Research article

Phylogeny and phylogeography of *Barbonymus schwanenfeldii* (Cyprinidae) from Malaysia inferred using partial *cytochrome b* mtDNA gene

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ABSTRACT. A study on the genetic variation of *Barbonymus schwanenfeldii* (Bleeker) populations from Malaysia was done using partial sequencing of *cytochrome b* mtDNA gene. Samples were collected from various localities in Peninsular Malaysia, Sarawak and Sabah, including the Indonesian (Kalimantan) type of *B. schwanenfeldii* from Kapit, Sarawak. Phylogenetic relationship inferred using neighbour joining, maximum likelihood and maximum parsimony methods generally divided samples into two major groups, consistent with their geographic origin: one widespread group consisted of *B. schwanenfeldii* (locally known as Lampam sungai) from Peninsular Malaysia while another particular group clustered *B. schwanenfeldii* (locally known as Tengadak) from Sarawak. Within the Peninsular Malaysia, our current data supported the validation of two main divisions: central and southern division (CS; consisting of Serting River, Muar River and Padang Piol, Jerantut, Pahang) and north west and north east division (NWE; consisting of Pulau Banding, Perak and Tasik Timah Tasoh, Perlis). The low distance values between haplotype sequences (0.00 to 1.01) and low nucleotide diversity, P_i (0.005) showed a very close genetic relationship between samples, enforcing their taxonomic validation as belonging to a single taxon. However, the low level of gene flow ($N_m = 0.07$) and high population structuring ($F_{st} = 0.88$) between Peninsular Malaysia and Sarawak could be correlated with the separation of Borneo Island from mainland Peninsular during last Pleistocene Epoch.

Keywords: *Barbonymus schwanenfeldii*, partial *cytochrome b*, population genetics.

INTRODUCTION

The greatest diversity of freshwater fish is found in tropical South America, Africa and South East Asia (Helfman *et al*., 1997). The fish diversity of South East Asia area was once affected during the Pleistocene Epoch of the Neogene Period. The Pleistocene was characterized by multiple episodes of glaciation, which caused ice to advance and retreat approximately every 100,000 years beginning about 2 million years ago (Dott & Prothero, 1994). Biogeographically, Malaysia is situated in the Oriental region. Peninsular Malaysia, southern Thailand, southern Indo-China, Sumatra, Java and Borneo were once part of the submerged Sunda Shelf (Mohsin & Ambak, 1983). In recent times, Peninsular Malaysia is separated from Borneo by the South

China Sea. Malaysian Borneo is made up of Sarawak and Sabah. The relatively high geographic distance between mainland of Peninsular Malaysia and both Sabah and Sarawak could be related to the biogeography theory of the Pleistocene events. Borneo Island is biogeographically located near the Wallacea region, the area between Wallace's line and Weber's line. In this case, we predict that genetic drift might have led to the genetic differentiation in mtDNA lineages between freshwater fish from Peninsular Malaysia and Borneo Island, after the Shelf submerged. According to Halliday (1993), genetic drift may become the major source of genetic variation between some populations.

Family Cyprinidae is currently the largest family of freshwater fish in terms of its abundant genera and species throughout Malaysia. According to Mohsin & Ambak (1983), the Malaysian region is considered as the southern centre for distribution of primary freshwater fish. Generally, freshwater fish from the genus *Puntius* are the most abundant species in Malaysia and can be found in almost every water body (Inger & Chin, 1962; Mohsin & Ambak, 1983; Zakaria Ismail, 1990). In this study, *Puntius schwanenfeldii* (Bleeker), *Barbodes schwanenfeldii* (Bleeker) or recently known as *Barbonymus schwanenfeldii* (Bleeker) was chosen to test the glacial effects on the freshwater fish diversity during the Pleistocene low sea levels. *Barbonymus schwanenfeldii* is known as “lampam sungai” in Peninsular Malaysia and “tengadak” in Sarawak. It is a pretty river-dwelling fish and morphologically quite similar to *Puntius gonionotus* (Java borb fish). This fish is widely distributed in all rivers and lakes in Peninsular Malaysia particularly in Pahang, Perak, Kelantan, Terengganu and Selangor (Mohsin & Ambak, 1991) and indigenous to upper and mid-zone of Rejang River system, as well as Limbang and Batang Ai rivers (Litis *et al*., 1997).

