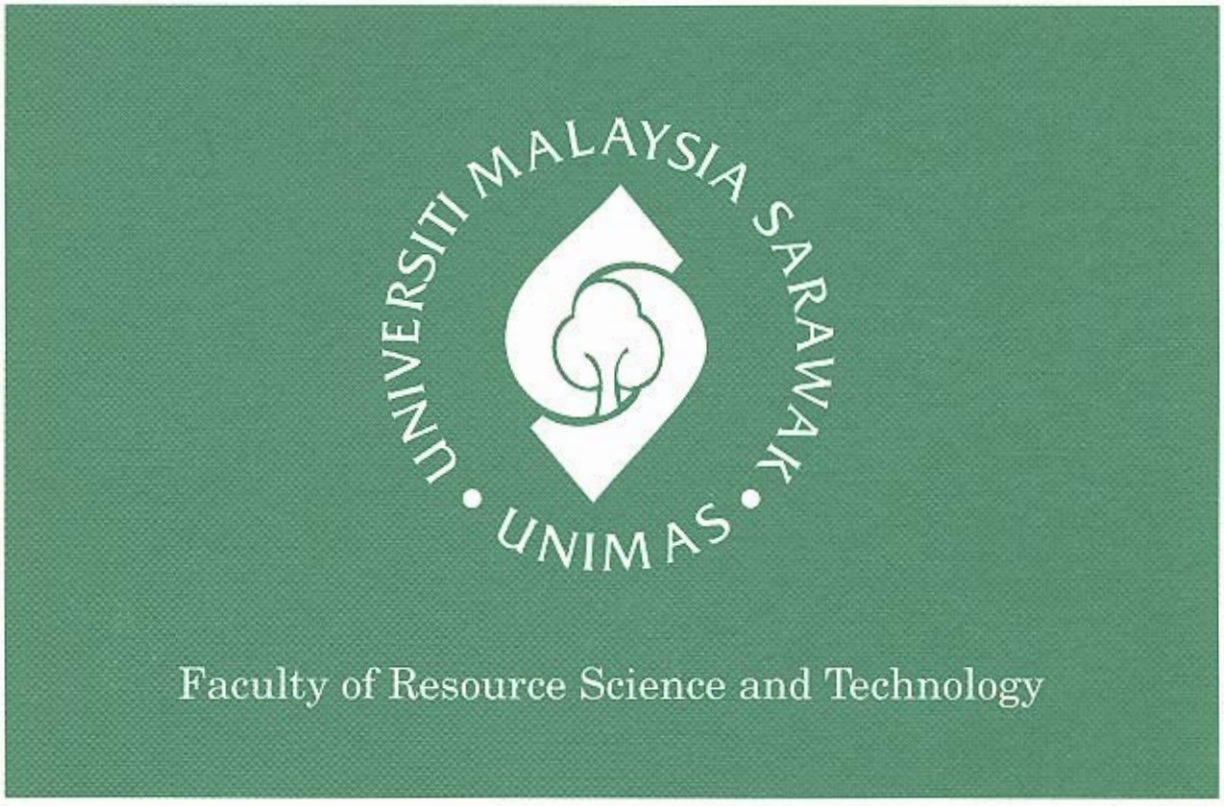


IDENTIFICATION OF GENETIC MARKERS BETWEEN *BARBONYMUS SCHWANENFELDII* (BLEEKER 1850) AND *BARBONYMUS GONIONOTUS* (BLEEKER 1850) USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AND SEQUENCING ANALYSIS OF *CYTOCHROME C OXIDASE* I (COI) MITOCHONDRIAL DNA FRAGMENT

Sabrina Haron

Bachelor of Science With Honours
(Resource Biotechnology)
2004

Faculty of Resource Science and Technology

IDENTIFICATION OF GENETIC MARKERS BETWEEN *Barbonymus schwanenfeldii* (Bleeker 1853) and *Barbonymus gonionotus* (Bleeker 1850) USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AND SEQUENCING ANALYSIS OF *CYTOCHROME C OXIDASE* I (COI) MITOCHONDRIAL DNA FRAGMENT

SABRINA HARON

This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

2004

IDENTIFICATION OF GENETIC MARKERS BETWEEN *Barbonymus schwanenfeldii* (Bleeker 1853) and *Barbonymus gonionotus* (Bleeker 1850) USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AND SEQUENCING ANALYSIS OF *CYTOCHROME C OXIDASE* I (COI) MITOCHONDRIAL DNA FRAGMENT

SABRINA HARON

This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

University Malaysia Sarawak
Faculty of Resource Science and Technology
2004

IDENTIFICATION OF GENETIC MARKERS BETWEEN *Barbonymus schwanenfeldii* (Bleeker 1853) and *Barbonymus gonionotus* (Bleeker 1850) USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AND SEQUENCING ANALYSIS OF *CYTOCHROME C OXIDASE* I (COI) MITOCHONDRIAL DNA FRAGMENT

Sabrina Binti Haron

Resource Biotechnology Programs
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

This study was done to identify genetic markers between two *cyprinids* fish (*Barbonymus schwanenfeldii* and *Barbonymus gonionotus*). The analyses were carried out using PCR-RFLP and DNA sequencing of *cytochrome c oxidase* I (COI) gene. Samples from Peninsular Malaysia were collected from eight sites in Negeri Sembilan (36 samples) and one site from Pahang River (one sample). Samples from Sarawak were collected from Kapit (four samples) and Betong (three samples). While samples from Sabah was collected from Kinabatangan (five samples). RFLP analysis produces four restrictions enzyme (*AluI, Csp6I, BsuRI, and PvuII*) diagnostic between *Barbonymus schwanenfeldii* and *Barbonymus gonionotus*. Phylogenetic analysis showed that *B. schwanenfeldii* and *B. gonionotus* are reciprocally monophyletic thus supporting their taxonomic status as distinct species. Besides, the clustering of samples in both species into Peninsular Malaysia and Borneo clades respectively showed their geographical structuring possibly related to genetic drift resulted from separation of Borneo from Peninsular Malaysia in the late Pleistocene epoch.

Key words: Genetic marker, *cytochrome c oxidase* I (COI), PCR-RFLP, DNA sequencing and phylogenetic relationship.

ABSTRAK

Kajian ini telah dibuat untuk mengenalpasti penanda genetik diantara dua ikan siprinid (Barbonymus schwanenfeldii dan Barbonymus gonionotus). Analisis genetik telah dibuat menggunakan kaedah PCR-RFLP dan analisis penjujukan DNA pada gen cytochrome c oxidase I (COI). Sampel dari Semenanjung Malaysia telah diambil dari lapan lokasi di Negeri Sembilan (36 sampel) dan satu lokasi di Sungai Pahang (satu sampel). Sampel dari Sarawak telah diperolehi dari Kapit (empat sampel) dan Betong (tiga sampel), manakala sampel dari Sabah telah diperolehi dari Kinabatangan (lima sampel). Analisis PCR-RFLP menggunakan empat enzim pembatas (AluI, Csp6I, BsuRI, dan PvuII) dapat membezakan diantara Barbonymus schwanenfeldii and Barbonymus gonionotus. Analisis filogenetik menunjukkan bahawa B. schwanenfeldii and B. gonionotus merupakan spesies yang berlainan dan seterusnya menyokong status taksonomi kedua-dua spesies. Disamping itu, kelompok yang berbeza dihasilkan diantara sampel dalam kedua-dua spesies berdasarkan stuktur geografi (Peninsular Malaysia dan Borneo), berkemungkinan berkaitan dengan hanyutan genetik akibat pemisahan Borneo dari Tanah Besar Asia yang berlaku semasa akhir era "Pleistocene".

