Chapter 3

Results

1. Preparation of Mitochondria

The hepatopancreas of giant freshwater prawn was used for isolating mitochondria. This tissue was large and soft so that it was easy for homogenization. The muscle tissue was difficult to homogenize and the mitochondria was destroyed. The relatively low abundance of mitochondria in muscle demanded large amount of tissues which was necessary to separate muscle fibrils from mitochondria by centrifugation at very large volume. That limited the number of samples which can be processed simultaneously and requires somewhat specialized equipment.

The mitochondria from a soft brown pellets were obtained by differential centrifugation, low speed and high speed, should be considered "crude mitochondria" (Fig 4) and relatively pure with simple repeated washing (two or three times) in homogenizing medium. In other case, the purification of crude mitochondria were necessary not only on size but also on density that could be performed by sucrose gradients centrifugation which should be considered "purified mitochondria". (Fig 5)

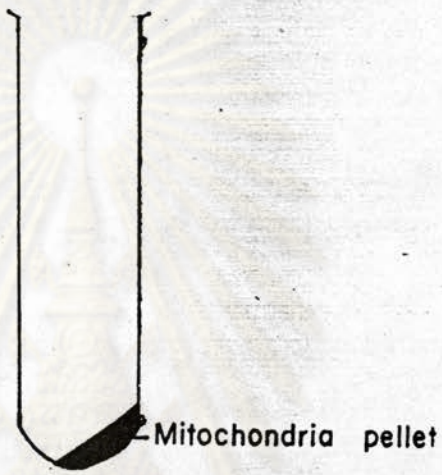


Figure 4 The crude mitochondria pellet was isolated by differential centrifugation

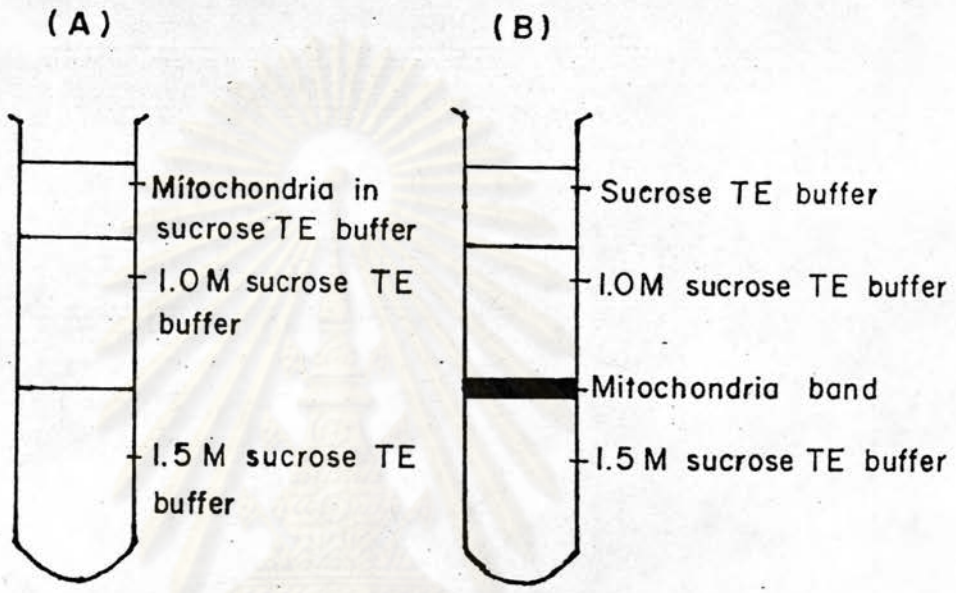


Figure 5 The purified mitochondria was isolated by sucrose gradient centrifugation

(A) before centrifugation
(B) after centrifugation

2. Analysis of Mitochondria

The enzyme succinate dehydrogenase was found exclusively in inner membrane of mitochondria, consequently, any centrifugal fraction that exhibited succinate dehydrogenase activity would be expected to contain mitochondria. The prawn mitochondria fraction was obtained by differential centrifugation. This fraction was used to assay succinate dehydrogenase activity as the marker enzyme for mitochondria. By Phenazine Methosulfate Method, this indicated that this fraction exhibited succinate dehydrogenase activity which decreased absorbance at 600 nm. (Fig 6)

This reaction could be explained;

$$\text{Succinate} + \text{PMS} \xrightarrow{\text{succinate dehydrogenase}} \text{Fumarate} + PMSH_2$$

$$PMSH_2 + \text{DCIP} \longrightarrow \text{PMS} + DCIPH_2$$

The succinate dehydrogenase can also catalyze the oxidation of succinate by a number of artificial electron acceptors. The PMS, Phenazine Methosulfate, was used as an intermediate electron carrier and DCIP, 2, 6 Dichlorophenol-indophenol, was a final acceptor.

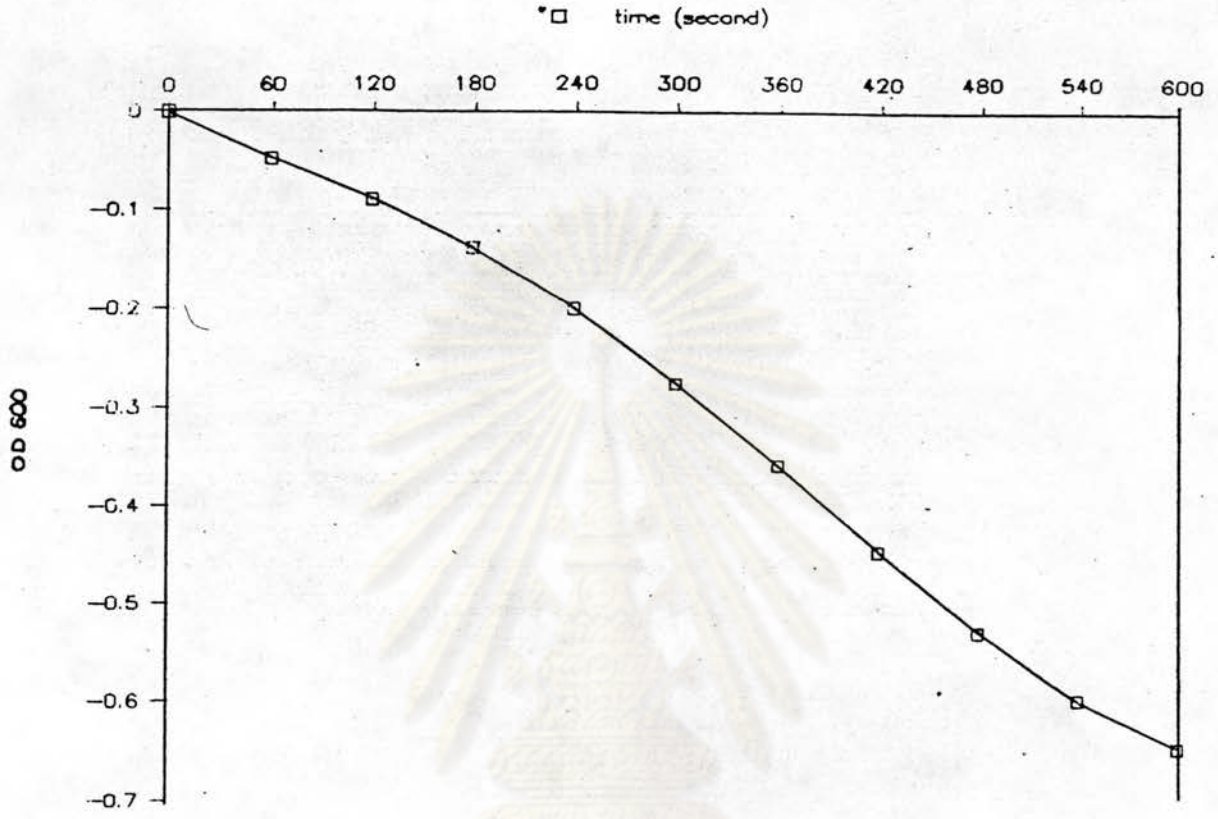


Figure 6 Kinetic of enzyme succinate dehydrogenase of mitochondria pellet from hepatopancreas of M. rosenbergii

3. Mitochondria DNA extraction

3.1 Cesium Chloride-Ethidium Bromide Gradients Method

The Cesium Chloride-Ethidium Bromide Gradients Method can separate two DNA bands, upper and lower bands, (Fig 7). The characterization of both DNA bands were analyzed on 0.7% agarose gel electrophoresis. There was no clear difference in these DNA bands (Fig 8A). When both DNA bands were digested with some restriction enzymes and separated on 0.7% agarose gel electrophoresis. It was found that the lower band DNA generated fragments at 10.2 and 5.0 Kb with Ava 1, 16.0 and 4.5 Kb with Bam H1 and 20.0 Kb with Eco R1 digestion. In contrast, the upper band DNA did not show any discrete fragments (Fig 8B). These indicated that the upper band DNA was nuclear DNA which had heterogeneous fragments and the lower band DNA was mitochondria DNA which contained homogeneous ones.

3.2 Rapid Alkaline Method

The mitochondria DNA extracted from crude mitochondria by using rapid alkaline method. It was showed the smear band (Fig 9) and this become less when purified mitochondria was used (Fig 10). This indicated that the purified mitochondria contained less contamination of nuclear DNA. The total yield of mitochondria DNA which extracted from purified mitochondria was measured by OD 260 nm approximately 800 ng/g tissue. After digestion mitochondria DNA with restriction enzymes and analyzed on 0.7% agarose gel electrophoresis. It was found discrete band at 10.2, 5.0 and 1.8 Kb with Ava 1 , 16.0 and 4.5

Kb with Bam H1 and 20.0 Kb with Eco R1 digestion (Fig 10). These digestion patterns were similar to those of mitochondria DNA obtained from lower band DNA of Cesium chloride-Ethidium bromide gradient method.

3.3 Phenol Extraction Method

The mitochondria DNA extraction from crude mitochondria and purified mitochondria using phenol extraction was similar to those obtained from rapid alkaline method. The mitochondria DNA obtained from purified mitochondria contained less nuclear DNA contamination than crude mitochondria (Fig 11, 12). The digestion patterns of this mitochondria DNA, which was extracted from purified mitochondria, appeared discrete band at 16.0 and 4.5 Kb with Bam H1 and 20.0 Kb with Eco R1 (Fig 12) which was similar to those of mitochondria DNA obtained from the two method as above.

This extract method of mitochondria DNA from purified mitochondria by Phenol extraction gave high yield of mitochondria DNA, the total yield of mitochondria DNA was measured by OD 260 nm approximately 3 ug/g tissue, and less nuclear DNA contamination. It was considered to be "purified-mitochondria DNA" which was suitable for cloning and used as a probe for screening the recombinant DNA.