The sequence evolution of mitochondrial DNA of freshwater fish has been relatively well studied in the phylogeographic structure and in clarifying the phenomena of postglacial colonization (Dodson *et al*., 1995; Wang *et al*., 2000). Fish mitochondrial genomes are effectively inherited maternally, haploid, apparently non-recombining and therefore correspond exactly to the model of bifurcating evolutionary tree (Stepien & Kocher, 1997). Overall mtDNA substitution rates are estimated 5-10 times greater than in 'single-copy' nuclear DNA, and commonly occur in transition or transversion form (Hartl & Clark, 1989; Hoelzel & Dover, 1991).

In general, molecular analyses of *cytochrome b* partial sequence were done in this study as an additional approach to study the Pleistocene impacts by comparing the genetic differentiation between *B. schwanenfeldii* from Peninsular Malaysia region with Sarawak and Sabah as the Borneo representatives. The *cytochrome b* is probably the best-studied mtDNA gene in fishes (Zhu *et al*., 1998; Jansson & Öst, 1997) and this region is used to analyze relationships among species, phylogeographic questions and population genetic structuring. Wang *et al*. (2000) used *cytochrome b* gene to assess the genetic and phylogeographic structure of *Acrossocheilus paradoxus* populations from Taiwan. In addition, a number of *B. schwanenfeldii* from Kalimantan might have been transferred to Kapit, Sarawak and had been put together into the population of local *B. schwanenfeldii*. Thus, the phylogenetic study was applied to test the taxonomic assumption of the Indonesian type *B. schwanenfeldii* as distinct from other *B. schwanenfeldii* populations from Sarawak, due to the minor difference in colour observed between the two types of *B. schwanenfeldii* (Stephen M. Sungan, personal comment).

MATERIALS AND METHODS

Sample Description and Location

Samples of *B. schwanenfeldii* were collected from several populations in Peninsular Malaysia, Sarawak and Sabah (Table 1, Figure 1). *Barbonymus schwanenfeldii* fish from Kapit, Sarawak were identified as Indonesian type fish as they are believed to have originated from Kalimantan. Fish were sampled using a variety of fishing methods, including seine net, gill nets, cast nets and fishing rod. Fresh samples of local type *B. schwanenfeldii* fish and pure Indonesian type *B. schwanenfeldii* fish from Kapit, Sarawak were stored at -80°C freezer for long-term storage. The other samples were preserved in 95% ethanol and stored at -20°C until genetic analyses were performed.

DNA Extraction

Total DNA was extracted from muscle tissue using modified Cetyl trimethylammonium bromide (CTAB) method (Grewe *et al*., 1993) with the presence of Proteinase K. Pelleted DNA was re-dissolved in 100 µL of sterilized distilled water. Quality and approximate yield were determined by electrophoresis in a 1% agarose gel containing ethidium bromide at 90 V for 30 min. Isolated genomic DNA were used for mtDNA analysis.

Polymerase Chain Reaction (PCR)

Approximately 450 base pairs (bp) section of the mtDNA genome from the *cytochrome b* gene was successfully amplified using standard polymerase chain reaction (PCR) procedures. Two universal primers of *cytochrome b* were used: GludG-L (5'-TGACTTGAARAACCAYCGTTG-3', forward) and CB2-H (5'-CCCTCAGAATGATATTTGTCCTCA-3', reverse) (Palumbi *et al*., 1991). Each thermal cycle amplification was performed in 50 µL reaction volume containing 31.75 µL sterilized distilled water, 0.25 µL of *Taq* DNA polymerase, 5.0 µL of 10X reaction buffer, 1.0 µL of dNTP (10 mM), 3.0 µL of Magnesium Chloride (25 mM) and 2.5 µL of each primer (10 µM). Approximately 4.0 µL of the DNA was added to each PCR cocktails. 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 (30 cycles) for elongation, and 7 min at 72°C for final elongation. Master mix was used for large quantities of PCR reactions. The amplified products were visualized on 1% agarose gel containing ethidium bromide, run for approximately 30 min at 90 V and photographed under UV light. A digested lambda DNA (GeneRuler™ 1 kb DNA Ladder) was used as a standard size marker (Fermentas). PCR products were purified prior to sequencing using purification kit (Promega).