Kata kunci: Penandaan genetik, cytochrome c oxidase I (COI), PCR-RFLP, penjujukan DNA dan hubungan filogenetik.

INTRODUCTION

Rapid development in fish biotechnology particularly the application of molecular techniques has been applied in various fields of fish biology including taxonomy, systematic and aquaculture. Genetic or molecular markers are progressively being used to identify samples or stocks of fish from natural populations, usually in conjunction with morphological analysis. Molecular genetic techniques have been developed into powerful tools to analyze genetic relationship and genetic diversity among fishes (Stepien *et al.*, 1997).

Mitochondrial genome possesses some characteristics that have made it attractive for studies of population structure, phylogenetic and conservation of endangered species (Avise, 1994). It has been used widely employed in systematic studies and ideal for animal species identification. The combination of mitochondrial DNA with morphology or nuclear markers has proven useful to resolve questions in taxonomy and systematic of many organism including fish (Ward and Grewe, 1994). Among the regions of mtDNA analyzed in crustaceans, *cytochrome c oxidase* I (COI) is a powerful marker for differentiating at both the interspecific and intraspecific levels (Palumbi and Benzie, 1991). *Cytochrome c oxidase* I (COI) control the last step of food oxidation and it is located in the inner membrane of mitochondria and bacteria.

Restriction fragment length polymorphism (RFLPs) of mitochondrial DNA has become a powerful tool in animal population genetic studies (Meyer *et al.*, 1995). For example, RFLPs has been applied to examine phylogenetic relationship among *Thunnus* species (Chow *et al.*, 1993) and population structure of *Fundulus heteroclitus* (Villasenor *et al.*, 1990).

Barbonymus schwanenfeldii (Bleeker, 1853) is a common and popular indigenous fish from the genus *Barbonymus*. In Peninsular Malaysia *B. schwanenfeldii* (Figure 2) are locally known as lampam sungai (Litis *et al.*, 1997), while in Sarawak it is known as tengadak (Mohsin and Ambak, 1991). It is very widely distributed in all rivers and lake in South Asia including Mekong and Chao Phraya valley, Malay Peninsular, Sumatra and Borneo (Kottelat *et al.*, 1993).

Barbonymus gonionotus (Bleeker, 1850) or locally known as lampam jawa is one of the popular freshwater fish in Malaysia. *B. gonionotus* (Figure 3) is an introduced species from Java (Indonesia) but has established itself throughout Malaysia freshwater system (Mohsin and Ambak, 1991). In Asia, this fish can be found in Mekong and Chao Phraya valley, Malay Peninsular, Sumatra and Java (Kottelat *et al.*, 1993). It is one of the most well known fishes and is caught in large quantities for domestic consumption.

B. gonionotus resembles *B. schwanenfeldii* in shape and form but lack the body markings exhibited in the latter. The dorsal fin in living specimens of *B. schwanenfeldii* is red with a large black and the caudal fin is red with a black band on each lobe. While, the dorsal and caudal fins of *B. gonionotus* are gray to gray-yellow.

Due to the close morphological similarities between the two species and their on growing importance as popular freshwater fishes for aquaculture as well as aquarium, it is importance to initiate genetic study to identify potential genetic markers between the two species. These markers will be useful for species identification as well as breeding programme between the two species for genetic improvements.

OBJECTIVE

This research is designed to identify genetic markers between *B. schwanenfeldii* and *B. gonionotus* using PCR-RFLPs of *cytochrome c oxidase* I (COI) mtDNA genes. We also aim to classify their phylogenetic relationship, thus give insight on their taxonomic status.

MATERIAL AND METHODS

Sample description and location

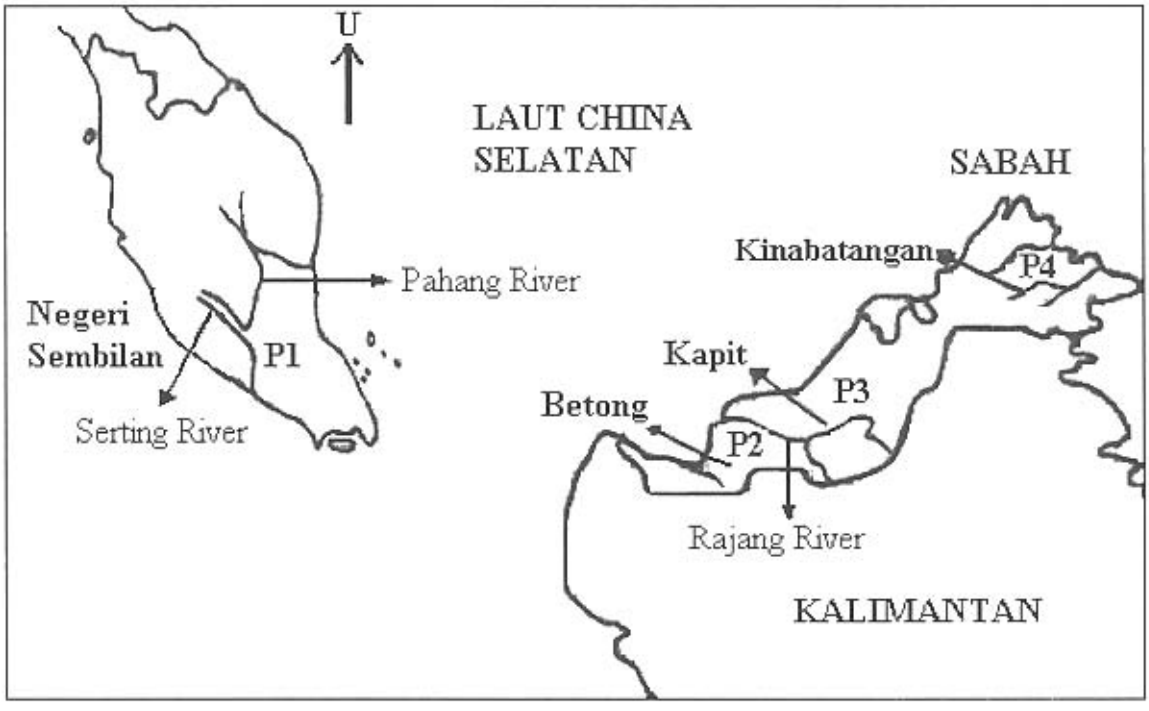


Figure 1: Study site in Peninsular Malaysia and Borneo

Fish samples were collected from nine locations in Peninsular Malaysia (Serting River and Padang Piol in Jerantut), Sarawak (Betong and Kapit) and Sabah (Kinabatangan River) (Table 1). Most samples were preserved in 70 % ethanol while a few frozen samples are stored at -20 ^{o}C until used for DNA extraction. Figure 1 showed the locations where the fishes were caught. Further information of Negeri Sembilan (Serting River) site referred to Appendix 1.