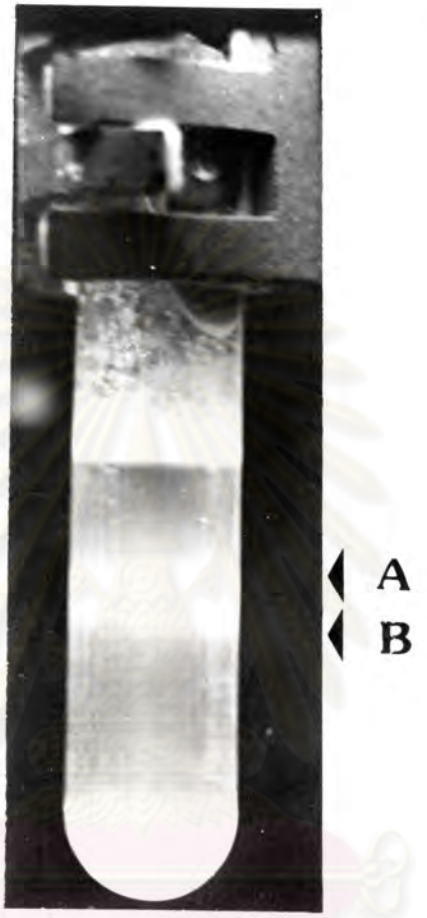


Figure 7 A photograph under UV wavelength of the centrifuge tube showed band of DNA which was extracted by Cesium Chloride-Ethidium bromide gradient method

A = upper band DNA

B = lower band DNA

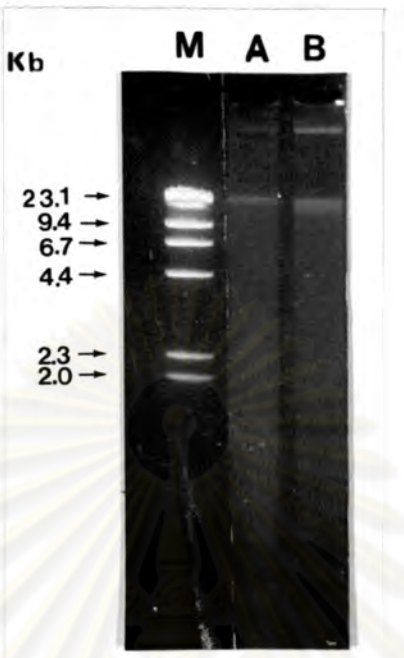
Figure 8 (A) Ethidium bromide staining pattern of two band DNA which was extracted by Cesium Chloride-Ethidium bromide gradient method and analyzed on 0.7% agarose gel electrophoresis

(B) Ethidium bromide staining pattern of two band DNA which was digested with various restriction enzymes and analyzed on 0.7% agarose gel electrophoresis (arrow indicate the descreat band)

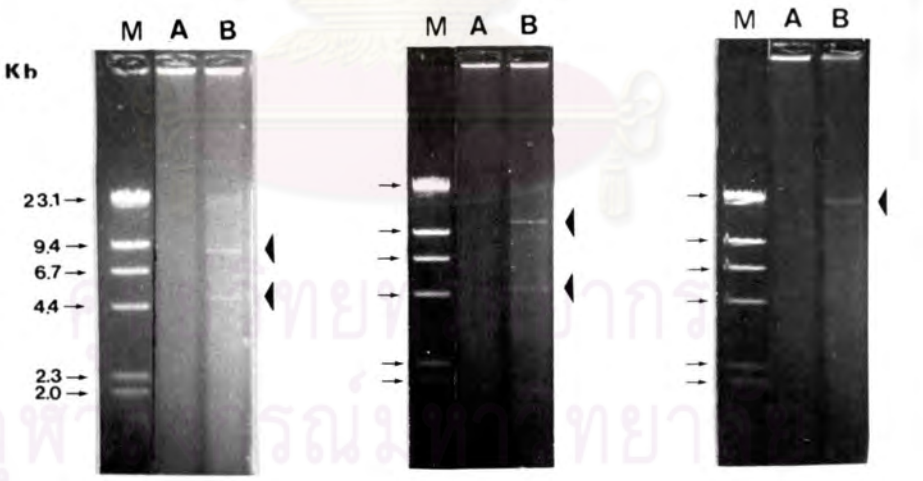
M = λ DNA cut with Hind 111 is a size marker

A = upper band DNA

B = lower band DNA


Kb
M A B
23.1
9.4
6.7
4.4
2.3
2.0

A


M A B
Kb
23.1
9.4
6.7
4.4
2.3
2.0
M A B
M A B
Ava I
BamH I
EcoR I

B

Figure 8

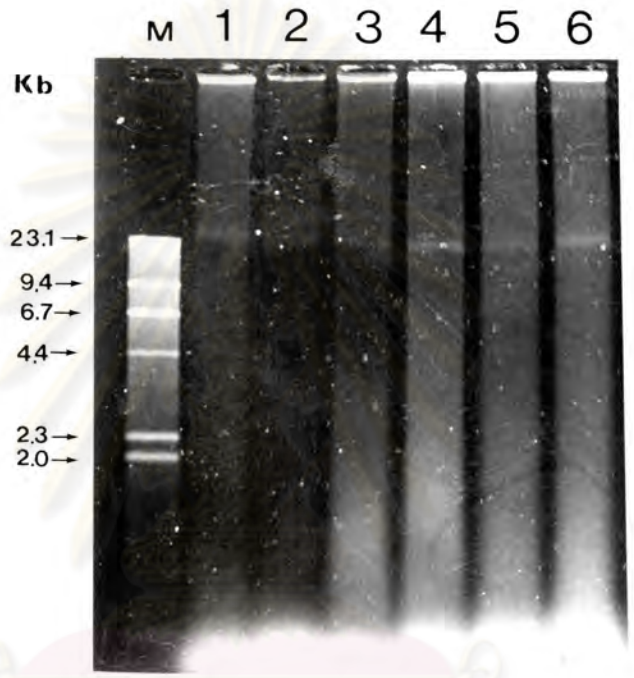


Figure 9 Ethidium bromide staining pattern of mtDNA which was extracted by rapid alkaline method from crude mitochondria and analyzed on 0.7% agarose gel electrophoresis

M = λ DNA cut with Hind 111 is a size marker

1-6 = mtDNA extracted from each individual

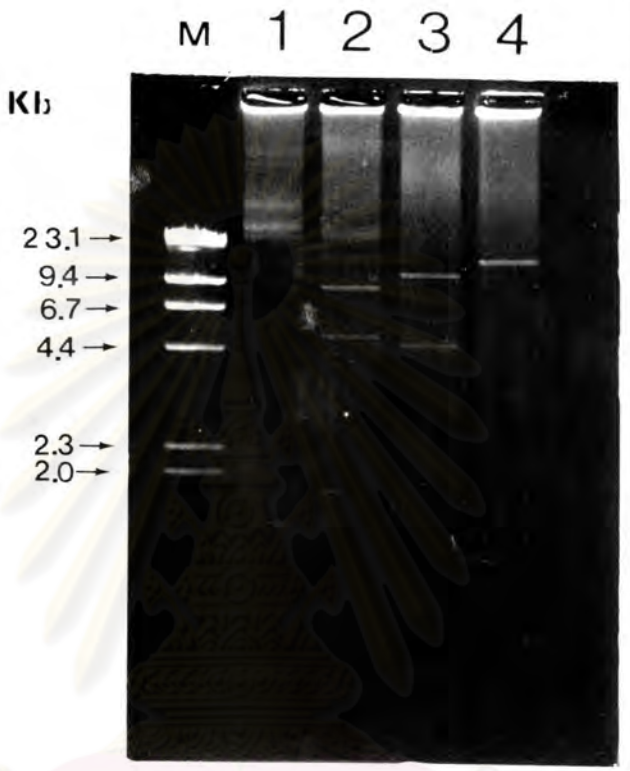


Figure 10 Ethidium bromide staining pattern of mtDNA which was extracted by rapid alkaline method from purified mitochondria and analyzed on 0.7% agarose gel electrophoresis

M = λ DNA cut with Hind 111 is a size marker

1 = uncut mtDNA

2 = mtDNA digested with Ava 1

3 = mtDNA digested with Bam H1

4 = mtDNA digested with Eco R1

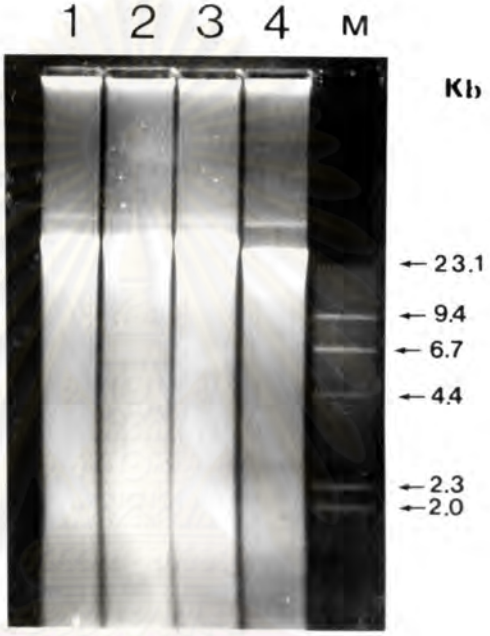


Figure 11 Ethidium bromide staining pattern of mtDNA which was extracted by phenol extraction from crude mitochondria and analyzed on 0.7% agarose gel electrophoresis

M = λ DNA cut with Hind 111 is a size marker

1-4 = mtDNA extracted from each individual

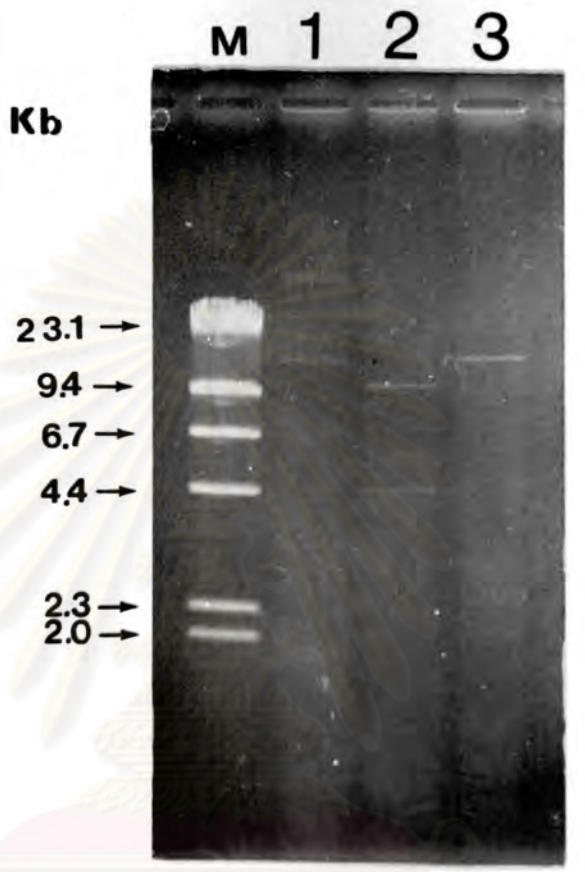


Figure 12 Ethidium bromide staining pattern of mtDNA which was extracted by phenol extraction from purified mitochondria and analyzed on 0.7% agarose gel electrophoresis

M = λ DNA cut with Hind 111 is a size marker

1 = uncut mtDNA

2 = mtDNA digested with Bam H1

3 = mtDNA digested with Eco R1

4. Restriction enzyme cut pattern of mtDNA for cloning

The mtDNA was digestd with restriction enzyme Sau 3A1 for generating small fragments. The restriction enzyme cut pattern of these fragments were characterized by 1.2% agarose gel electrophoresis. These fragments were about 0.2-2.0 Kb. (Fig 13). The ligation of the resulting fragments into Bam H1 site of vector pUC 12 as described in Materials and Methods (see section 7) by the cohesive 5' termini of Sau 3A1 digested mtDNA was compatable to the 5' protruding end of the vector.

5. Transformation

After the ligation, the recombinant DNA molecules were transformed into E. coli strain JM 107 in which they were propagated. The DMSO treatment method was employed to prepare competent cells which were capable to being transformed by plasmid DNA. The 2,500 recombinant clones, which could be identified by their white colonies from about $2x10^4$ transformants were obtained.

The transformation of the intact vector pUC 12 was employed as a positive control and plasmid free host cells which had been plated on ampicilin-containing agar plate were used as negative control. The positive control gave transformation efficiency about $1x10^7$ transformants/ug plasmid DNA and in negative control the cells were not grown.