DNA Sequencing

Purified PCR products were pelleted and air-dried before sending for sequencing. Sequencing was done using the BigDye® Terminator v3.0 Cycle Sequencing kit (ACGT). Cycle sequencing reaction was done in a programmable cycler (Tpersonal Combi Thermocycler). Cycle sequencing reaction was done for 35 cycles of 96°C: 10 sec, 55°C: 5 sec, 60°C: 4 min hold and proceeded to Ethanol/ Sodium Acetate precipitation. Rapid thermal ramp was 1°C/sec. Sequencing was carried out on ABI 377 automated sequencer (PE Applied Biosystem).

Statistical Analysis

CHROMAS (version 1.45; Technelysium Pty. Ltd., Tewantin Qld 4565, Australia) program was used to display fluorescence-based DNA sequence analysis results. Multiple sequence alignment for forward reactions of *cytochrome b* partial sequences of *B. schwanenfeldii* was done by using CLUSTAL X program (version 1.81; Thompson *et al*., 1997), and subsequently

Table 1. Description of *Barbonymus schwanenfeldii* samples from several populations in Malaysia.

No.	Region	Study site	n	Abbreviation	Gen Bank no.
1	Sabah	Tenom (BSFT)	1	BSFT1	-
2	Sarawak (S)	Kapit (BSFKS)	14		
		(a) Local type (BSFK)	4	BSFK11	AY243354
				BSFK12	AY355431
				BSFK14	AY355432
				BSFK15	AY462409
		(b) Indonesian type (BSFI) - originated from Kalimantan, Indonesia	10	BSF11	AY355434
				BSFI4	AY462413
				BSFI9	AY355436
				BSFI13	AY462412
				BSFI14	AY355433
				BSFI15	AY462414
				BSFI16	AY462410
				BSFI20	AY462411
				BSFI21	AY462408
				BSFI22	AY355435
3		Paku River, Betong, Sri Aman (BSFB)	2	BSFB1	AY355418
				BSFB2	AY355437
				BSFB3	AY355438
4		Bau (BSFBP)	1	BSFBP1	AY462415
5	Peninsular Malaysia (PM)	Serting River			
		Jempol Pump Station, Serting Hilir (BSFJ)	2	BSFJ1	AY243355
				BSFJ2	AY355425
		Pulapah Lama Road, Negeri Sembilan (BSFPL)	4	BSFPL1	AY355428
				BSFPL2	AY355423
				BSFPL4	AY355429
				BSFPL5	AY355424
		Triang Selatan 2, Serting Hilir (BSFTS)	3	BSFTS1	AY355430
				BSFTS2	AY355421
				BSFTS3	AY355422
		Ayer Hitam (BSFAH)	1	BSFAH1	AY462416
6		Padang Piol, Jerantut, Pahang (BSFPP)	4	BSFPP1	AY355419
				BSFPP2	AY355426
				BSFPP3	AY355427
				BSFPP4	AY355420
7		Pulau Banding, Perak (BSFPB)	3	BSFPB1	AY462404
				BSFPB2	AY462405
				BSFPB3	AY462406
8		Tasik Timah Tasoh, Perlis (BSFTT)	1	BSFTT1	AY462407
9		Muar River, Johor (BSFM)	2	BSFM1	AY576455
				BSFM3	AY576456
		TOTAL	**39**		

* Location and individual abbreviations are used in figures, tables and text.