Table 1: Sample collection for extraction, DNA sequence analysis and PCR-RFLP analysis of *cytochrome c oxidase* I (CO1) mtDNA gene.

Location	Study site	Species	Abbreviation	Collection	Sequence analysis	PCP-RFLP
Peninsular Malaysia	Pulapah Lama Hulu Serting Pam Air Jempol Kolam Serting5 St.8 Jln.Penarek	*Barbonymus Gonionotus*	GPL (1-3) GHS (1-4) GJ (1-3) GKS (3,5) GJP (1-9)	21	4	5
	Pulapah Lama Triang Selatan Jempol Ayer Hitam Jerantut Pahang	*Barbonymus Schwanenfeldii*	SFPL (1-6) SFTS (1-4) SFJ (1-4) SAH 1 SFPP 4	16	7	5
Borneo (Sabah & Sarawak)	Kinabatangan	*Barbonymus Gonionotus*	BGK (1-5)	5	2	5
	Betong Kapit	*Barbonymus Schwanenfeldii*	TBSFB (1,2,3) TBSFK(5,6,10,20)	7	7	5
			Total	49	20	20

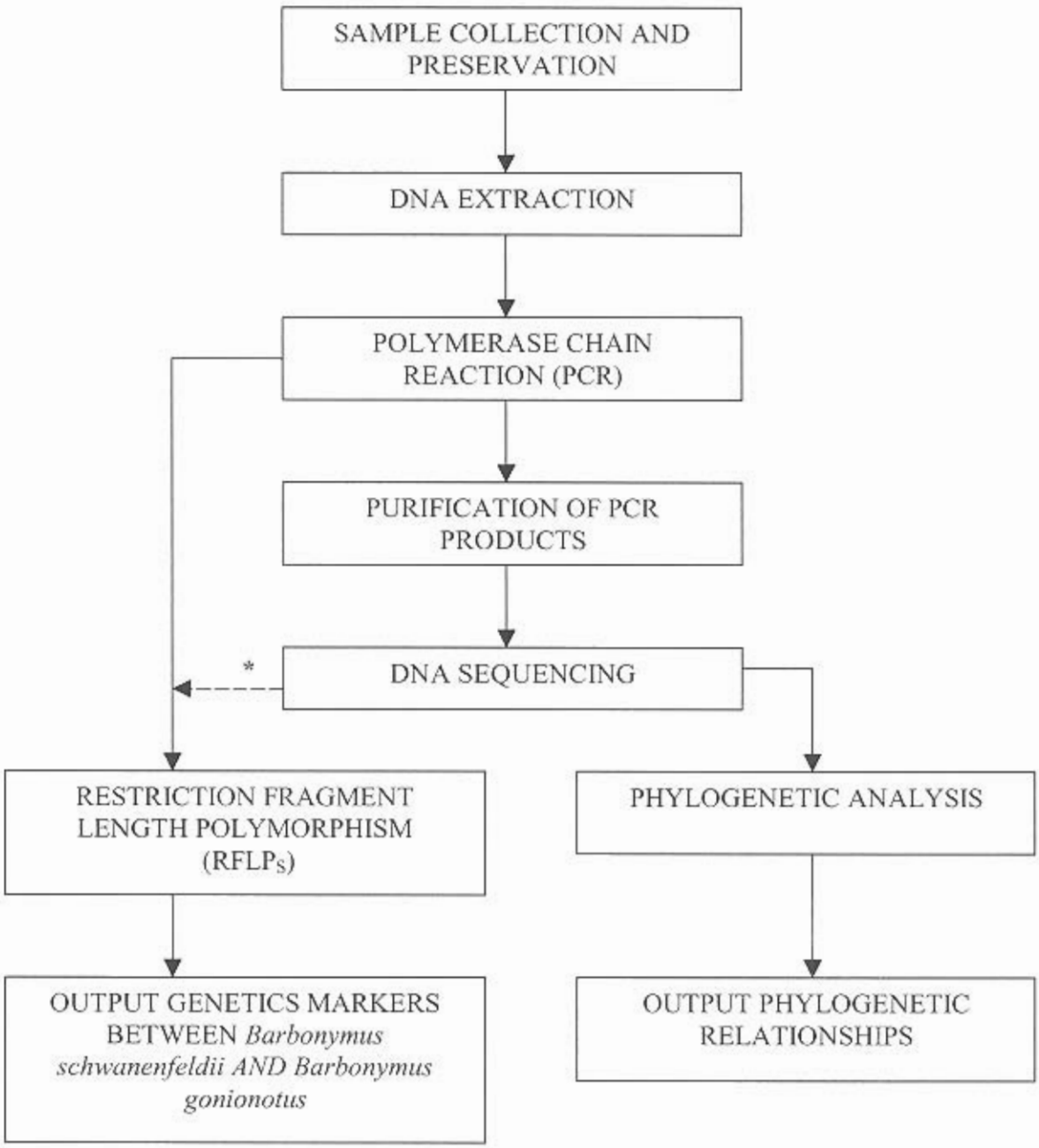
Subfamily : Cyprininae
Genus : *Barbonymus*
Species : *Barbonymus schwanenfeldii*
Local Names : Tengadak (Sarawak) or Lampam Sungai (Peninsular Malaysia)

Figure 2: Sample of *Barbonymus schwanenfeldii*

Subfamily : Cyprininae
Genus : *Barbonymus*
Species : *Barbonymus gonionotus*
Local Names : Lampam Jawa

Figure 3: Sample of *Barbonymus gonionotus*

Figure 4: Flow chart of methodology



* Data from DNA sequencing were use to identify specific restriction site between *B. schwanenfeldii* and *B. gonionotus.*

Extraction of genome DNA

Total DNA was extracted from muscles tissue using a modified CTAB method (see Grewe *et al.*, 1993). Tissue sample were transfer into 1.5 ml microcentrifuge tube containing 10 µl of Proteinase K and 700 µl CTAB. Then, the sample was incubated at 65°C until completely dissolved. Longer time was needed to ensure that whole tissue was dissolved. Chloroform isoamyl alcohol (24: 1) was subsequently added and mixed for 2 minutes before centrifuge at 15,000 rpm for 10 minute.

The upper aqueous phase containing DNA was transferred to a new tube, and an equal volume of 100 % cold ethanol was added. The sample was mixed by vortex, and centrifuge for about 10 minutes. The supernatant was removed into a new tube and approximately 600µl cold 70% ETOH and 25 µl 3m NaOAC or NaCl was added, followed by centrifugation at 15,000 rpm for 10 minute. Finally, the pellet was air-dried before resuspended in 70 µl of distilled water (dH_2O).

