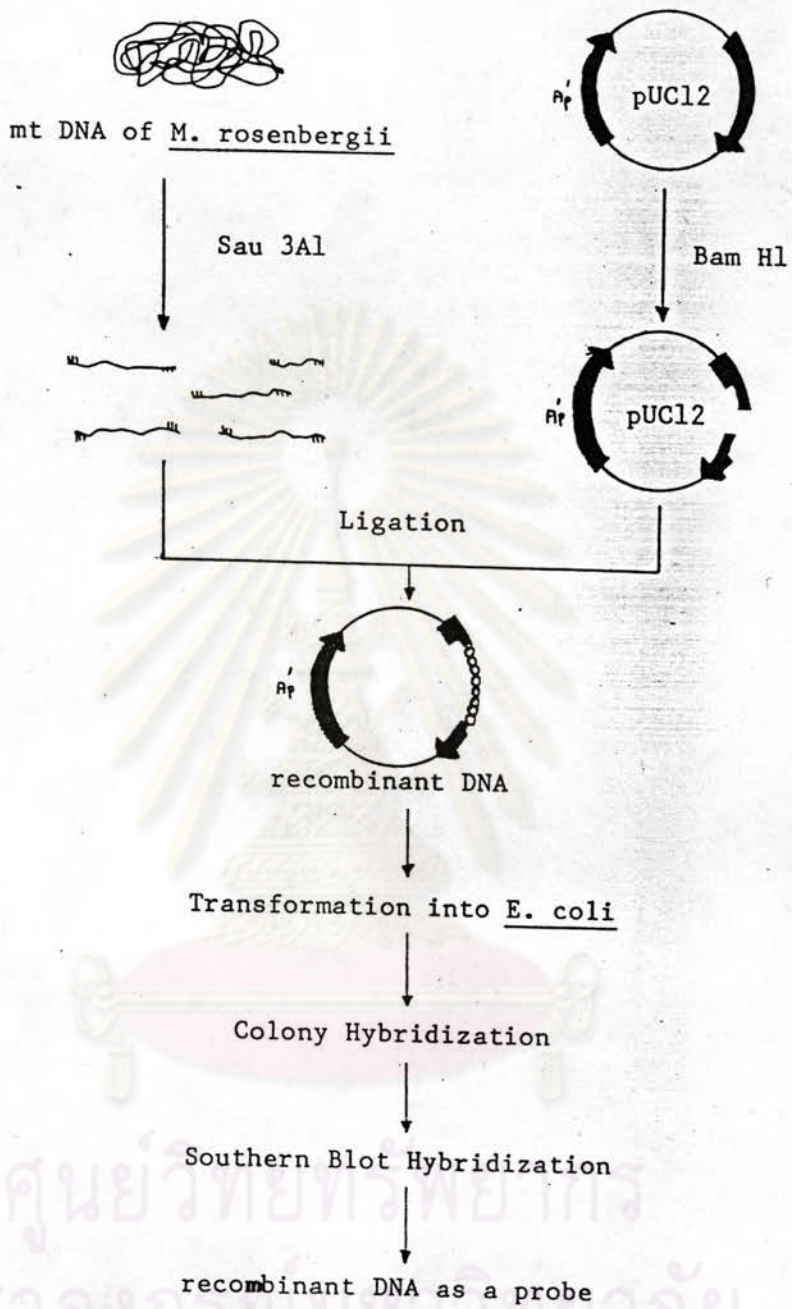

Figure 2 Cloning strategy of mtDNA fragments of Macrobrachium rosenbergii in E. coli

-SOB media: 1 liter containing

2% tryptone

0.5% yeast extract

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

-SOC media: 1 liter containing SOB plus 0.02 M glucose

-LB agar: 1 liter containing LB media plus 15 g of bacto agar (Difco)

-ampicilin

-DMSO (dimethyl sulfoxide)

-DTT: 2.25 M DTT in 40 mM potassium acetate pH 6.0

-TFB: 1 liter containing

100 mM rubidium chloride (Sigma)