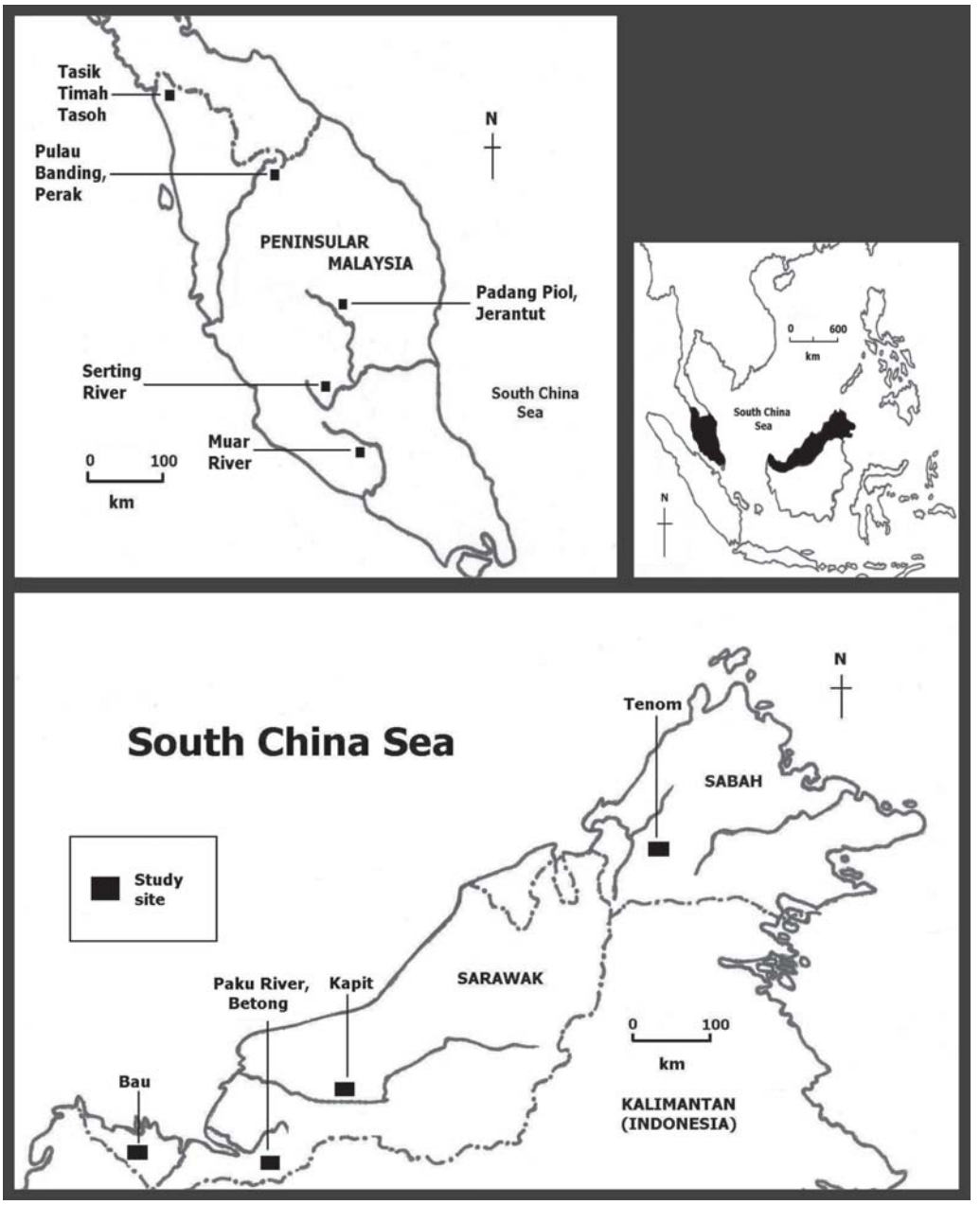

Figure 1. Small figure on the upper right shows the location of Malaysia region (filled) in South East Asia. The left figure shows the location map of *Barbonymus schwanenfeldii* study areas in the mainland of Peninsular Malaysia while the lower figure shows the location map of *B. schwanenfeldii* study areas in Sabah and Sarawak. Each study area is detailed in Table 1.

aligned by eye. A sequence of *Barbodes schwanenfeldii* from GenBank (GenBank accession number: AF180823) was chosen as corresponding sequence. Phylogenetic Analysis Using Parsimony (PAUP*) program version 4.0b10 (Swofford, 1998) was used to reconstruct neighbour joining tree (Saitou & Nei, 1987), maximum parsimony and maximum likelihood trees. This program was also utilized to examine other relevant analyses such as base frequencies. Kimura 2-parameter distance (1980) was selected, base on equal base frequencies and unequal ratio of transition to transversion (Ti:Tv). Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets. In addition, Molecular Evolutionary Genetics Analysis (MEGA version 2.1; Kumar *et al*., 2001) was used as comparison. The level of genetic diversity between, within and among populations and geographical regions were quantified by pairwise estimates of nucleotide diversity (P_i; Jukes & Cantor, 1969); level of gene flow (N_m) and F-statistic analysis (F_{st}) from Hudson *et al*. (1992). The parameters were analyzed using DNA Sequence Polymorphism (DNA SP) version 3.53 (Rozas & Rozas, 1999).