Polymerase Chain Reaction (PCR)

All samples of *B. schwanenfeldii* and *B. gonionotus* were used for DNA amplification of *cytochrome c oxidase* I (COI) gene sequencing. Thermal cycle amplification was performed in 50 µl reaction volume contained 31.75 µl sterilized water (ddH_2O), 4.0 µl DNA and 0.25 µl of *Taq* DNA polymerase (Promega), 5.0 µl 10X buffer (Promega), 1.0 µl of dNTP, 3.0 µl $MgCl_2$ and 2.5 µl of each primer. *Cytochrome c oxidase* I (COI) primer was used for this analysis is given below;

5'-CCTGCAGGAGGAGGAGGAGAYCC-3' (forward)

5'- CCAGAGATTAGAGGGAATCAGTG-3' (reverse), (Palumbi *et al.*, 1991).

The cycle of heating (adding raw materials) and cooling (incubating) is repeated until the DNA has been sufficiently amplified. Cycle parameters were 5 min at 96°C for initial denaturation, 45 sec at 95° C for denaturation, 1 min 30 sec at 47° C for annealing, 1 min 30 sec at 72° C for elongation and 7 min at 72 ° C for final elongation. For this analysis, the optimization of annealing temperature range between 44° C to 48° C. Gene Ruler™ 1 kb DNA Ladder was used as a standard size marker. PCR product was visualized by using 1.0%-1.2% agarose gel (containing ethidium bromide) for 1 hour at 90V.

DNA Sequencing

All PCR products were purified before subjected for sequencing. The cycle sequencing reaction was normally performed in a programmable thermal cycler (BiometraT-Personal). Cycle sequencing reaction was done for 25 cycles which is involving denaturation at 96 °C for 10 sec, annealing process at 55 °C and extension at 60 °C for 4 min and sequencing was done in an ABI 377 automated DNA sequencer. 14 samples of *B. schwanenfeldii* (seven from Peninsular Malaysia and seven from Borneo) and six samples of *B. gonionotus* (four from Peninsular Malaysia and two samples from Borneo) were used for sequencing analysis.

PCR-RFLP Analysis

Four amplified samples of *B. gonionotus* and four amplified samples of *B. schwanenfeldii* were digested with 10 restriction enzymes (*Alu*I, *BamH*I, *Eco*RI, *Hpa*II/*Msp*I, *Bsu*RI, *Csp*6I, *Ssp*I *and Rsa*I *and Pvu*II) to generate haplotypes. Data from DNA sequencing were used to identify specific restriction site between these species. Reaction mixture contains 4 µl distilled water, 4 µl of PCR product, 1 µl of 10X buffers and 1 µl of restriction enzyme. The reaction mixture was incubated for 4 hours or overnight at 37°C. After incubation, about 2.5 µl of loading dye was incubated into the mixture and preserved at –20°C to inactivated enzyme activity. Finally, digestion mixtures were run in 2.0 % agarose gel to check for digestion profiles.

Sequence analysis

CLUSTAL X (1.81) (Thompson *et al.*, 1997) software was used for multiple alignments of DNA sequences. CHROMAS software (version 1.45) was initially to display DNA sequence results. Besides, MEGA (Molecular Evolutionary Genetic Analysis) software version 2.1 (Kumar *et al.*, 2001) was utilized for phylogenetic analysis using Neighbour-Joining (N-J) (Saitou and Nei, 1987) and Maximum Parsimony (MP) method with bootstrap analysis of 1000 replication. DNA Sequence Polymorphism (DNASP) version 3.53 (Rozas and Rozas, 2001) was used to investigate nucleotide diversity, gene flow and population structure between populations and species.

RESULTS

DNA Extraction

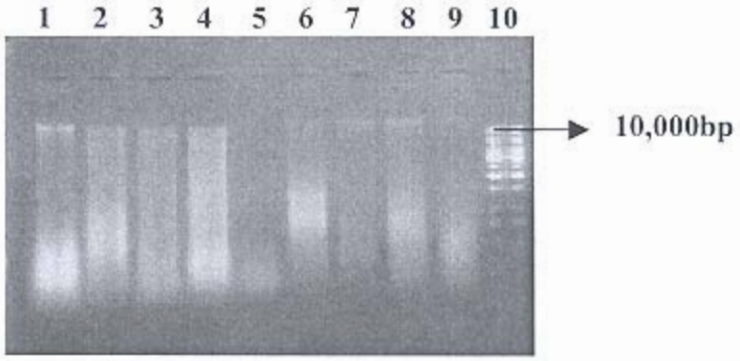


Figure 5: DNA extraction. Lanes 1-9 showed the bright extracted DNA bands. Lane 10 represents GeneRuler^{TM} 1 kb DNA Ladder as a standard size marker (Fermentas).

All sample *B. schwanenfeldii* and *B. gonionotus* from Peninsular Malaysia and Borneo produced high quality DNA. This result was appeared based on the visualization of a single band on Polaroid film (Figure 1). GeneRuler^{TM} 1 kb DNA Ladder was used as a standard size marker.

Polymerase Chain Reaction (PCR)

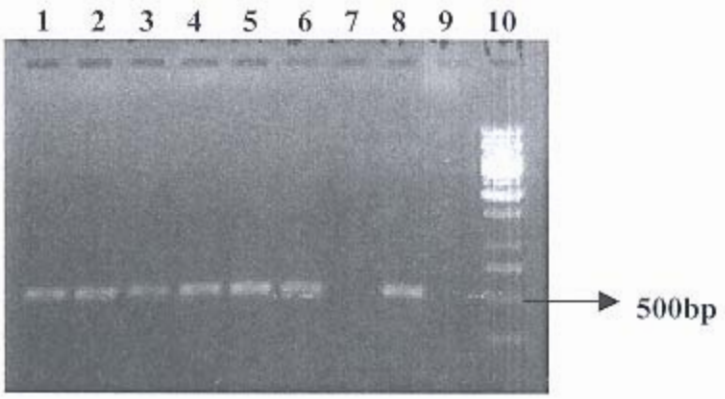


Figure 6: PCR products. Lanes 1-6 and lanes 8 indicate some successful amplification of *B. schwanenfeldii* and *B. gonionotus* sample. Lane 7 unsuccessful amplification and lane 9 was a negative control. Lane 10 represents GeneRuler^{TM} 1 kb DNA Ladder as a standard size marker.

All sample used for PCR gave a single product about 500 bp (Figure 2). Negative control was used in lane 9 as a guideline to examine contamination. PCR product was visualized under UV light and photograph of DNA band was taken on Polaroid film.

DNA Sequencing

20 samples of *B. schwanenfeldii* and *B. gonionotus* and one sample of out group *Helostoma temminckii* from Bakong, Sarawak were used for sequencing analysis. About 500-520 base pairs (bp) section of the *cytochrome c oxidase* I (COI) mtDNA region was utilized for analysis.