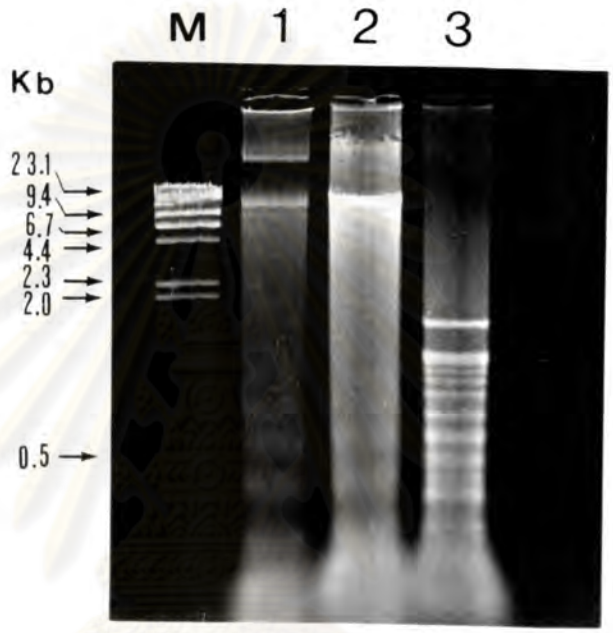


Figure 13 Ethidium bromide staining pattern of mtDNA which was digested with some restriction enzymes and analyzed on 1.2% agarose gel electrophoresis

M = λ DNA cut with Hind 111 is a size marker

1 = uncut mtDNA

2 = mtDNA digested with Eco R1*

3 = mtDNA digested with Sau 3A1

6. Colony hybridization

All of the 2,500 recombinant clones had colorless colonies. These colonies were picked up to nitrocellulose filter and hybridized with radiolabelling mtDNA for screening the inserted mtDNA fragments as described in Materials and Methods (see section 9.1) (Fig 14). The 51 colonies which produced intense signal were picked up onto a new nitrocellulose filter and they were rechecked by rehybridization with radiolabelling mtDNA again. It was found that all 51 recombinant colonies gave strong signal on autoradiogram (Fig 15).

7. Characterization of recombinant plasmid

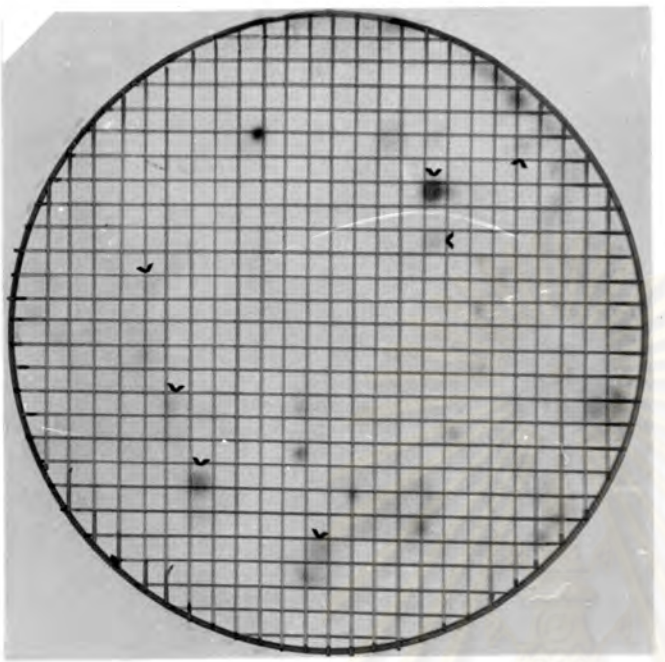
Recombinant plasmids number 1-51 were extracted using rapid alkaline method. The recombinant plasmids were analyzed on 0.7% agarose gel electrophoresis to check their forms and concentration (Fig 16,17). Then these recombinant plasmids were linearized by single digestion with restriction enzyme Eco R1 and analyzed on 0.7% agarose gel electrophoresis. The linear form of recombinant plasmids showed various size of inserted fragments because they had size variation and larger than linear form of vector pUC 12 (Fig 18a, 19a, 20a and 21a). The linear form of recombinant plasmids were transfered from agarose gel to nylon membrane using southern blot technique and were hybridized with radiolabelling mtDNA. The mtDNA probe can be hybridized with all 51 recombinant plasmids but little hybridized with vector pUC 12 (Fig 18b, 19b, 20b and 21b). Such results demonstrated that the inserted fragments of recombinant plasmids contained in mtDNA genome. Only 17 recombinant plasmids, which had different linear

size and strong signal, were double digested with Eco R1 and Xba 1 to separate inserted fragment and analyzed on 1.2% agarose gel electrophoresis for estimating size of inserted fragment (Fig 22a). Then these double digested recombinant plasmids were used for southern blot hybridization with radiolabelling mtDNA (Fig 22b). The 17 inserted fragments were hybridized with mtDNA probe in different intense signal.

The recombinant plasmids were selected for further study based on their strong hybridization with mtDNA probe. These clones were used as probe to detect restriction fragment length polymorphism (RFLP) of mtDNA from giant freshwater prawn by southern blot hybridization.

Figure 14 Two sample filters from autoradiogram of first colony hybridization with nick-translated mtDNA probe. The specific activity of the probe was 1.2×10^7 cpm/ug DNA

Arrow indicate colony with strong intensity signal in autoradiogram. From 2,500 white colonies, the 51 colonies were selected.



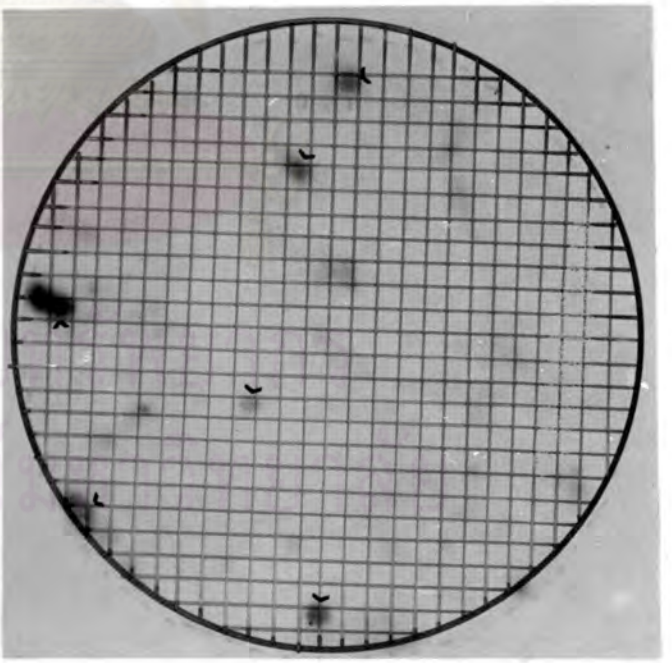
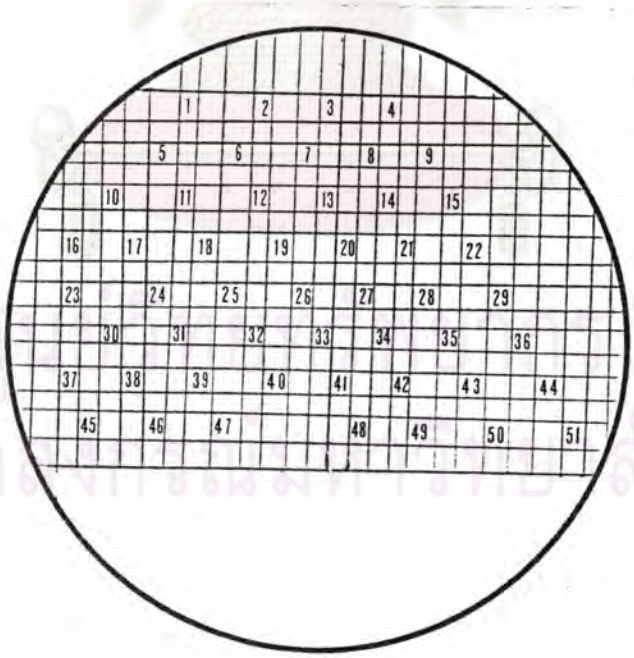


Figure 14

Figure 15 (A) Autoradiogram of second colony hybridization of 51 recombinant colonies with nick-translated mtDNA probe. The specific activity of the probe was 2.0×10^7 cpm/ug DNA

(B) The position of 51 recombinant colonies

A


1
2
3
4
5
6
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10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
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43
44
45
46
47
48
49
50
51

B

Figure 15

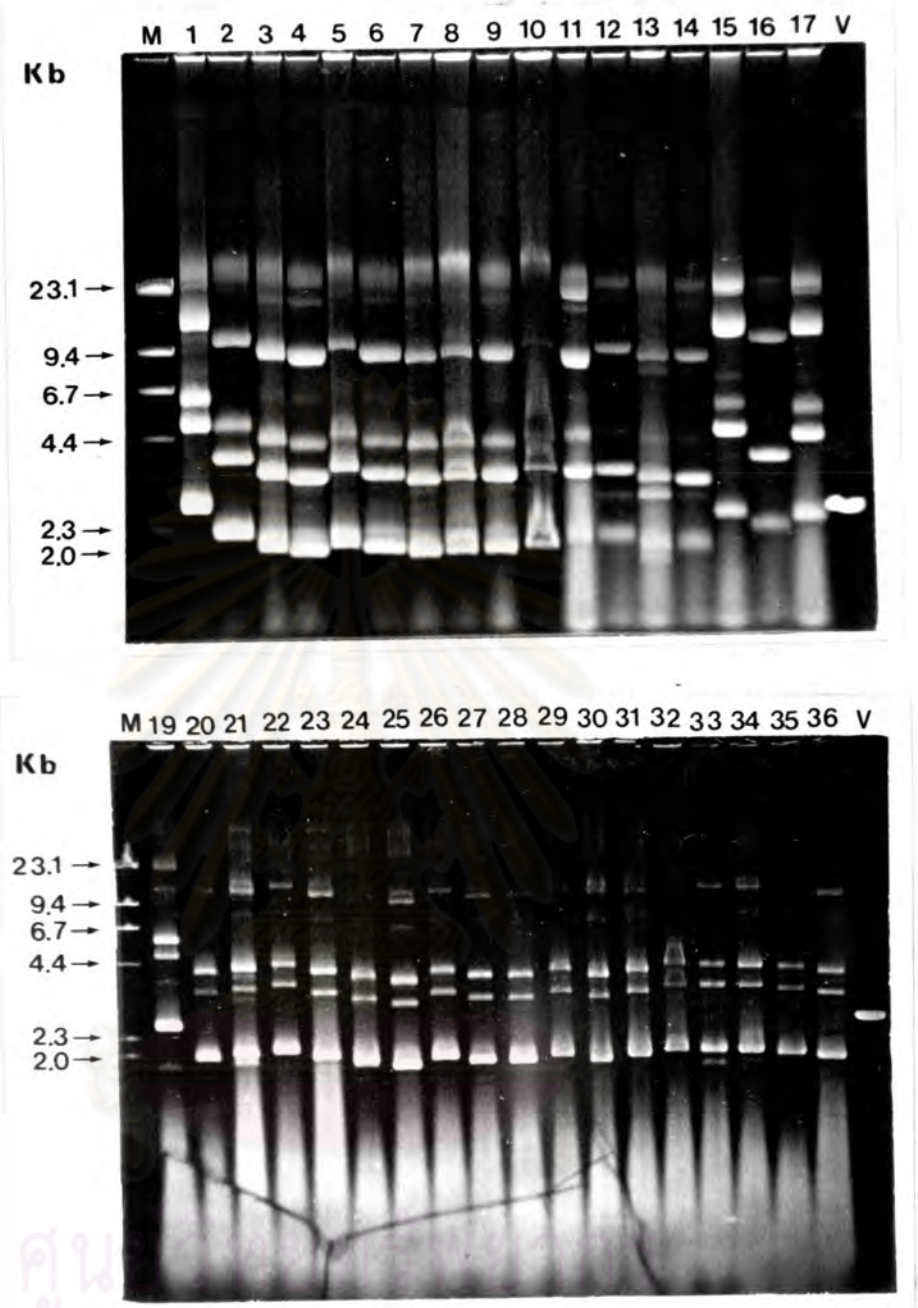


Figure 16 Ethidium bromide staining pattern of recombinant DNA which was extracted by rapid alkaline method and analyzed on 0.7% agarose gel electrophoresis