45 mM $MnCl_2.4H_2O$

10 mM $CaCl_2.2H_2O$

3 mM hexamine cobalt chloride (Fluka)

10 mM morpholinoethane sulfonic acid (Sigma)

-X-gal

(5-bromo-4-chloro-3-indolyl-B-D-galactoside)

-IPTG (isopropylthiogalactoside)

Method

E. coli JM 107 was streaked on LB agar supplemented 100 g/ml ampicilin. A single colony was transfered to 50 ml SOB medium and shaked at 37°C until the cells were grown to log phase about 3 hours (OD 550nm = 0.4-0.5). The culture was transfered to centrifuge tube (Falcon 2057) and chilled on ice for 10 minutes.

These cells were collected cells by centrifigation at 3,000 rpm at 4°C for 12 minutes (Hettich Universal). The pellet was resuspended in 4 ml TFB and left on ice for 10 minutes, then it was centrifuged at 3,000 rpm 4°C for 10 minutes. These cells were suspended in 1 ml of cold TFB and added 140 ul of DMSO. The solution was left on ice for 5 minutes, 140 ul of DTT were added and left on ice for 10 minutes. Then, the solution was added 140 ul of DMSO and cooled on ice for 5 minutes.

210 ul of competent cells were transfered to cold centrifuge tube and ligated product from section 7 (500 ng) was added. The solution was gentle mixed and left on ice for 30 minutes. After that it was heat shocked in water bath at 42°C for exactly 90 second and quick cooled. The mixture was added 800 ul SOC medium and incubated at 37°C for 1 hour in shaking incubator. The 200 ul of transformed cells were spreaded on LB agar with supplemented 100 ug/ml ampicilin, 50 ul of 20 mg/ml x-gal and 200 mM IPTG.

9. Screening for recombinant DNA

Three steps were used to screen recombinant DNA, namely, colony hybridization, repeat colony hybridization and southern blot hybridization. The purified-mitochondria DNA of M. rosenbergii from section 3.3 was used as probe for screening.

9.1 Colony hybridization (Grunstein and Hogness, 1975)

9.1.1 Cell lysis

Materials

1. LB agar
2. Chemicals and solutions
   -0.5 N NaOH
   -1 M Tris-HCl pH 7.4
   -0.5 M Tris-HCl pH 7.4 and 1.5M NaCl
3. Nitrocellulose filter (82 mm 54 85/21 0.45 m Schleicher & Schuell)
4. Sterile toothpicks
5. recombinant clones from section 8

Method

The white bacterial colonies from section 8 were picked by sterile toothpicks and stabbed onto the nitrocellulose filter, which was placed onto an LB plate containing 100 ug/ml ampicilin, and then onto a master plate (no filter) in an identical position. Both plates were incubated at 37°C for overnight. The filter was transfered to another LB plate containing 20 ug/ml chloramphenical and incubated at 37°C for 5-6 hours to amplify the copy number of recombinant plasmid but the master plate was kept at 4°C to maintain cells.

The filter was removed from plate and placed (colony uppermost) on 0.75 ml of 0.5 N NaOH, which it was poured onto Saran Wrap, and left for 2-3 minutes. The filter was carefully

lifted up and blotted dry on 3MM paper (Whatman). This step for lyse cells and denature DNA was repeated about 3 times. The filter was neutralized 3 times by transfered to 0.75 ml of 1 M Tris-HCl pH 7.4 for 5 minutes and blotted dry. After that the filter was placed on 0.75 ml of 0.5 N Tris-HCl pH 7.4-1.5 M NaCl 3 times for 5 minutes to fix DNA on the filter. The filter was blotted dry in betwen two piece of 3MM paper and baked at 80° C for 2 hour. The filter was stored at 4° C in sealed plastic bag until hybridization.