PCR-RFLP Analysis

From the RFLP analysis, only four enzymes *(Alu*I, *Bsu*RI, *Csp*61, *and Pvu*II) produced cutting fragments observed diagnostic between *B. schwanenfedlii* and *B. gonionotus*. The other enzymes did not exhibited any cutting fragments. Table 2 revealed the observed restriction site of the 10 enzymes on agarose gel and the expected restriction site based on sequence data. *Ssp*1 enzymes produced similar cutting profile between the two species (Table 2).

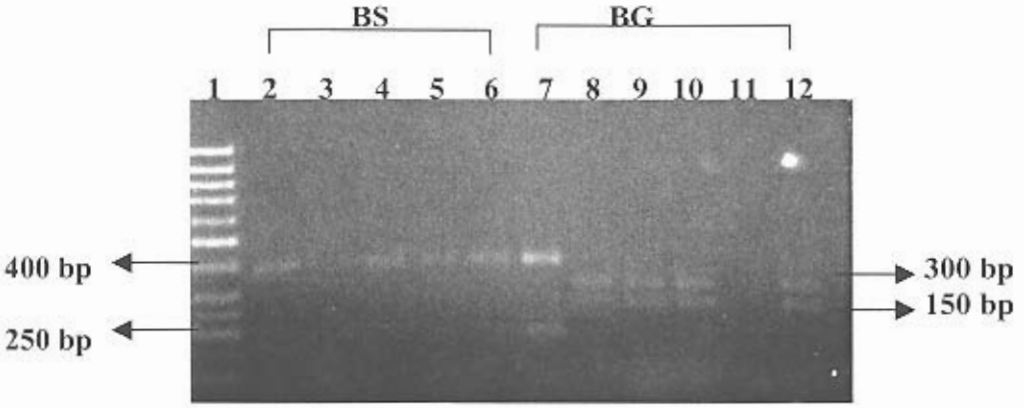


Figure 7: Result of *Alu*I restriction enzyme digestion between *B. schwanenfeldii* and *B. gonionotus*. Lanes 2-7 represent *B. schwanenfeldii* (BS) bands and lanes 8-12 showed *B. gonionotus* (BG) bands. Lane 1 represents GeneRuler^{TM} 50 kb DNA Ladder as a standard size marker.

Figure 7 showed digestion profile of *Alu*I enzyme, with differences in restriction fragment pattern between *B. schwanenfedlii* (lane 2-7) and *B. gonionotus* (lane 8-12). Two fragments (length 250 bp and 400 bp) were observed in *B. schwanenfedlii* while two fragments (length 150 bp and 300 bp) were observed in *B. gonionotus.*

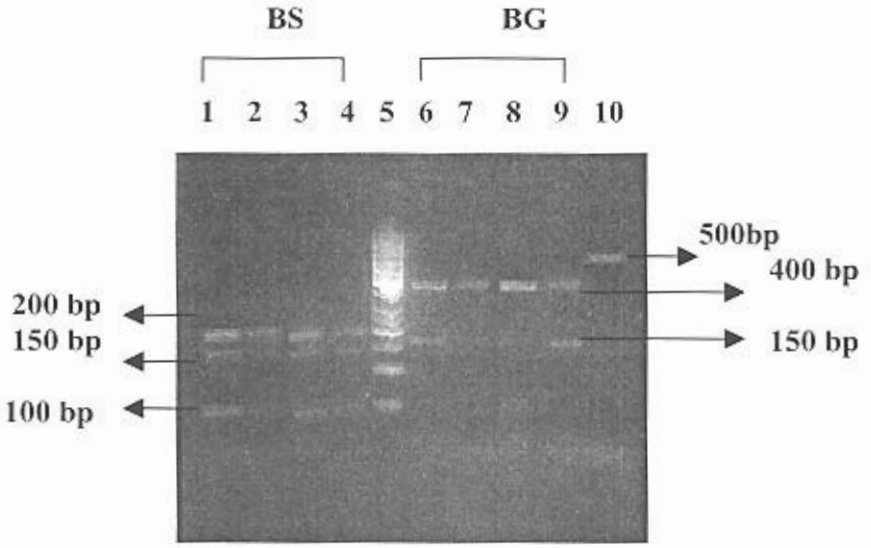


Figure 8: Result of *BsuR*I restriction enzyme digestion between *B. schwanenfeldii* and *B. gonionotus*. Lanes 1-4 represent *B. schwanenfeldii* (BS) bands and lanes 6-9 showed *B. gonionotus* (BG) bands. Lane 5 represents GeneRuler^{TM} 50 kb DNA Ladder as a standard size marker and lane 10 was uncut DNA.

*BsuR*I enzymes produced three fragments (200bp, 150bp, 100 bp) in *B. schwanenfeldii* and only two fragments in *B. gonionotus* (400 bp, 150 bp). Figure 8 showed result of *BsuR*I digestion

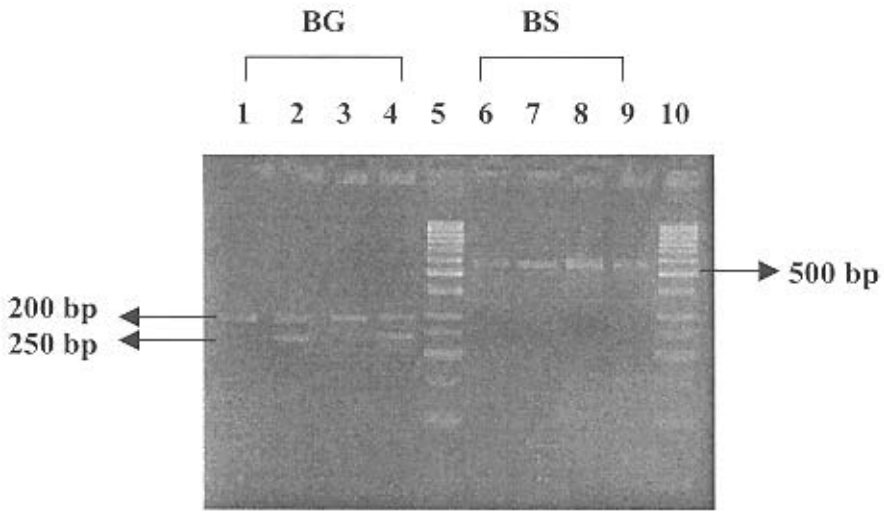


Figure 9: Result of *Pvu*II restriction enzyme digestion between *B. schwanenfeldii* and *B. gonionotus*. Lanes 1-4 present of *B. gonionotus* (BG) result and lanes 6-9 showed *B. schwanenfeldii* (BS) bright bands. Lane 10 represents GeneRuler™ 50 kb DNA Ladder as a standard size marker.

*Pvu*II enzyme also showed differentiation in digestion profile between the 2 species. It revealed three fragments (30 bp, 200 bp and 250 bp) in *B. gonionotus*, but was uncut in *B. schwanenfeldii*. The 30 fragment was too small and DNA cut not been seen during visualization or photograph. The confirmation of digestion profiles was made using DNA sequence data (expected size).