RESULTS

Genetic Diversity and Population Structure

Partial sequences of 450 base pairs (bp) of *cytochrome b* mtDNA gene were obtained from 38 individuals of *B. schwanenfeldii* and after excluding alignment gaps, 399 bp were aligned prior to DNA sequence analyses. All sequences were registered with GenBank (Table 1). However, as a precautionary step to minimize bias, one sequence of *B. schwanenfeldii* from Tenom, Sabah was excluded due to the low number of sample from Sabah population, i.e. one sample. Alignment was made against a sequence of *Helostoma temminckii* from Bakong, Sarawak, as an outgroup. Out of 399 sites, seven sites were considered as variable sites (1.75%), including five parsimony-informative characters (1.25%). Three hundred and ninety two out of 399 sites were conserved (98.25%). There were six haplotypes (N_{Hap}); only one haplotype within the Sarawak region and five haplotypes within the Peninsular region, with haplotype (gene) diversity 0% and 70% respectively. Within the Peninsular region, four haplotypes originated from central and southern division (CS; consisting of Serting River, Muar River and Padang Piol, Jerantut, Pahang) and a single haplotype from north west and north east division (NWE; consisting of Pulau Banding, Perak and Tasik Timah Tasoh, Perlis). The current divisions were modified from and based on regional partition by Mohsin & Ambak (1983, 1991; Figure 2).

The Kimura 2-parameter distance values (results were not shown) between sequences were remarkably low, with a range between 0% and 1.0%. The distance values within the Peninsular Malaysia samples ranged from 0% to 1.0%. Within the Borneo region, the average distance value was 0%, suggesting identical mtDNA lineage. Moreover, the distance values between the Peninsular region and Sarawak population as the sub-Borneo representative ranged between 0.8% and 1.0%. The standard deviation values likely supported the Kimura 2-parameter distance values between samples of *B. schwanenfeldii*.

Likewise, low nucleotide diversity, P_i was calculated within regions, ranging from 0% to 0.3% (Table 2). BSFTS has the highest nucleotide diversity (0.3%) with three haplotypes (haplotype diversity=1.00). Within the Peninsular region, the average P_i was 0.2%, relatively higher than within the Sarawak region (0%). However, the nucleotide diversity between Peninsular and Sarawak regions may be considered as similar, due to the small difference in percentage (0.5%). Most samples from central and southern division (CS) shared a single and identical haplotype. The average P_i