M = λ DNA cut with Hind 111 is a size marker

1-36 = number of recombinant DNA

V = vector pUC12 which was linearlized by Bam H1

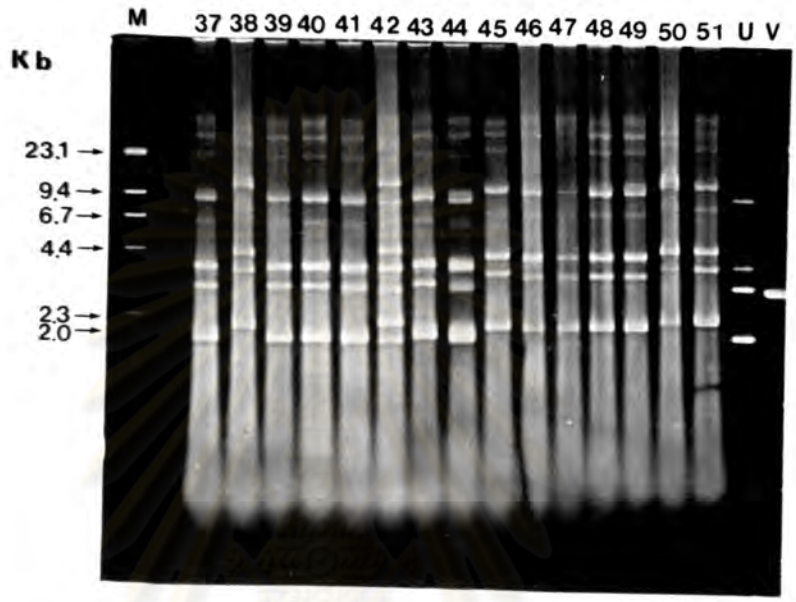


Figure 17 Ethidium bromide staining pattern of recombinant DNA which was extracted by rapid alkaline method and analyzed on 0.7% agarose gel electrophoresis

M = λ DNA cut with Hind 111 is a size marker

37-51 = number of recombinant DNA

U = vector pUC12 was uncut

V = vector pUC12 which was linearlized by Bam H1

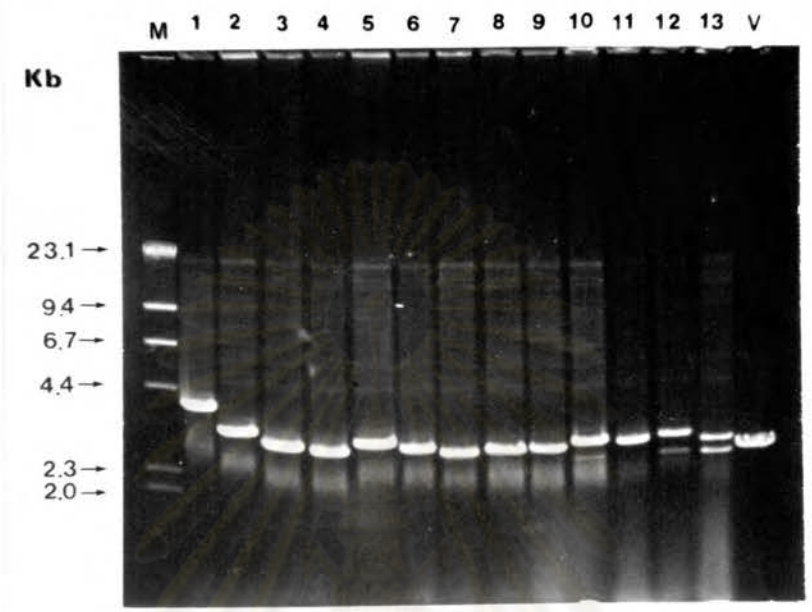
Figure 18 (A) Ethidium bromide staining pattern of recombinant DNA which was digested with Eco R1 and analyzed on 0.7% agarose gel electrophoresis.

(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated mtDNA probe. The specific activity of the probe was 2.7×10^7 cpm/ug DNA

M = λ DNA cut with Hind 111 is a size marker

1-13 = number of recombinant DNA

V = vector pUC12 which was linearlized by Bam H1


M 1 2 3 4 5 6 7 8 9 10 11 12 13 V
Kb
23.1
9.4
6.7
4.4
2.3
2.0

A


1 2 3 4 5 6 7 8 9 10 11 12 13 V

B

Figure 18

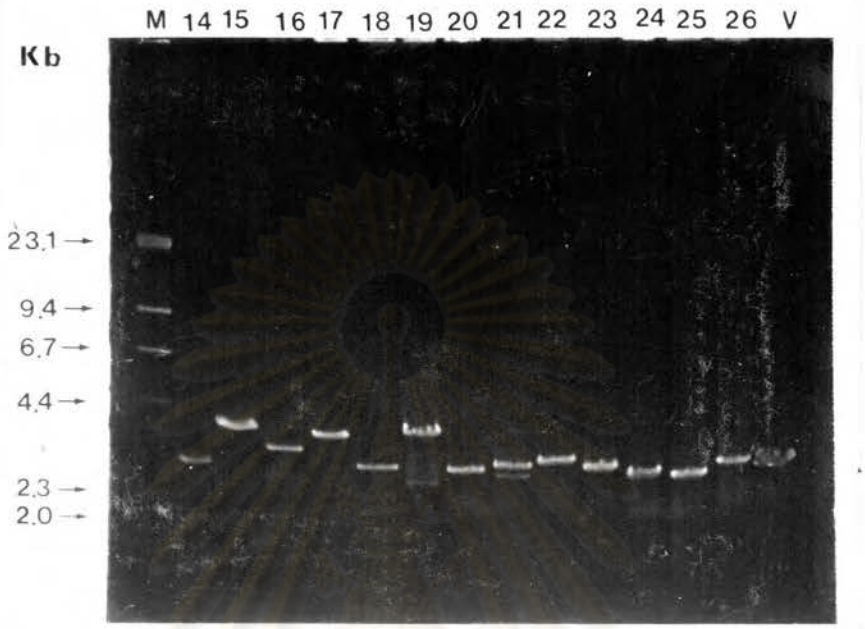
Figure 19 (A) Ethidium bromide staining pattern of recombinant DNA which was digested with Eco R1 and analyzed on 0.7% agarose gel electrophoresis.

(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated mtDNA probe. The specific activity of the probe was 3.0×10^7 cpm/ug DNA

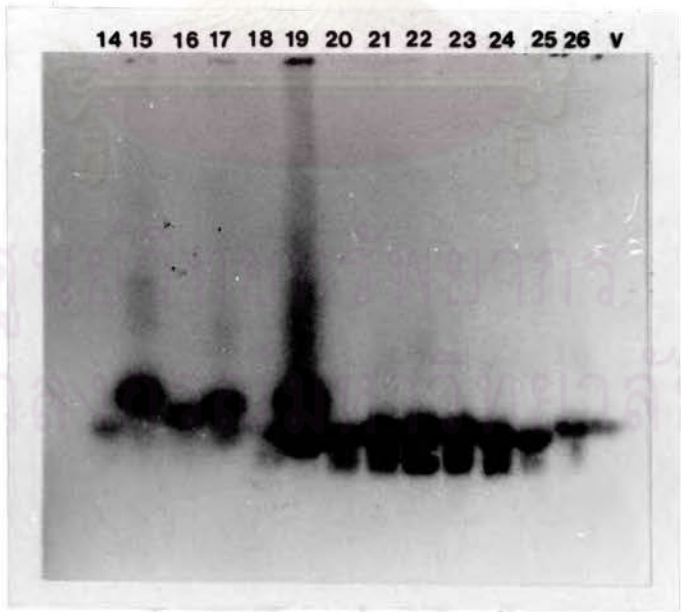
M = λ DNA cut with Hind 111 is a size marker

14-26 = number of recombinant DNA

V = vector pUC12 which was linearlized by Bam H1


M 14 15 16 17 18 19 20 21 22 23 24 25 26 V
Kb
23.1
9.4
6.7
4.4
2.3
2.0

A


14 15 16 17 18 19 20 21 22 23 24 25 26 V

B

Figure 19

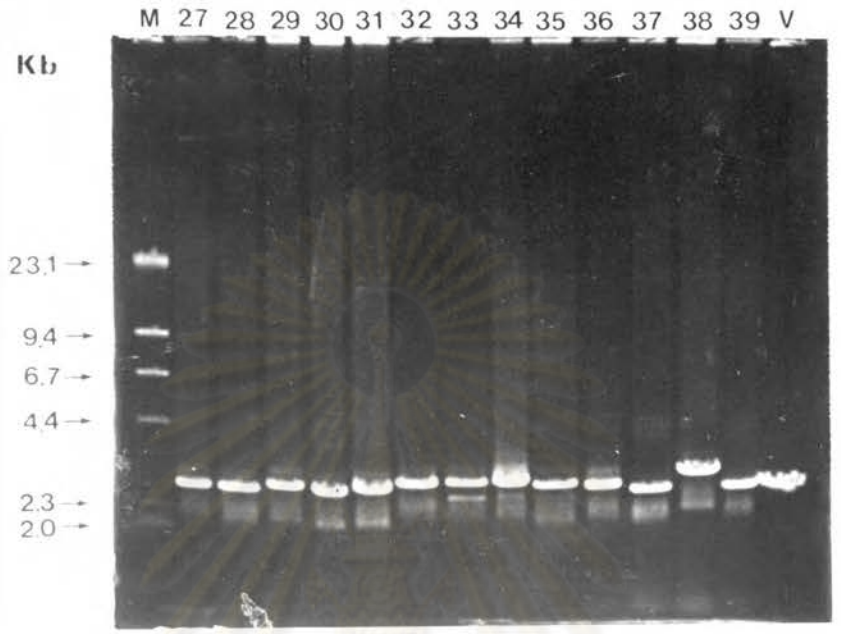
Figure 20 (A) Ethidium bromide staining pattern of recombinant DNA which was digested with Eco R1 and analyzed on 0.7% agarose gel electrophoresis.

(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated mtDNA probe. The specific activity of the probe was 2.0×10^7 cpm/ug DNA

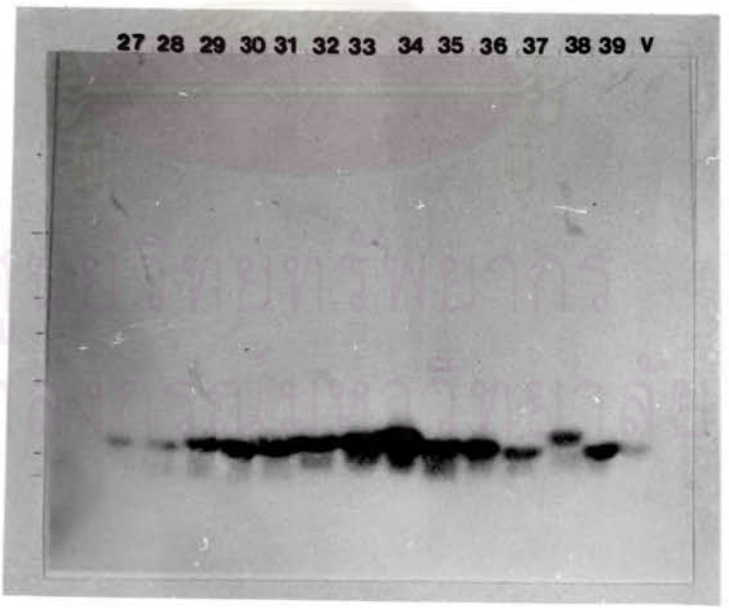
M = λ DNA cut with Hind 111 is a size marker

27-39 = number of recombinant DNA

V = vector pUC12 which was linearlized by Bam H1


M 27 28 29 30 31 32 33 34 35 36 37 38 39 V
Kb
23.1
9.4
6.7
4.4
2.3
2.0

A


27 28 29 30 31 32 33 34 35 36 37 38 39 V

B

Figure 20

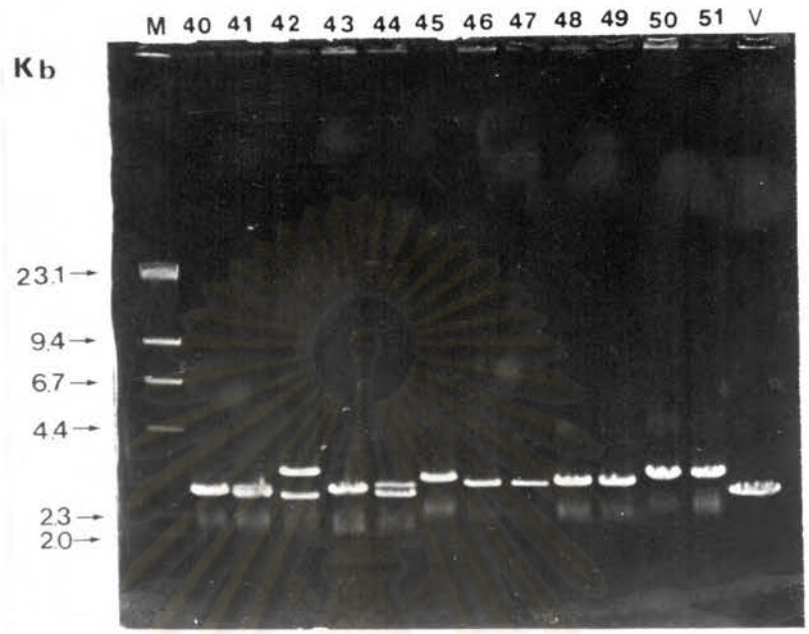
Figure 21 (A) Ethidium bromide staining pattern of recombinant DNA which was digested with Eco R1 and analyzed on 0.7% agarose gel electrophoresis.