Fourthly, *Csp*61 produced two fragments in *B. schwanenfeldii* (500 bp, 30 bp) but was also uncut (no restriction site) in *B. gonionotus*. The 30 bp fragments were also too small and very difficult to see in gel or photograph. (Confirmed by sequence data).

Table 2: The restriction fragment composition of haplotypes from PCR-RFLP analysis (observed) and DNA sequence (expected)

Enzymes	*B. schwanenfeldii*		*B. gonionotus*	
	Gel electrophoresis (observed bp)	Sequence data (expected bp)	Gel electrophoresis (observed bp)	Sequence data (expected bp)
* ***AluI***	150	141	250	242
AG^CT	400	383	300	278
* ***BsuRI***	100	131	150	152
GG^CC	150	157	400	368
	200	236	-	-
* ***Csp6I***	30	27	-	-
G^TAC	500	497		
* ***PvuII***	30	26	-	-
CAG^CTG	200	180		
	250	247		
SspI				
AAT^ATT	250	245	250	242
BamHI				
G^GATCC	-	-	-	-
EcoRI				
G^AATTC	-	-	-	-
HpaII/MspI				
C^CGG	-	-	-	-
RsaI	-	-	-	-
GT^AC				

Keys:- 1) ^ =The cleavage of restriction site in the DNA sequence.
2) - = No restriction site in the DNA sequence
3) * = Diagnostics restriction enzymes

Phylogenetic Analysis

For phylogenetic analysis, both Neighbour-joining tree and Maximum Parsimony tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis, version 2.1) software. Figure 10 showed the phylogenetic tree using Neighbour-Joining method (with 1000 replicates). The tree strongly supported the reciprocally monophyletic between *B. schwanenfeldii* and *B. gonionotus* with strong bootstrap value (100%) in both species. Subsequently, all samples from both species were separated into two different clades according to their geographical origin (Peninsular Malaysia vs Borneo). In *B. schwanenfeldii,* the geographical clustering was fairly supported (60% for Peninsular Malaysia and 74% for Borneo) while in *B. gonionotus* the clustering was strongly supported (68% for Peninsular Malaysia and 94% for Borneo).

Figure 11 showed the phylogenetic tree based on Maximum Parsimony method. The top clustering of Maximum Parsimony tree consist two major clades with (100%) for *B. schwanenfeldii* and *B. gonionotus*. The tree also supported the reciprocally monophyletic between *B. schwanenfeldii* and *B. gonionotus* (100% bootstrap value). Similarly, samples from both species were also clustered according to their geographical locations (Peninsular and Borneo). The geographical separation was strongly supported in *B. gonionotus* (76% for Peninsular and 87% for Borneo) but was less clear in *B. schwanenfeldii* samples from Peninsular (2 subclades). Samples from Jempol and Padang Piol formed one group, while sample from Triang Selatan and Pulapah Lama formed another group.

Neighbour-joining analysis

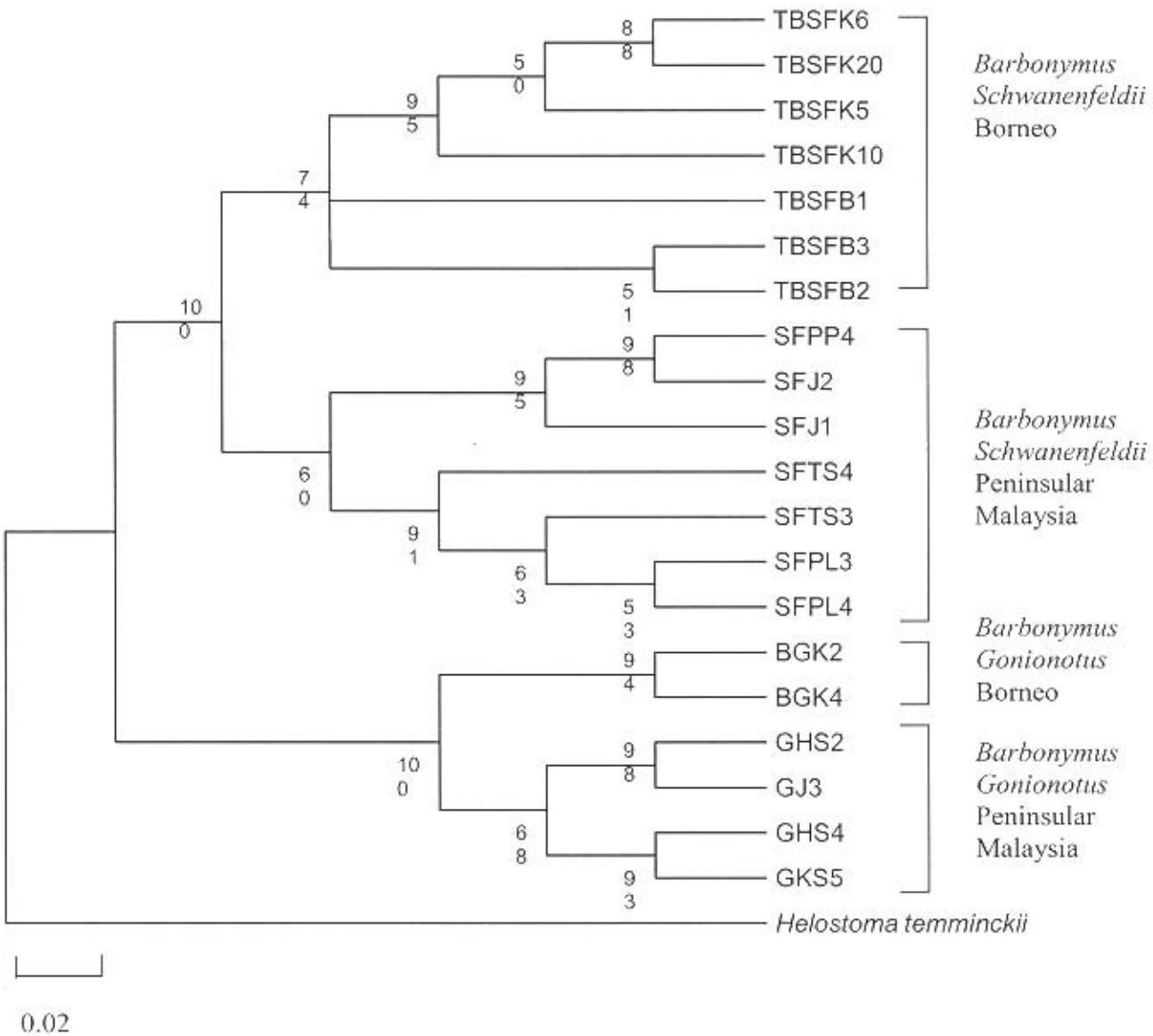


Figure 10: Bootstrap 50% majority – rule consensus tree Neighbour-joining tree analysis with 1000 replicates. *Helostoma temminckii* from Bakong was used as an out group. BGK1, BGK2: *B. gonionotus* Borneo; GJ3, GHS2, GHS4 and GKS5: *B. gonionotus* Peninsular; T-BSFB1, T-BSFB2, T-BSFB3, T-BSFK5, T-BSFK6, T-BSFK10 and T-BSFK20: *B. schwanenfeldii* Borneo; SFJ1, SFJ2, SFPL3, SFPL4, SFTS3, SFTS4 and SFPP4: *B. schwanenfeldii* Peninsular; HTBR: *H. temminckii* from Bakong, Sarawak.