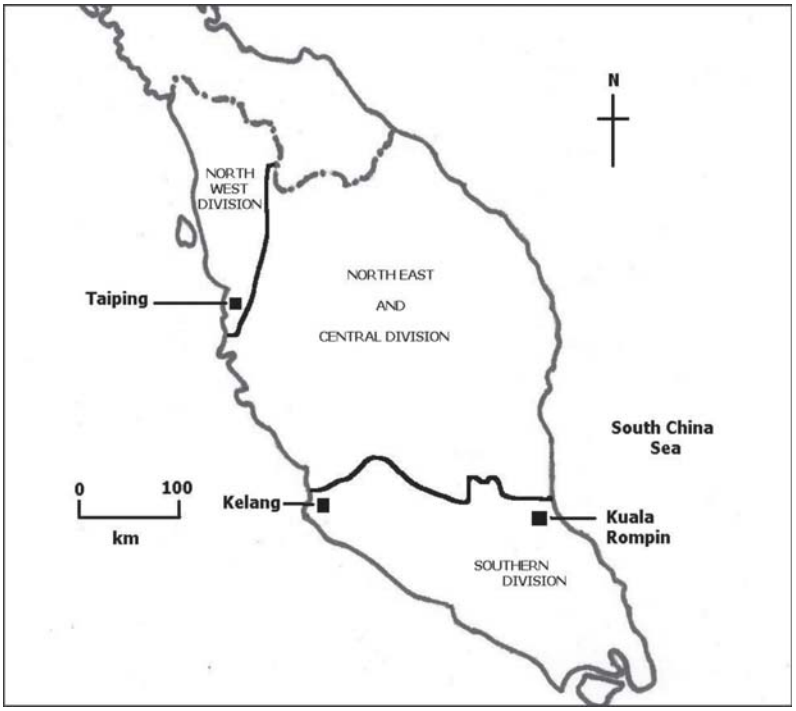

Figure 2. Three main divisions of Peninsular Malaysia. Adapted from Mohsin & Ambak (1983, 1991)

Table 2. Calculation of nucleotide diversity, P_i (JC) (Jukes & Cantor, 1969) within populations and geographical regions of *Barbonymus schwanenfeldii* in Malaysia. Each study area is detailed in Table 1.

Study site	**Nucleotide diversity, P_i (JC) %**
Sarawak	0.0
Kapit	0.0
Bau	0.0
Peninsular Malaysia	0.2
Central and Southern Division (CS)	0.2
North West and North East Division (NWE)	0.0
Jempol Pump Station, Serting Hilir (CS)	0.0
Pulapah Lama Road, Negeri Sembilan (CS)	0.1
Triang Selatan 2, Serting Hilir (CS)	0.3
Padang Piol, Jerantut, Pahang (CS)	0.0
Muar River (CS)	0.0
Pulau Banding, Perak (NWE)	0.0
Malaysia	0.5

of CS and NWE were 0.2% and 0% respectively, with 0.2% in difference. Overall, the nucleotide diversity of *B. schwanenfeldii* throughout Malaysia was 0.5%, featuring a very close similarity among haplotypes.

The extent of gene flow level between Peninsular and Sarawak regions was low (Nm = 0.07, Table 3). The outcome was expected based on the recent separation of Borneo Island from Peninsular Malaysia by South China Sea. Consequently, the population structuring between both regions, which has been estimated from F-statistic analysis was high (F_{st} = 0.88). These two types of data gave valuable clarification towards the Pleistocene events. Within the Peninsular region, the level of gene flow (Table 3) between NWE and CS was low (N_m= 0.15) with high population structuring (F_{st}= 0.76), supporting parts of the regional partition by Mohsin & Ambak (1983, 1991).

Phylogenetic Analysis

A total of 39 partial sequences (399 bp) of *cytochrome b* mtDNA gene were aligned; including one sequence of *Helostoma temminckii* as an outgroup to root the phylogenetic trees. The neighbour joining tree using bootstrap test (Figure 3) of *B. schwanenfeldii* populations revealed a major clade of *B. schwanenfeldii* samples from Sarawak (96% bootstrap value) while *B. schwanenfeldii* samples from Peninsular Malaysia region were basal to the Sarawak clade. Within the Peninsular Malaysia, samples from NWE formed a particular cluster, with 65% bootstrap value. Likewise, the maximum parsimony and maximum likelihood tree using bootstrap test (Figure 3) of *B. schwanenfeldii* populations revealed almost the same pattern, with 96% and 93% bootstrap value for Sarawak clade, respectively; 59% and 64% bootstrap value for NWE cluster, respectively. In view of the level of genetic diversity, the clustering showed by both neighbour joining and maximum parsimony trees suggested the possibilities of genetic drift impacts on Borneo populations that subsequently led to the genetic variation in *B. schwanenfeldii* mtDNA genome. These data were corresponding to the low level of gene flow (N_m = 0.07) and high population structuring (F_{st} = 0.88) between mainland of Peninsular Malaysia and Sarawak as the Borneo representative.

In addition, the individuals of the Indonesian type *B. schwanenfeldii* mixed with the local type *B. schwanenfeldii* samples from Sarawak (Figure 3). Regarding the results of genetic distance and nucleotide diversity (P_i), the phylogenetic pattern apparently showed that the Indonesian type *B. schwanenfeldii* shared the same mtDNA lineages with the local type *B. schwanenfeldii* from Sarawak. In other words, the identical genetic lineage between *B. schwanenfeldii* from Kalimantan and *B. schwanenfeldii* from Sarawak totally unraveled the taxonomic similarity between them.