(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated mtDNA probe. The specific activity of the probe was 4.3×10^7 cpm/ug DNA

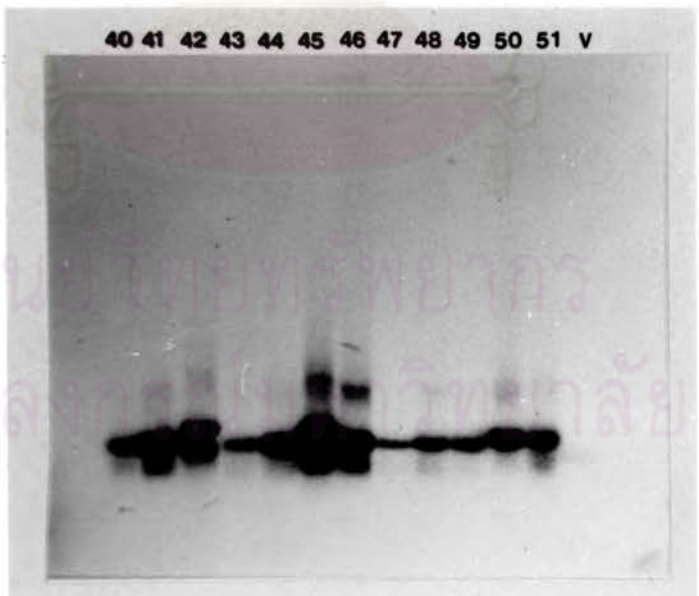
M = λ DNA cut with Hind 111 is a size marker

40-51 = number of recombinant DNA

V = vector pUC12 which was linearlized by Bam H1


M 40 41 42 43 44 45 46 47 48 49 50 51 V
Kb
23.1
9.4
6.7
4.4
2.3
2.0

A


40 41 42 43 44 45 46 47 48 49 50 51 V

B

Figure 21

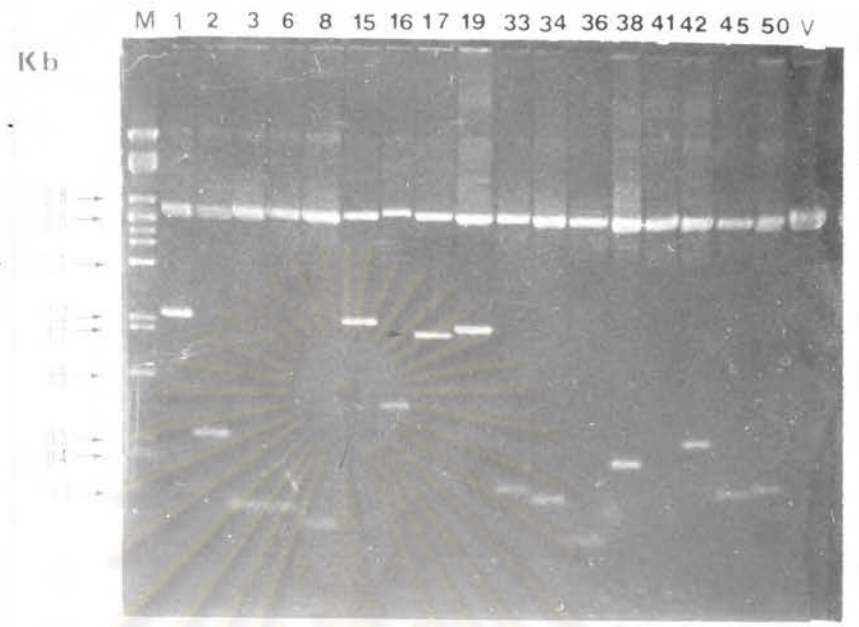
Figure 22 (A) Ethidium bromide staining pattern of recombinant DNA which was digested with Eco R1/Xba 1 and analyzed on 0.7% agarose gel electrophoresis

(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated mtDNA probe. The specific activity of the probe was 4.6x10 cpm/ug DNA

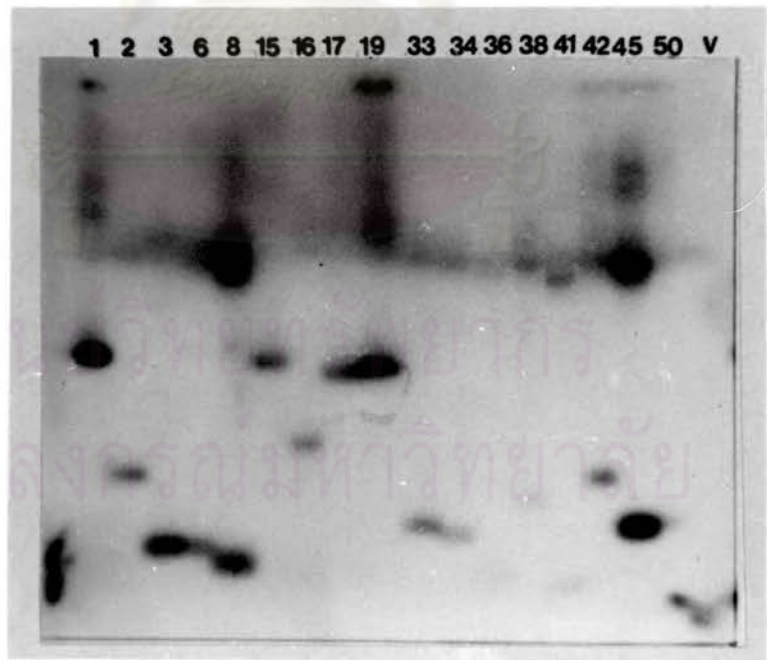
M = λ DNA cut with Pst 1 is a size marker

1-50 = number of recombinant DNA

V = vector pUC12 which was linearlized by Bam H1


M 1 2 3 6 8 15 16 17 19 33 34 36 38 41 42 45 50 V
Kb

A


1 2 3 6 8 15 16 17 19 33 34 36 38 41 42 45 50 V

B

Figure 22

8. RFLP of mtDNA in prawn from various resources

The mitochondria DNA (mtDNA) extracted from a hepatopancreas of individual prawn as described in Materials and Methods (see section 10) was obtained about 50-100 ug. The ratio of OD 260:280 nm was 1.8-2.0 which revealed good quality of DNA. The mtDNA of a prawn was digested with 4 restriction enzymes, Sau 3A1, Eco R1, Hae 111 and Hha 1. The digested mtDNA was analyzed on 1.2% agarose gel electrophoresis and stained with ethidium bromide showing unclear discrete band (Fig 23a). After southern blot hybridization with recombinant plasmid no 1 (probe 1) and recombinant plasmid no 45 (probe 45), both recombinant plasmids gave discrete band and strong signal with Sau 3A1 digestion (Fig 23b and 23c, respectively).

Then mtDNA was extracted from each individual prawn which was caught from various resources, Kung Kam Thong Farm, Bangpakong River and Kraburi River. These mtDNA were digested with restriction enzyme Sau 3A1 and analyzed on 1.5% agarose gel electrophoresis. It demonstrated no difference in these resources from ethidium bromide staining pattern (Fig 24a). When conducting southern blot hybridization with probe 1 and probe 45, the result of autoradiogram showed the same discrete band of probe 45 but probe 1 showed variation of discrete band in other resources (Fig 24b). New samples from these resources were repeated hybridization with probe 1 alone. The prawn from Kung Kam Thong Farm and Bangpakong River demonstrated discrete band at about 1.1 kb. but the prawn from Kraburi River demonstrated discrete band at about 0.7 kb. which was homologous with probe 1 (Fig 25).

Figure 23 (A) Ethidium bromide staining pattern of mtDNA which was digested with various restriction enzymes and analyzed on 1.2% agarose gel electrophoresis

(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated probe 1. The specific activity of the probe was 3.0x10 cpm/ug DNA

(C) Autoradiogram of rehybridization from filter in panel (B) with nick-translated probe 45. The specific activity of the probe was 4.0x10 cpm/ug DNA

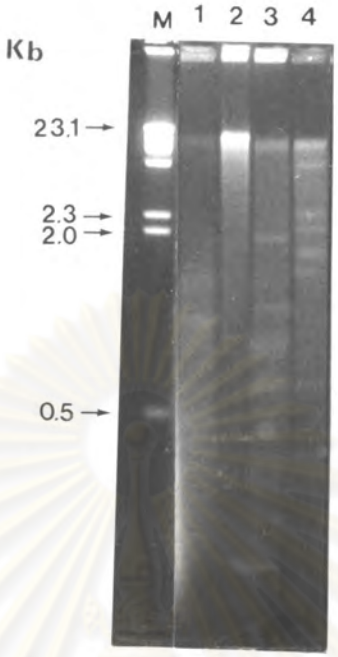
M = λ DNA cut with Hind 111 is a size marker

1 = mtDNA digested with Sau 3A1

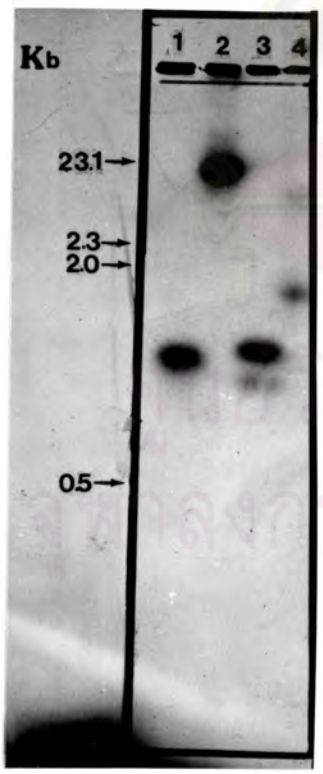
2 = mtDNA digested with Eco R1

3 = mtDNA digested with Hae 111

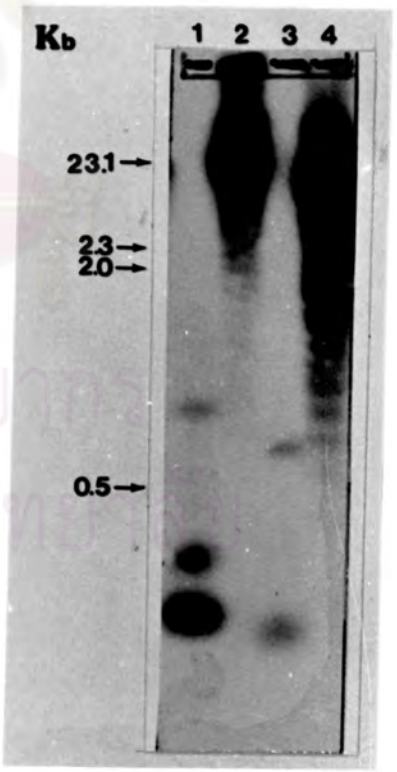
4 = mtDNA digested with Hha 1



A



B



C

Figure 23

Figure 24 (A) Ethidium bromide staining pattern of extracted mtDNA from each individual which was digested with Sau 3A1 and analyzed on 1.5% agarose gel electrophoresis