Maximum Parsimony analysis

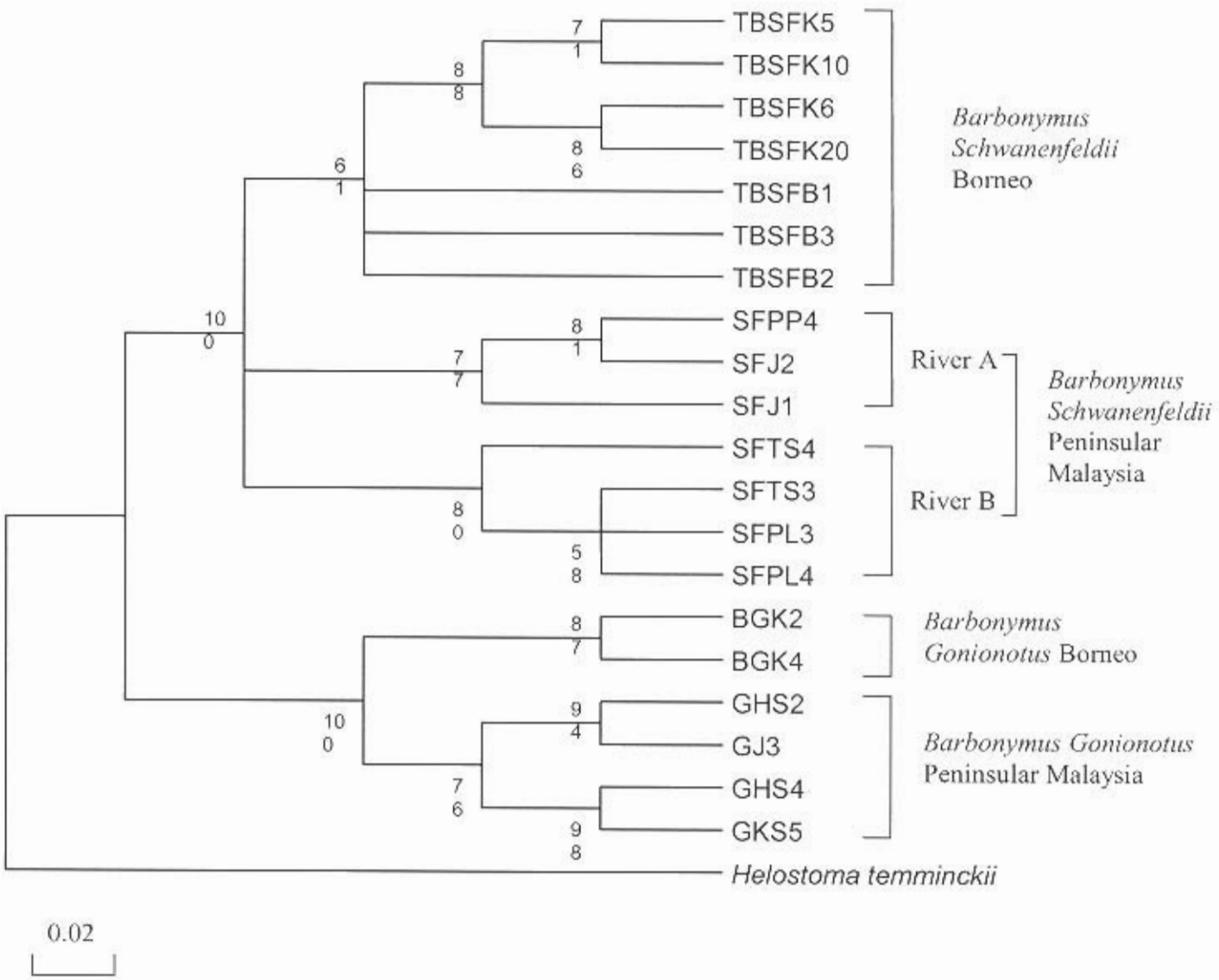


Figure 11: Bootstrap 50% majority – rule consensus tree of Maximum Parsimony with analysis with 1000 replicates. *Helostoma temminckii* from Bakong was used as an out group. BGK1, BGK2: *B. gonionotus* Borneo; GJ3, GHS2, GHS4 and GKS5: *B. gonionotus* Peninsular; T-BSFB1, T-BSFB2, T-BSFB3, T-BSFK5, T-BSFK6, T-BSFK10 and T-BSFK20: *B. schwanenfeldii* Borneo; SFJ1, SFJ2, SFPL3, SFPL4, SFTS3, SFTS4 and SFPP4: *B. schwanenfeldii* Peninsular; HTBR: *H. temminckii* from Bakong, Sarawak. River A is referred to Jempol River and river B is referred to Serting River.

Diversity and Population Structure.

Table 3: Genetic divergence between *Barbonymus gonionotus* and *Barbonymus schwanenfeldii* sample.

Species		Value Pi(t)
Barbonymus gonionotus :	(Jukes and Cantor)	
	Borneo	0.00616
	Peninsular Malaysia	0.03812
	Borneo vs Peninsular Malaysia	0.03553
	Total sample	**0.03662**
Barbonymus schwanenfeldii :	(Jukes and Cantor)	
	Borneo	0.02190
	Peninsular Malaysia	0.01466
	Borneo vs Peninsular Malaysia	0.02723
	Total sample	**0.02674**
Barbonymus gonionotus vs	***Barbonymus schwanenfeldii***	**0.08451**

Table 3 shows the analysis of genetic divergence between and within *B. gonionotus* and *B. schwanenfeldii* samples. Concerning to Jukes and Cantor, genetic divergence for *B. gonionotus* (0.03662) and *B. schwanenfeldii* (0.02674) samples was low. While, analysis between *B. gonionotus* and *B. schwanenfeldii* was apparently low (8.5%). Hence, it showed that both species is much closed with each other. Subsequently, for *B. schwanenfeldii* and *B. gonionotus* the value of nucleotide divergence between Borneo and Peninsular samples was also low (2.7% for *B. schwanenfeldii* and 3.6% for *B. gonionotus*).

Table 4: Shows the value of pairwise distance of Kimura 2-Parameter (below diagonal) and value of nucleotide diversity (above diagonal).