9.1.2 Nick Translation of mtDNA probe (Rigby, 1977)

Materials

1. α-^{32}P dATP (Amersham; triethylammonium salt, 6000 Ci/mmol, 10 uCi/ml or Dupont; tetra (triethylammonium) salt, 5000 Ci/mmol, 20 uCi/ml)
2. Nick translation buffer
   50 mM Tris-HCl pH 7.5
   10 mM $MgCl_2$
   50 ug/ml nuclease-free BSA
   1 mM DTT
3. 10 umol/ul of dCTP, dGTP, dTTP
4. 500 ng mtDNA
5. 1 ng/ ul of DNase 1 (Sigma)
6. 1 unit of E. coli polymerase 1
7. 250 mM EDTA pH 8.0
8. distilled water
9. TE buffer
10. GF/A filter paper (Whatman)
11. 10% TCA, 5% TCA (trichloroacetic acid)

12. 95% ethanol

13. Sephadex G-50 (Pharmacia)

Method

The reaction mixture, 25 ul, contained 500 ng of mitochondria DNA, 2.5 ul nick translation buffer, 10 uM of each dGTP, dCTP and dTTP, 40 uCi of α-^{32}P dATP, 1 ng of DNase 1 and 1 unit of E. coli polymerase 1 and make volume to 25 ul by distilled water. The solution was incubated at 15°C until α-^{32}P dATP was maximum incorporated into the DNA.

Kinetics of the reaction was followed by periodic measurement of radioactivity of 1 ul of reaction mixture spotted on GF/A filter paper (Whatman) which was soaked with 10% TCA. The filter was washed twice with 5% TCA and 95% ethanol, dried and put into a scintillation vial containing 10 ml distilled water and counted in liquid scintillation counter (Beckman LS-100). Percentage of incorporation could be determined by comparision with the radioactivity of 1 ul of reaction mixture spotted on unwashing GF/A filter paper. When maximum incorporation was obtained, the reaction was stopped by addition of an equal volume of 250 mM EDTA pH 8.0, and heat at 65°C for 10 minutes. The labelled mtDNA was separated from free deoxynucleotide by passing through 1 ml column filled with Sephadex G-50, 50 ul of TE buffer were added and the colomn centrifuged for 1 minute. The eluate was collected for mtDNA probe.

9.1.3 DNA hybridization

Materials

1. Prehybridization (PHB) or Hybridization (HB) solution contained
   -50% formamide (99% Fluka)
   -5x SSC (1x standard saline citrate was 15 mM NaCl, 15 mM tri-sodium citrate pH 7.5)
   -5x Denhardt's solution (50x Denhardt solution was 10% Ficoll 400 (Pharmacia), 1% polyvinyl pyrolidone (Calbiochem), 1% bovine serum albumin (BSA Fraction v)(Sigma), 50 mM EDTA and 200 mM Tris-HCl pH 7.5)
   -Salmon sperm DNA (sonicated)
   -mtDNA probe

Method

The nitrocellulose filter (Scheicher & Schuell) or nylon membrane (Gene Screen Plus, Dupont) was transfered to plastic bag then added 7-10 ml of PHB solution and 100 ug/ml of sonicated salmon sperm DNA, which was heat denatured at 100°C for 10 minutes and quick cooled for 2-3 minutes. If the nylon membrane was used, it was contained 2% SDS. After mixed the solution, all air bubbles were removed and the plastic bag was sealed and incubated at 42°C overnight.

After the nitrocellulose filter or nylon membrane was prehybridized, it was squeezed out PHB solution and added new PHB solution about 5-7 ml and contained 2% SDS if the nylon membrane was used. Then the 100 ug/ml of heat denatured salmon sperm DNA

and heat denatured mtDNA probe (specific activity about $1\text{-}5x10^7$ cpm/ ug DNA) were added. After the bag was removed all air bubble, it was sealed with plastic sealer and incubated at 42°C for 36-48 hour in shaker.

9.1.4 Washing to removed non-hybridized probe

Materials

1. 20x SSC: 1 liter was contained
   - -3 M NaCl
   - -0.3 M Trisodium citrate pH 7.0
2. 10% SDS

Method

All of the HB solution was squeezed out and the filter was washed twice with 3x SSC at room temperature with gently shaking for 30 minutes. The filter was washed twice at 62°C with gently shaking in 0.1x SSC-0.1% SDS for 45 minutes and dried on 3MM paper (Whatman) and put into sealed-plastic bag.