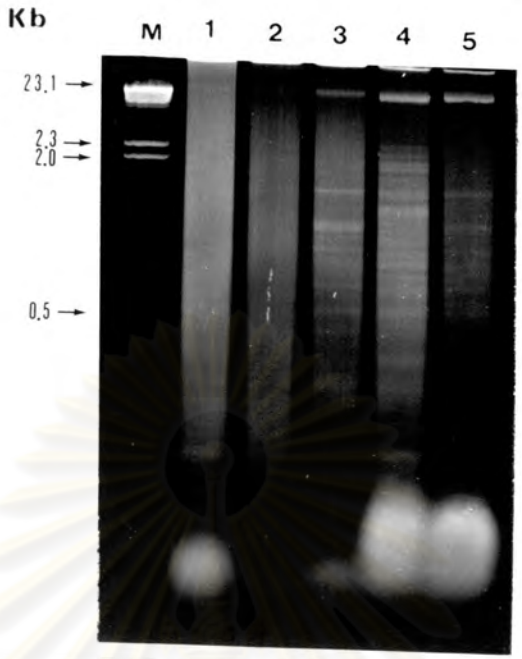
(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated probe 1 and probe 45. The specific activity of each was 3.0x10 cpm/ug DNA

M = λ DNA cut with Hind 111 is a size marker

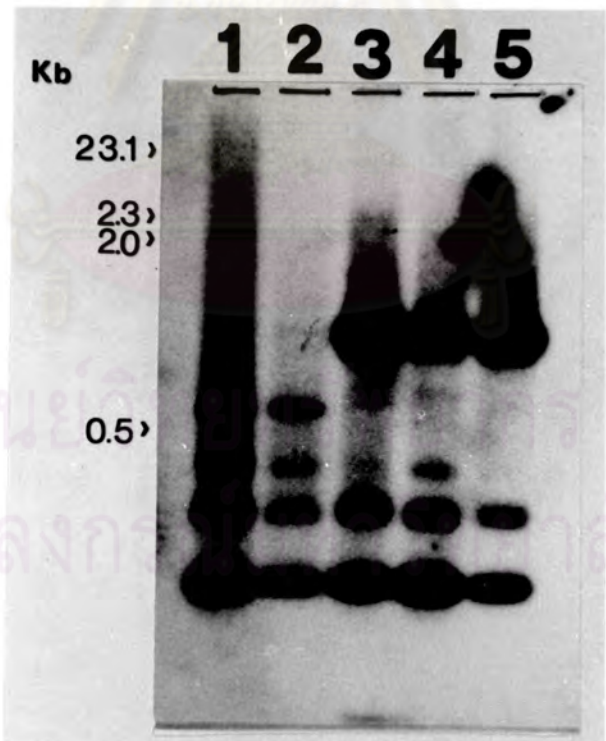
1-2 = The prawn from Kraburi River

3 = The prawn from Bangpakong River

4-5 = The prawn from Kung Kam Thong Farm



A



B

Figure 24

Figure 25 (A) Ethidium bromide staining pattern of extracted mtDNA from each individual which was digested with Sau 3A1 and analyzed on 1.2% agarose gel electrophoresis

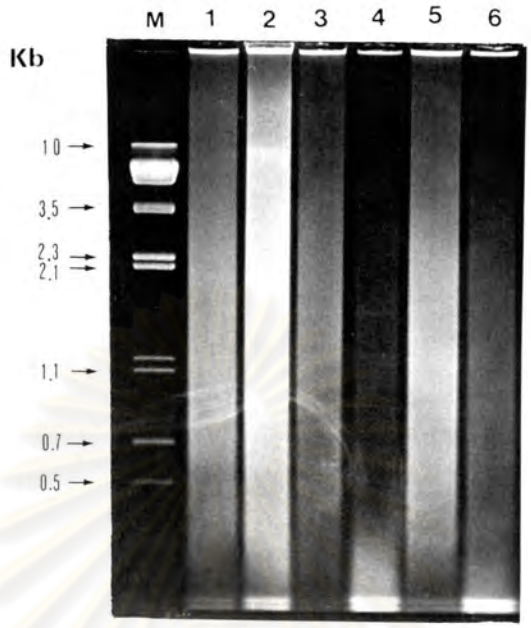
(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated probe 1. The specific activity of the probe was 3.0x10 cpm/ug DNA

M = λ DNA cut with Dra 1 is a size marker

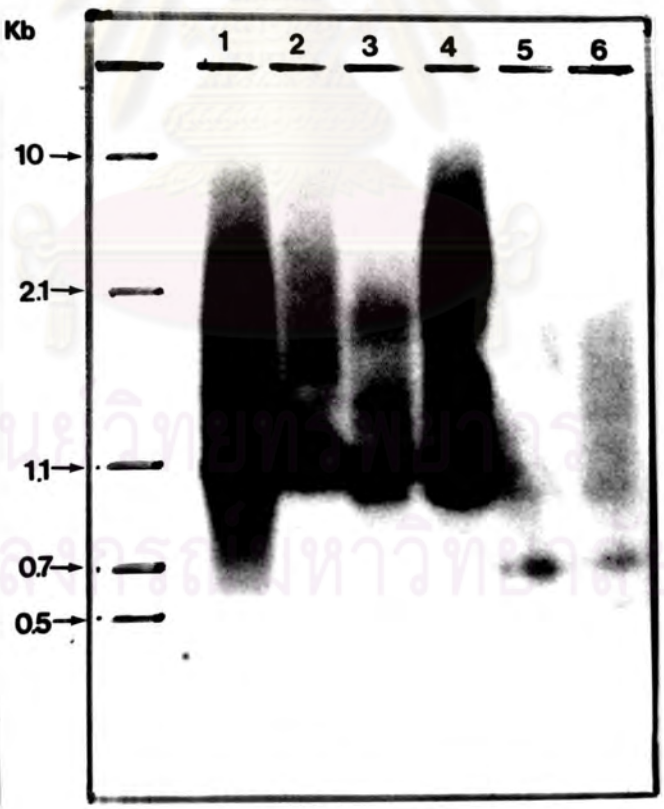
1-2 = The prawn from Kung Kam Thong Farm

3-4 = The prawn from Bangpakong River

5-6 = The prawn from Kraburi River



A



B

Figure 25

The uncut form mtDNA of individual prawn from Bangpakong River and Kraburi River were analyzed on 0.7% agarose gel electrophoresis and subsequently stained with ethidium bromide. The mtDNA pattern were smear owing to contamination of nuclear DNA (Fig 26a). After southern blot hybridization with probe 1, some discrete bands were appeared the same in both resources (Fig 26b). This indicated that the discrete band was homogeneous DNA and specific with probe 1.

9. RFLP of mtDNA within resource

For 12 and 16 sample of prawns were collected from Bangpakong River and Kraburi River, respectively. The mtDNA were extracted from each individual, digested with restriction enzyme Sau 3A1 and subsequently analyzed on 1.2% agarose gel electrophoresis. The ethidium bromide staining pattern of all mtDNA samples were not different (Fig 27a, 28a, 29a and 30a). After southern blot hybridization with probe 1, it appeared some discrete band having single prominent band at 1.1 kb. of Bangpakong River's prawns and 0.7 kb. of Kraburi River's prawns while some individual from each resource showed various patterns in minor bands (Fig 27b, 28b, 29b and 30b).

Figure 26 (A) Ethidium bromide staining pattern of extracted mtDNA from each individual and analyzed on 0.7% agarose gel electrophoresis

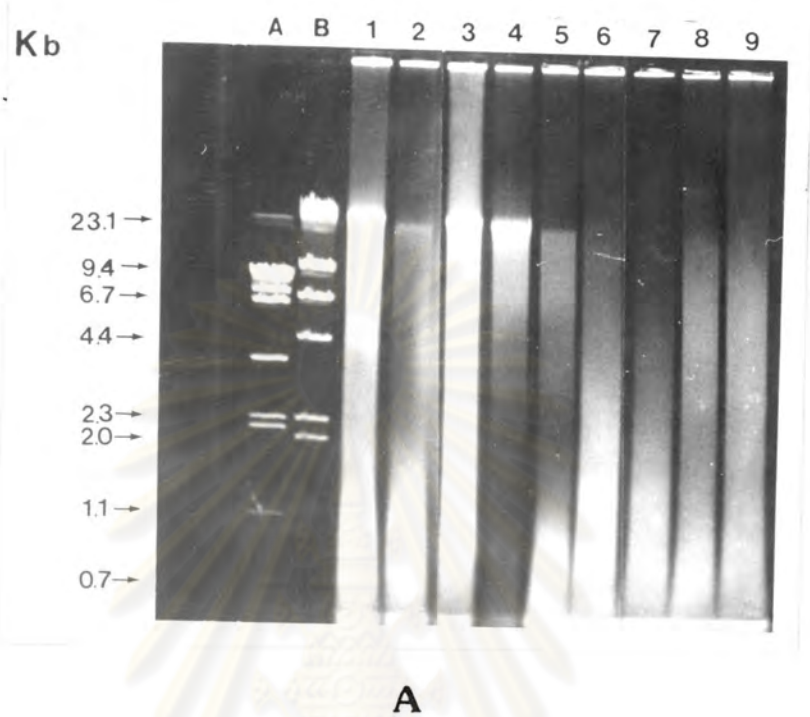
(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated probe 1. The specific activity of the probe was 2.5x10 cpm/ug DNA

A = λ DNA cut with Dra 1 is a size marker

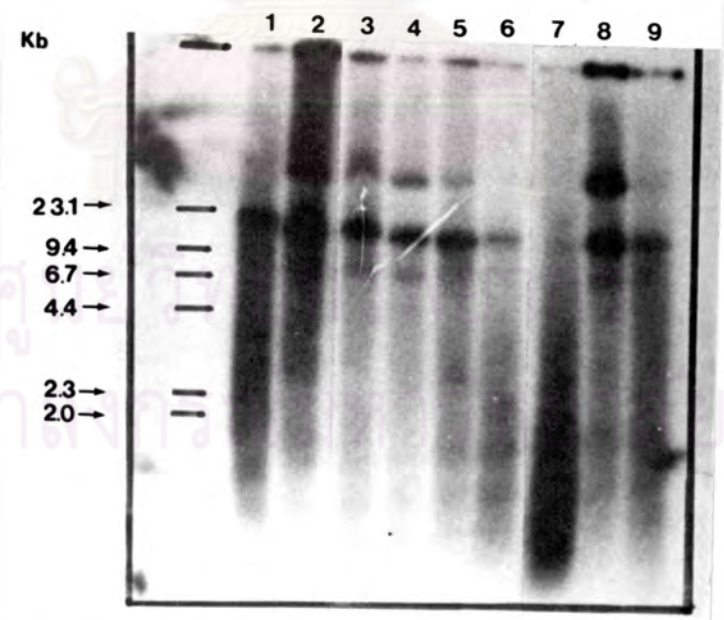
B = λ DNA cut with Hind 111 is a size marker