Generally, the phylogenetic relationship inferred using the neighbour joining, maximum likelihood and maximum parsimony methods revealed two major groups: the widespread group of *B. schwanenfeldii* samples from Peninsular Malaysia, which is basal, and a particular group of *B. schwanenfeldii* samples from Sarawak support the historical theory of Pleistocene events. Within the Peninsular Malaysia there are currently two main valid divisions: central and southern division (CS; consisting of Serting River, Muar River and Padang Piol, Jerantut) and north west and north east division (NWE; consisting of Pulau Banding, Perak and Tasik Timah Tasoh, Perlis). The *B. schwanenfeldii* sequences of NWE differed from CS populations with one transition (Ti=1). Furthermore, the phylogenetic relationship did not support the taxonomic assumption of the pure Indonesian (Kalimantan) type *B. schwanenfeldii* as a distinct from other *B. schwanenfeldii* populations from Sarawak.

Table 3. Calculation of nucleotide diversity, Pi (JC) (Jukes & Cantor, 1969), level of gene flow, Nm (Hudson *et al.*, 1992) and population structuring, Fst (Hudson *et al.*, 1992) within and between geographical regions of *Barbonymus schwanenfeldii* in Malaysia. NWE - North West and North East Division, Peninsular Malaysia; CS - Central and Southern Division, Peninsular Malaysia.

	Nucleotide diversity, Pi (JC) (Jukes & Cantor, 1969)	**Level of gene flow, Nm (Hudson *et al.*, 1992)**	**Population structuring, Fst (Hudson *et al.*,1992)**
Peninsular Malaysia	0.2	-	-
NWE vs CS	0.2	0.15	0.76
Sarawak	0.0	-	-
Peninsular Malaysia vs Sarawak	0.5	0.07	0.88