9.1.5 Autoradiography

Materials

1. x-ray film
2. developer (Kodax x-ray developer)
3. fixer (Kodax x-ray fixer)

Method

The washed filter was exposed to x-ray film in an x-ray cassette with intensifying screen and left at -70°C for appropriate time. The signal was observed after washing the expose film in developer and fixer as follow:

The x-ray cassette was removed from -70°C freezer and allowed to warm up to 30 minutes. The film was removed from the x-ray cassette in the darkroom, rinsed with water and immersed in developer for 2-3 minutes until the signal was seen. The film was washed with water and immersed in fixer until the film was clear. Finally the film was washed in water before air drying at room temperature.

9.1.6 Rehybridization of DNA probe

Materials

1. 0.4 N NaOH
2. 0.1x SSC, 0.1% SDS and 0.2 M Tris-HCl pH 7.5

Method

The nylon membrane was incubated in 200 ml of 0.4 N NaOH at 42°C for 30 minutes with gentle agitation. Then it was incubated in 200 ml of 0.1x SSC, 0.1% SDS and 0.2 M Tris-HCl pH 7.5 at 42°C for 30 minutes. After that the nylon membrane was blotted dry between 3MM paper and autoradiograph for an appropriate period of time to determine. If sufficient probe was removed, it was prehybridized and hybridized again or stored at

4°C.

9.2 Southern blot hybridization (Southern, 1975)

Materials

1. Nylon membrane (Dupont) cut to dimension of agarose gel
2. 20x SSC: 1 liter contained
   3 M NaCl
   0.3 M trisodium citrate pH 7.0
3. 0.5 N NaOH-0.5 M NaCL
4. 1 M Tris-HCl pH 8.0-1.5 M NaCl
5. Whatman 3MM filter paper

Methods

9.2.1 Gel treatment

The agarose gel was gently shaken in 200 ml of 0.5 N NaOH-1.5 M NaCl on shaker at room temperature for 30 minutes, DNA was denature. After that the gel was neutralized by shaking in 200 ml of 1 M Tris-HCl pH 8.0-1.5 M NaCl at room temperature for 30 minutes.

9.2.2 Nylon membrane treatment

Nylon membrane was cut to a size of 0.3 centrimeter larger than the gel and marked the orientation. After soaking the membrane in sterile distilled water, it was soaked with 10x SSC for 30 minutes.

9.2.3 Transfer of DNA to membrane

Three centrimeter pile of Whatman 3MM paper was soaked with 10x SSC, and laid on Saran Wrap. The gel was laid on top of the paper. All air bubbles between the gel and the paper were removed. A nylon membrane was placed on top of the gel and all air bubbles were removed. Ten centrimeter pile of Whatman 3MM paper cut to a size 0.5 centrimeter larger than the gel was placed on top of the nylon membrane. The 10x SSC solution was added to saturate the Whatman 3MM paper which was under the gel. The blotting set was completely covered with Saran Wrap to prevent evaporation of the solution, and a weight was placed on the blotting set (Fig. 3). The transfer step was complete after 12-24 hour. The nylon membrane was removed and shaken in 0.4 N NaOH for 40-60 second to completely denature of the DNA on the membrane. Then, the bounded DNA was neutralized in 0.2 M Tris-HCl pH 7.5-2x SSC for a few minutes. The gel was stained in 2.5 ug/ml ethidium bromide to check for completeness of transfer. The nylon membrane was air dried on Whatman 3MM paper and kept in seal-plastic bag at $4^{o}C$ until used.