1-4 = number of each individual prawn from Bangpakong River

5-9 = number of each individual prawn from Kraburi River


Kb
A B 1 2 3 4 5 6 7 8 9
23.1
9.4
6.7
4.4
2.3
2.0
1.1
0.7

A


Kb
1 2 3 4 5 6 7 8 9
23.1
9.4
6.7
4.4
2.3
2.0

B

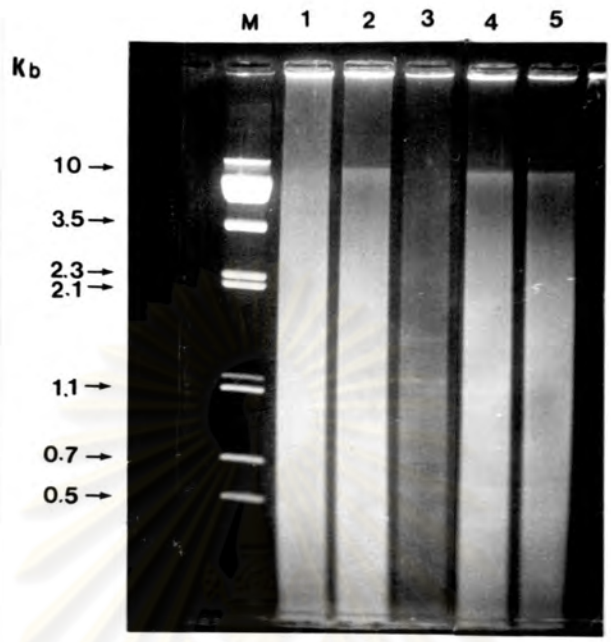
Figure 26

Figure 27 (A) Ethidium bromide staining pattern of extracted mtDNA from individual prawn of Bangpakong River which was digested with Sau 3A1 and analyzed on 1.2% agarose gel electrophoresis

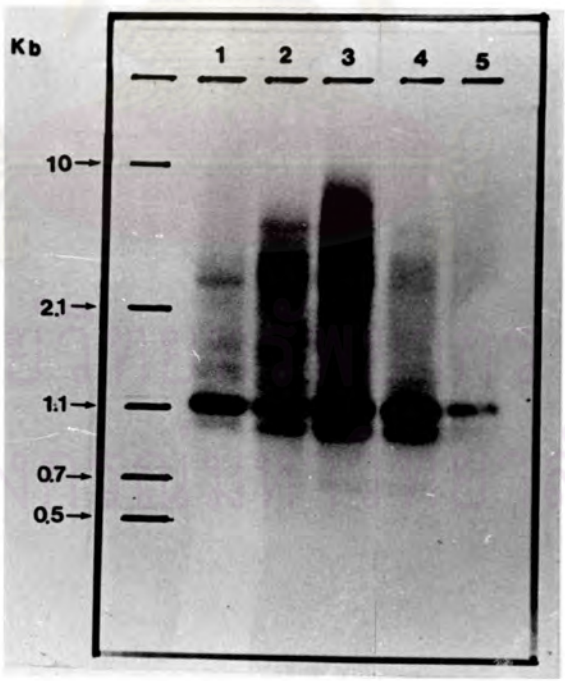
(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated probe 1. The specific activity of the probe was 3.8x10 cpm/ug DNA

M = λ DNA cut with Dra 1 is a size marker

1-5 = number of individual prawn



A



B

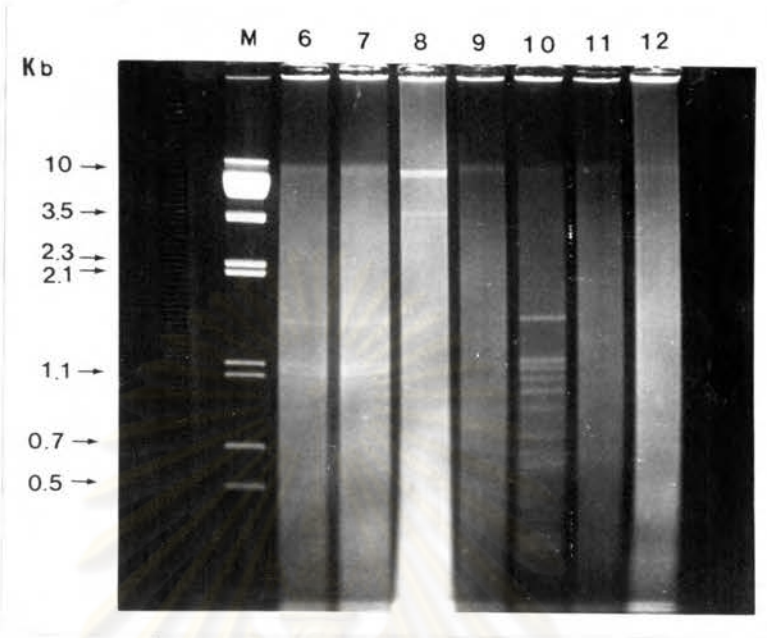
Figure 27

Figure 28 (A) Ethidium bromide staining pattern of extracted mtDNA from individual prawn of Bangpakong River which was digested with Sau 3A1 and analyzed on 1.2% agarose gel electrophoresis

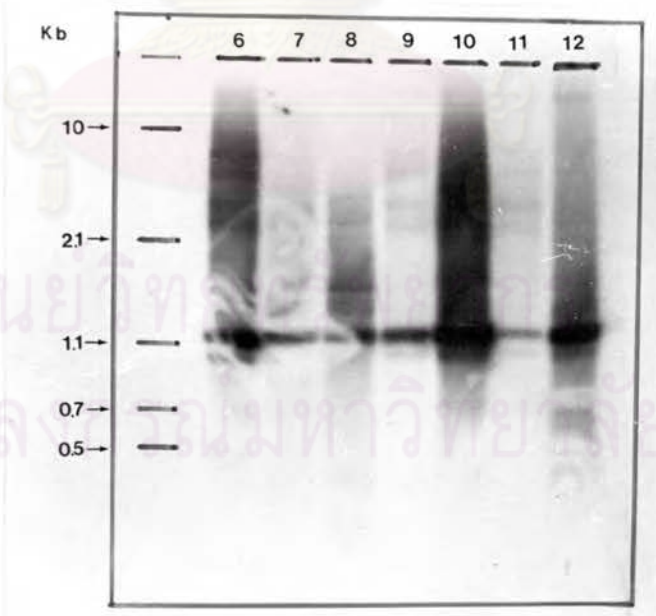
(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated probe 1. The specific activity if the probe was 4.5x10 cpm/ug DNA

M = λ DNA cut with Dra 1 is a size marker

6-12 = number of individual prawn



A



B

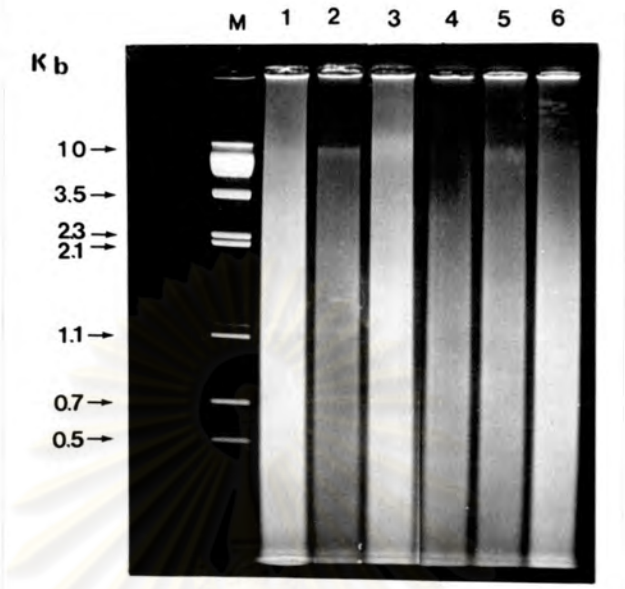
Figure 28

Figure 29 (A) Ethidium bromide staining pattern of extracted mtDNA from individual prawn of Kraburi River which was digested with Sau 3A1 and analyzed on 1.2% agarose gel electrophoresis

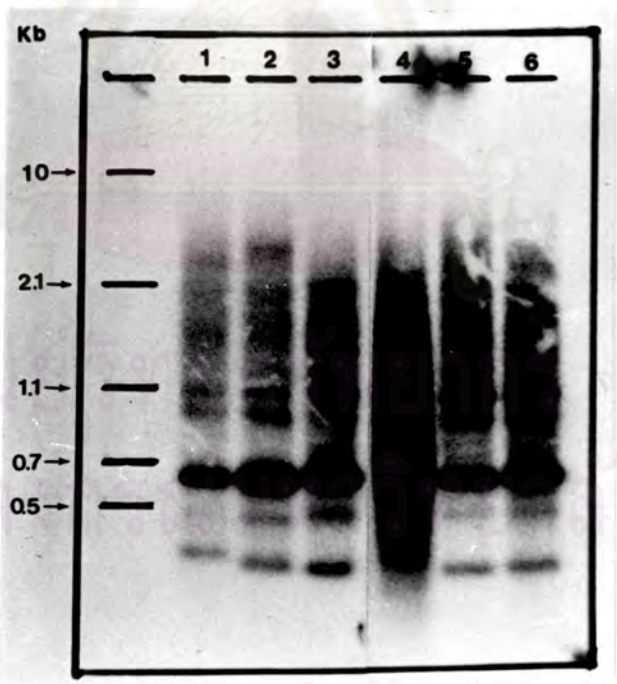
(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated probe 1. The specific activity of the probe was 4.0x10 cpm/ug DNA

M = λ DNA cut with Dra 1 is a size marker

1-6 = number of individual prawn



A



B

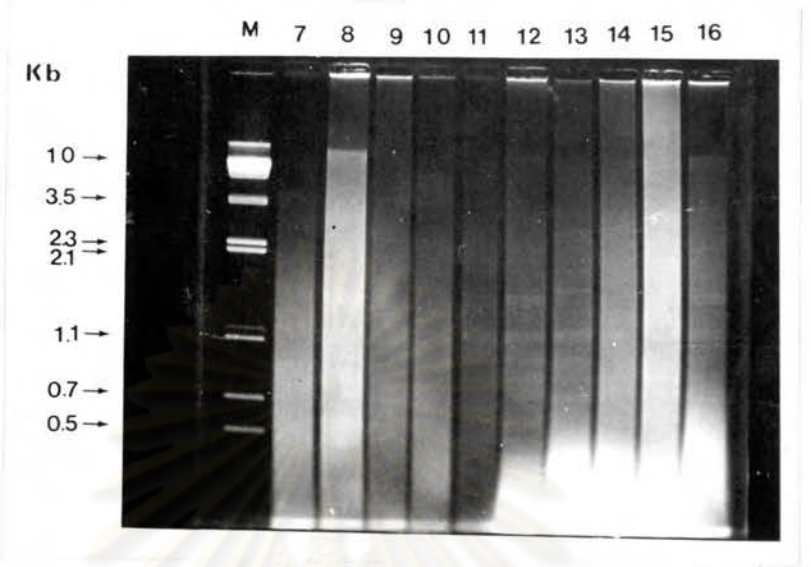
Figure 29

Figure 30 (A) Ethidium bromide staining pattern of extracted mtDNA from individual prawn of Kraburi River which was digested with Sau 3A1 and analyzed on 1.2% agarose gel electrophoresis

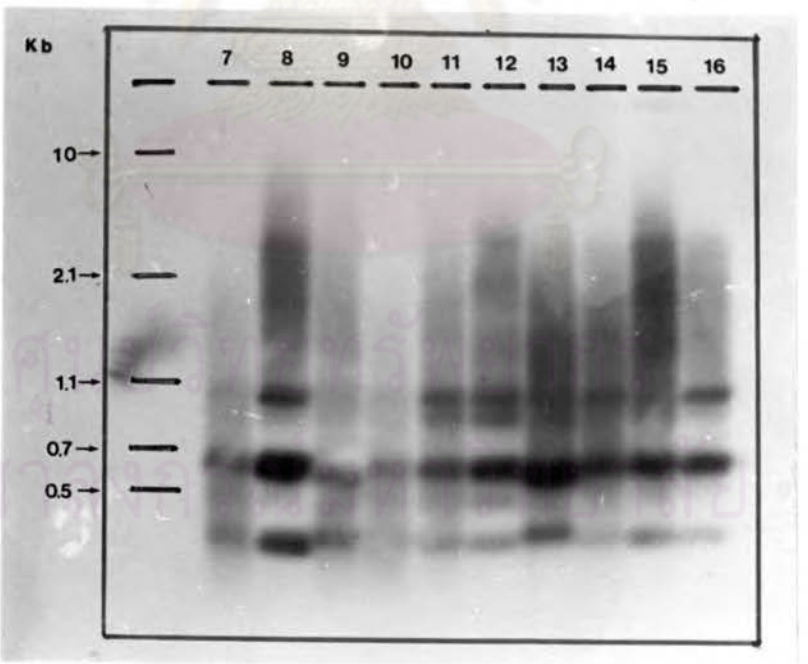
(B) Autoradiogram of southern blot hybridization from gel in panel (A) with nick-translated probe 1. The specific activity of the probe was 3.3x10 cpm/ug DNA

M = λ DNA cut with Dra 1 is a size marker

7-16 = number of individual prawn


M 7 8 9 10 11 12 13 14 15 16
Kb
10
3.5
2.3
2.1
1.1
0.7
0.5

A


Kb
7 8 9 10 11 12 13 14 15 16
10
2.1
1.1
0.7
0.5

B

Figure 30

10. Restriction map of probe 1

The probe 1 was digested with 9 restriction enzymes which was 6 nucleotide recognition enzymes. These enzymes were selected to characterize the insert fragment. These enzymes gave single discrete band in the same position about 3.8 Kb on 1.0% agarose gel electrophoresis (Fig 31). This resulting indicated that it was no restriction enzyme site in inserted fragment because these restriction enzymes were one recognition in vector pUC 12.