	1 BGK2	2 BGK4	3 GHS2	4 GJ3	5 GHS4	6 GKS5	7 TBSFB1	8 TBSFB2	9 TBSFB3	10 TBSFK5
1	-	0.006	0.029	0.025	0.035	0.061	0.137	0.154	0.133	0.143
2	0.006	-	0.033	0.029	0.039	0.063	0.142	0.161	0.140	0.151
3	0.025	0.029	-	0.037	0.029	0.061	0.153	0.170	0.149	0.151
4	0.029	0.033	0.004	-	0.029	0.061	0.157	0.174	0.153	0.155
5	0.036	0.040	0.029	0.029	-	0.037	0.150	0.166	0.148	0.152
6	0.064	0.066	0.064	0.064	0.038	-	0.174	0.188	0.170	0.177
7	0.156	0.163	0.176	0.182	0.170	0.202	-	0.029	0.008	0.014
8	0.176	0.186	0.197	0.203	0.191	0.221	0.029	-	0.022	0.029
9	0.150	0.160	0.171	0.176	0.167	0.196	0.008	0.023	-	0.017
10	0.164	0.174	0.174	0.179	0.173	0.206	0.015	0.030	0.017	-
11	0.194	0.199	0.204	0.210	0.203	0.237	0.036	0.054	0.040	0.023
12	0.166	0.176	0.176	0.181	0.175	0.208	0.015	0.032	0.017	0.002
13	0.174	0.185	0.184	0.190	0.183	0.216	0.021	0.038	0.025	0.006
14	0.145	0.155	0.166	0.171	0.165	0.194	0.025	0.040	0.021	0.030
15	0.151	0.161	0.172	0.177	0.168	0.197	0.032	0.047	0.027	0.036
16	0.161	0.171	0.179	0.185	0.173	0.208	0.032	0.047	0.032	0.028
17	0.158	0.169	0.174	0.179	0.168	0.203	0.029	0.044	0.029	0.025
18	0.161	0.171	0.176	0.182	0.171	0.205	0.029	0.044	0.029	0.023
19	0.153	0.164	0.169	0.174	0.166	0.198	0.029	0.045	0.029	0.025
20	0.154	0.164	0.174	0.180	0.171	0.200	0.034	0.049	0.030	0.039
21	0.233	0.243	0.245	0.250	0.248	0.270	0.219	0.241	0.219	0.230

	11 TBSFK6	12 TBSFK10	13 TBSFK20	14 SFJ1	15 SFJ2	16 SFPL3	17 SFPL4	18 SFTS3	19 SFTS4	20 SFPP4
1	0.166	0.145	0.151	0.129	0.133	0.142	0.140	0.142	0.136	0.135
2	0.169	0.152	0.158	0.136	0.141	0.149	0.147	0.149	0.143	0.143
3	0.174	0.153	0.159	0.145	0.150	0.156	0.152	0.154	0.148	0.152
4	0.178	0.157	0.163	0.149	0.154	0.160	0.156	0.158	0.152	0.156
5	0.174	0.154	0.160	0.146	0.148	0.152	0.148	0.150	0.146	0.150
6	0.199	0.178	0.184	0.168	0.171	0.179	0.175	0.177	0.171	0.173
7	0.035	0.014	0.020	0.024	0.031	0.031	0.029	0.029	0.029	0.033
8	0.052	0.031	0.037	0.039	0.045	0.045	0.029	0.043	0.043	0.047
9	0.039	0.017	0.025	0.020	0.027	0.031	0.029	0.029	0.029	0.029
10	0.023	0.002	0.006	0.029	0.035	0.027	0.025	0.023	0.025	0.038
11	-	0.027	0.023	0.052	0.058	0.045	0.043	0.041	0.043	0.060
12	0.027	-	0.010	0.029	0.035	0.031	0.029	0.027	0.029	0.037
13	0.023	0.010	-	0.035	0.041	0.029	0.029	0.025	0.027	0.043
14	0.054	0.030	0.036	-	0.006	0.023	0.020	0.020	0.016	0.008
15	0.061	0.036	0.043	0.006	-	0.020	0.023	0.027	0.018	0.006
16	0.047	0.032	0.030	0.023	0.021	-	0.002	0.006	0.006	0.023
17	0.045	0.030	0.030	0.021	0.023	0.002	-	0.029	0.004	0.025
18	0.043	0.027	0.025	0.021	0.027	0.006	0.002	-	0.006	0.029
19	0.045	0.030	0.028	0.017	0.019	0.006	0.004	0.006	-	0.021
20	0.063	0.038	0.045	0.008	0.006	0.023	0.025	0.029	0.021	-
21	0.253	0.229	0.230	0.224	0.231	0.239	0.236	0.239	0.231	0.233

* BGK1, BGK2: *B. gonionotus* Borneo; GJ3, GHS2, GHS4 and GKS5: *B. gonionotus* Peninsular; TBSFB1, TBSFB2, TBSFB3, TBSFK5, TBSFK6, TBSFK10 and TBSFK20: *B. schwanenfeldii* Borneo; SFJ1, SFJ2, SFPL3, SFPL4, SFTS3, SFTS4 and SFPP4: *B. schwanenfeldii* Peninsular; HTBR: *H. temminckii* from Bakong Sarawak.

Table 4 showed the value of pairwise distance (below diagonal) and value of nucleotide diversity (above diagonal) in all samples. For *B. gonionotus*, the highest value for nucleotide diversity was 0.063 (between Kolam Serting 5 and Kinabatangan 4) while the lowest value was 0.006. For *B. schwanenfeldii* the highest value was 0.060 (between Padang Piol and Kapit 6), while the lowest value was 0.02 (between Kapit 10 and Kapit 5).

Regarding to pairwise distance, the highest value for *B. gonionotus* was 0.066 (between Kinabatangan and Kolam Serting 5) while the lowest value was 0.004 (between Hulu Serting 2 and Hulu Serting 4). In *B. schwanenfeldii*, the highest value is 0.061 (between Kapit 6 and Jempol 2), while the lowest value was 0.002 (between three site, Kapit 5 and Kapit 10, Pulapah Lama 3 and Pulapah Lama 4 and lastly between Pulapah Lama 4 and Triang Selatan).

Table 5: The value of population structure F_{st} (below diagonal) and gene flow or number of migrants, N_m (above diagonal).

Sample and site		*B. schwanenfeldii*		*B. gonionotus*	
		Borneo	**Peninsular Malaysia**	**Borneo**	**Peninsular Malaysia**
B. schwanenfeldii	**Borneo**	-	0.54	0.05	0.10
	Peninsular Malaysia	0.481	-	0.04	0.10
B. gonionotus	**Borneo**	0.907	0.921	-	0.59
	Peninsular Malaysia	0.828	0.838	0.457	-

Table 5 shows value of the population structure, (F_{st}) and gene flow (N_m). The level of gene flow (N_m) between Peninsular Malaysia and Borneo, was low (N_m = 0.54 for *B. schwanenfeldii* and N_m = 0.59 for *B. gonionotus*). Thus, the population structuring between samples from Peninsular Malaysia and Borneo in both species was high (F_{st} = 0.481 in *B. schwanenfeldii* and F_{st} = 0.457 in *B. gonionotus*). Therefore, the results showed a possibility of genetic drift among population of *B. schwanenfeldii* and *B. gonionotus* from Peninsular Malaysia and Borneo, resulted from geographical isolation by South China Sea.