9.2.4 Nick translation and Hybridization

After transfering the DNA fragments from the gel to nylon membrane, the nylon membrane was hybridized with radiolabelling mtDNA by Nick translation method (see section 9.1.2), for screening inserted fragments which were complementary with mtDNA and highly repetitive by DNA-DNA hybridization method (see section 9.1.3)

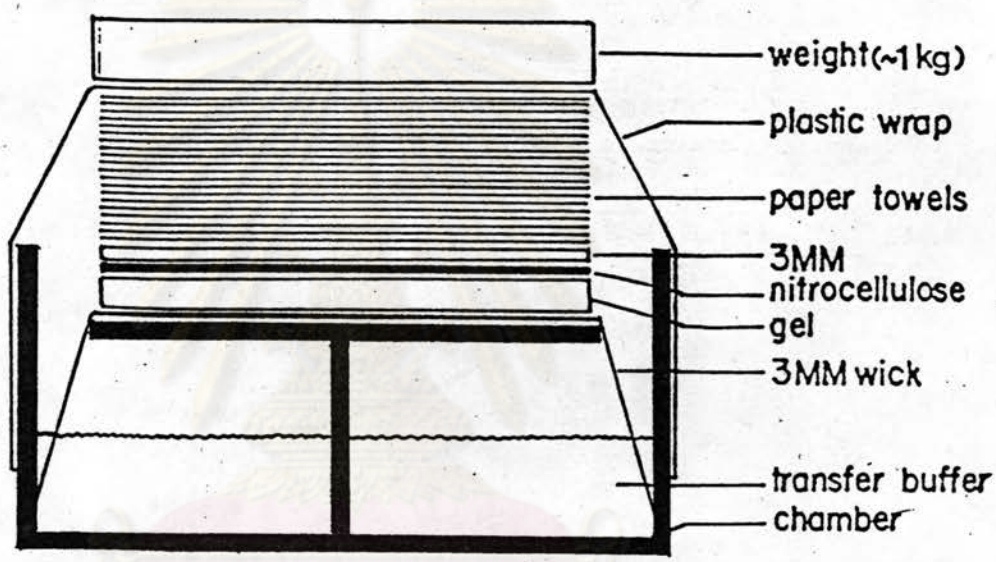

Figure 3 Method of transfer DNA from agarose gel to nitrocellulose or nylon membrane. (Southern blot)

10. Detection RFLP of mtDNA in each individual prawns

Materials

1. The hepatopancreas of M. rosenbergii which was caught from various resources
   -Bangpakong River, Chachoengsao Province.
   -Kraburi River, Ranong Province.
   -Kung Kam Thong Farm, Pathumthani Province.
2. Chemicals and solutions
   -1x pronase buffer: 10 mM Tris, 10 mM EDTA and 150 mM NaCl
   -20% SDS
   -10 mg/ml proteinase K in 1x pronase buffer
   -Water saturated distilled phenol containing 0.1% of 8-hydroxy quinoline
   -3% isoamyl alcohol in chloroform
   -diethyl ether
   -absolute ethanal
   -70% ethanal
   -TE buffer

Method

The crude mitochondria pellet was extracted from 2-3 g of hepatopancreas in each individual prawn as described in Materials and Methods (see section 1.1) but centrifuged at low speed, 3,000 rpm, 2 times before centrifugation at high speed.

The crude mitochondria pellet was resuspended in 500 ul of 1x pronase buffer. The suspension was added 25 ul of 20% SDS

and 25 ul of 10 mg/ml proteinase K in 1x pronase buffer and incubated at 37°C for 30 minutes. The solution was extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) 3 times. The aqueous phase was extracted twice with equal volume of chloroform:isoamyl alcohol (24:1) and extracted with equal volume of diethylether twice. The mtDNA was precipitated by adding 1/10 volume of 3 M sodium acetate pH 5.5 and 2 volumes of absolute ethanal and kept at -20°C for overnight. The mtDNA was pelleted by centrifugation at 10,000 rpm for 10 minutes and washed with 70% ethanal twice and dried by heat at 65°C for 15 minutes. The mtDNA pellet was dissolved in 20-50 ul TE buffer.

The mtDNA was digested with restriction enzyme Sau 3A1 (see section 6.4). After separating the mtDNA fragments on 1.2% agarose gel electrophoresis. The mtDNA fragments were transfered from agarose gel to nylon membrane by southern technique and hybridized with radiolabelling recombinant DNA (see section 9.2). The amount of recombinant DNA in the mixture was 200 ng and α-^{32}P dATP was 20 uCi.