In case of Sau 3A1 restriction enzyme which recognized 4 nucleotide, the enzyme can separate the inserted fragment from vector pUC12. It appeared one special band of inserted fragment about 1.1 Kb. When probe 1 was digested with the other 4 nucleotide recognition enzymes such, Bst U1, Hae 111, Hha 1 and Rsa 1 comparing to vector pUC 12 digested with the same enzyme along with probe 1 in order to check restriction site containing in probe 1. The one special band of inserted fragment appeared smaller than Sau 3A1 digestion (Fig 32). Therefore, these enzymes had recognition site in the inserted fragment. The two restriction enzymes digestion were performed to check oreintation of inserted fragment. Another enzyme was selected on the criteria that the enzyme can digest with single recognition in upper or lower site from point of Bam H1 recognition in vector pUC 12. Then size of inserted fragment was determined comparing to one enzyme digestion (Fig 33). The restriction map of probe 1 was shown in Fig 34.

Figure 31 Ethidium bromide staining pattern of probe 1 which was digested with six nucleotide recognition restriction enzymes

M = λ DNA cut with Bst E11 is a size marker

1 = probe 1 digested with Acc 1

2 = probe 1 digested with Ava 1

3 = probe 1 digested with Eco R1

4 = probe 1 digested with Hinc 1

5 = probe 1 digested with Hind 111

6 = probe 1 digested with Sma 1

7 = probe 1 digested with Sst 1

8 = probe 1 digested with Xba 1

9 = probe 1 digested with Pst 1

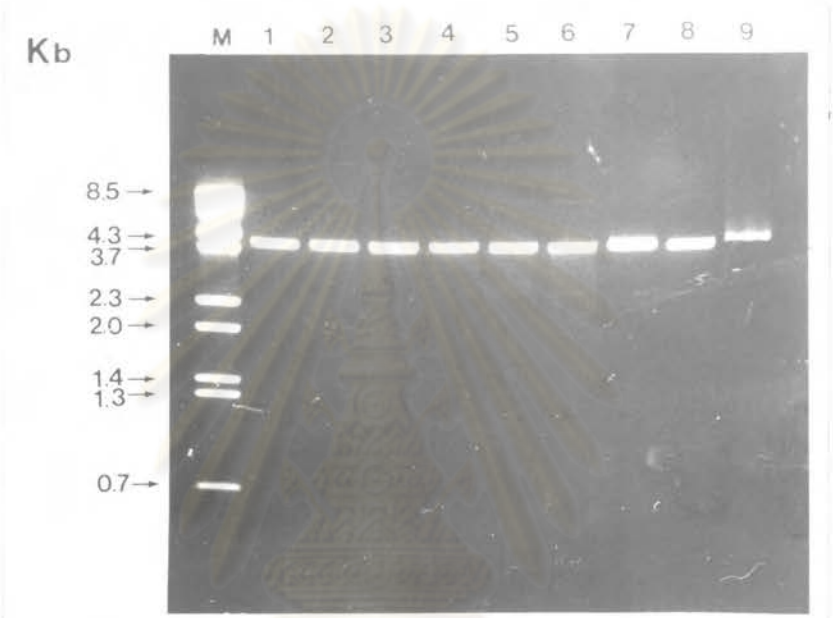


Figure 31

Figure 32 Ethidium bromide staining pattern of probe 1 which was digested with four nucleotide recognition restriction enzymes

M = λ DNA cut with Bst E11 is a size marker
1 = vector pUC12 digested with Bst U1
2 = probe 1 digested with Bst U1
3 = vector pUC12 digested with Hae 111
4 = probe 1 digested with Hae 111
5 = vector pUC12 digested with Sau 3A1
6 = probe 1 digested with Sau 3A1
7 = vector pUC12 digested with Rsa 1
8 = probe 1 digested with Rsa 1
9 = vector pUC12 digested with Hha 1
10 = probe 1 digested with Hha 1

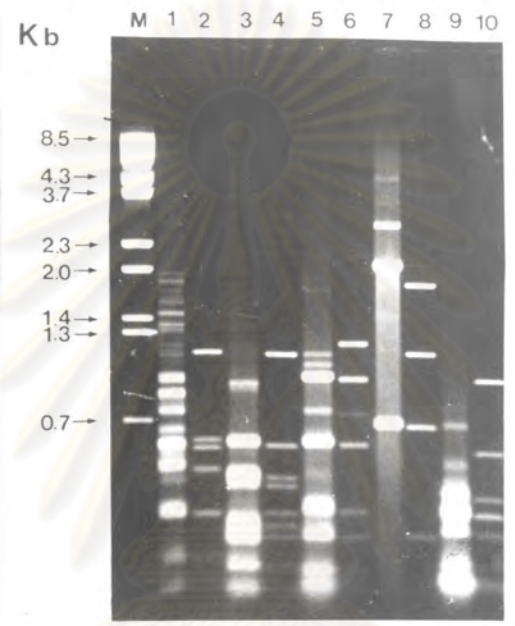


Figure 32

Figure 33 Ethidium bromide staining pattern of probe 1 which was double digested with restriction enzymes

A = λ DNA cut with Pst 1 is a size marker

B = λ DNA cut with Dra 1 is a size marker

1 = probe 1 digested with Bst U1

2 = probe 1 digested with Bst U1/Ava 1

3 = probe 1 digested with Hae 111

4 = probe 1 digested with Hae 111/Ava 1

5 = probe 1 digested with Hha 1

6 = probe 1 digested with Hha 1/Hind 111

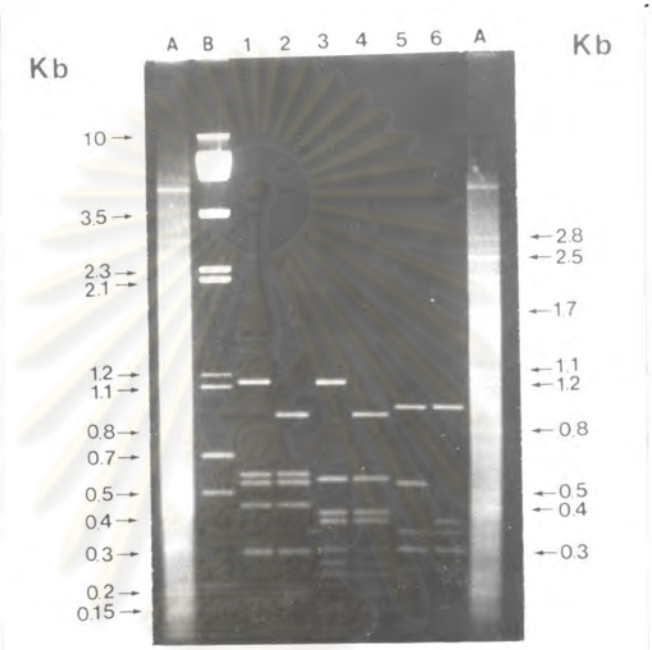


Figure 33

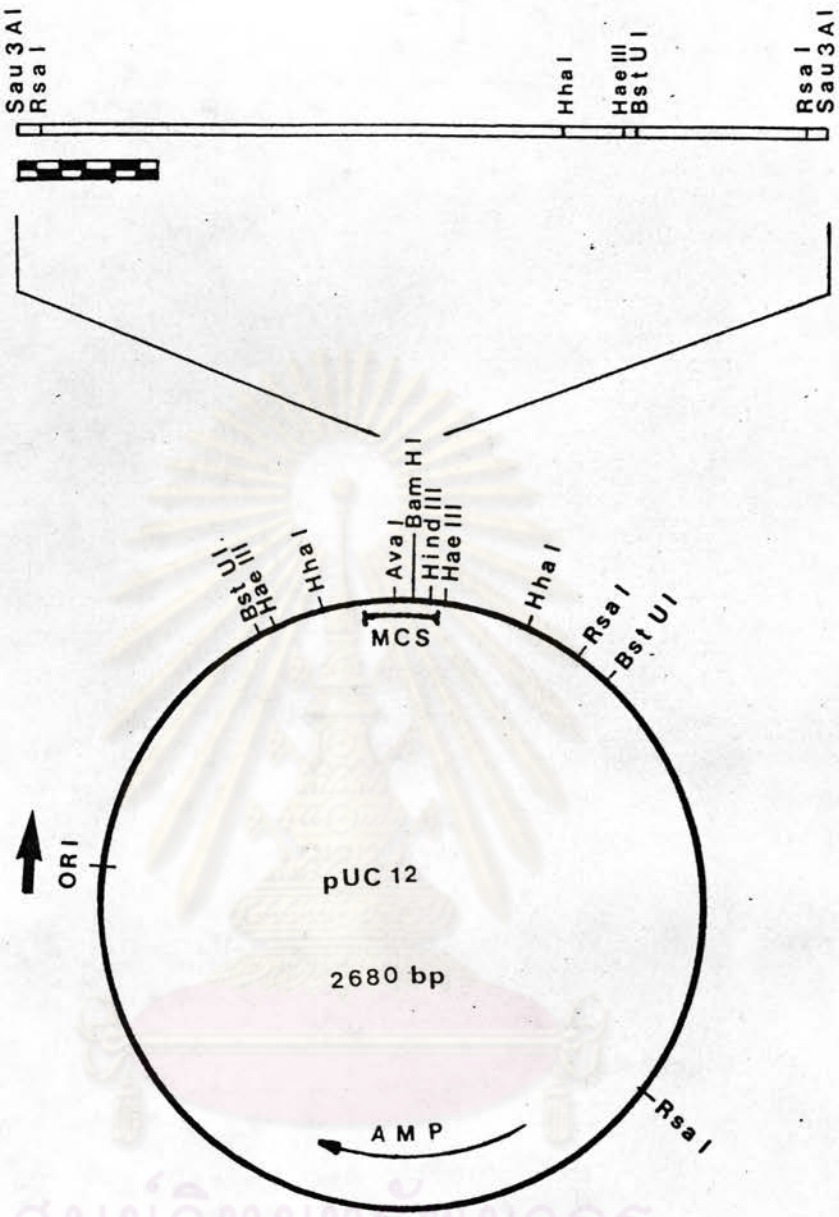


Figure 34 The restriction map of probe 1 oriented in pUC12 starting from Bam H1 restriction site

Striped box indicate distance of 200 bp

MCS = multiple cloning site of pUC12