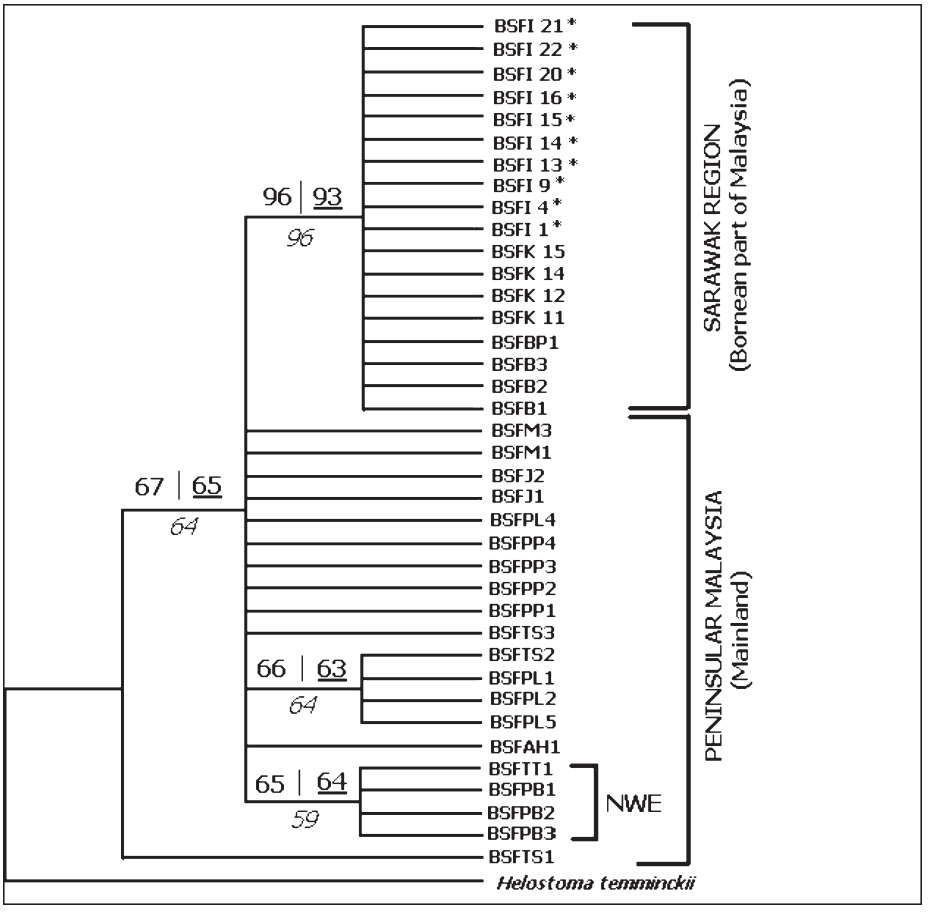

Figure 3. Phylogenetic tree (consensus tree) of the populations of *Barbonymus schwanenfeldii* inferred from *cytochrome b* mtDNA gene. Abbreviations refer to individuals in populations identified in Table 1. The tree was rooted with a sequence of *Helostoma temminckii* from Bakong, Sarawak. Kimura 2-parameter distance (1980) with 1000 replications was used. Indonesian (Kalimantan) type *B. schwanenfeldii* (BSFI) is labelled with asterisk symbol (*). Numbers at nodes indicate the bootstrap values in percentage (Bold– neighbour joining method; underline- maximum likelihood; italic- maximum parsimony method). NWE – north west and north east division.

DISCUSSION

Haplotype Diversity and Population Structure

Geographical isolation of allopatric populations is the major way of preventing gene flow between two populations, which allows an evolution of a genome adapted to local condition (Hall, 1993). Likewise, the low level of gene flow of *B. schwanenfeldii* between Peninsular Malaysia and Sarawak may be associated with the isolation of Borneo; the submersion of the Sunda River and the rise of the sea level after the Pleistocene Epoch have caused the rise in the sea level between 40 to 100 meters. This gradual separation of Borneo from the mainland of Peninsular Malaysia was suspected to have caused accumulative genetic drift. Genetic drift is likely to occur, particularly in small populations that are isolated from the main population of a species (Halliday, 1993).

The relatively high similarity in *B. schwanenfeldii* sequences between Peninsular Malaysia and Sarawak samples was in congruence with the Pleistocene glaciation (about 10,000 years ago; see Halliday, 1993). The results may reflect the formation of the Sunda River during the Pleistocene, which allowed the flow and migration of *B. schwanenfeldii* between the eastern part of the Peninsular Malaysia and the western parts of Borneo, until the submersion of the great river system (Mohsin & Ambak, 1983; Dodson *et al*., 1995). During this period of Pleistocene land bridge, there were great potential for the movement of freshwater fish between both regions. Furthermore, the high similarity in *B. schwanenfeldii* sequences between Peninsular Malaysia and Sarawak samples presumably may indicate remnants of identical haplotypes from both lineages, and they were essentially similar at one time before the separation (Inger & Chin, 1962, 2002). Dodson *et al*. (1995) found that *Hemibagrus nemurus* colonizing the Kapuas river of west Borneo, east Sumatra and south Peninsular Malaysia; tentatively identified as *H. hoevenii*, have formed the most morphologically and genetically distinct Sundaic clade suggesting the occurrence of gene flow between the areas. However, there was no report on *B. schwanenfeldii* in North Borneo (Inger & Chin, 1962, 2002). Interestingly, we managed to get one sample of *B. schwanenfeldii* from Tenom, Sabah and thus giving the possibility of recent introduction of the species into the northern part of Borneo. In terms of biogeography, Sabah rivers were never connected to Sunda River during the last Pleistocene (Figure 4; Voris, 2000).

All individuals of *B. schwanenfeldii* from Sarawak were represented by one haplotype and there was no genetic differentiation among them, suggesting an identical genetic inheritance between the Indonesian type individuals and individuals from Sarawak rivers. Even though relatively low values of genetic distances were calculated between individuals from mainland of Peninsular Malaysia and Sarawak, the status of Peninsular Malaysia *B. schwanenfeldii* as basal to the Sarawak clade in all the phylogenetic trees (Figure 3) and the presence of five different haplotypes in Peninsular Malaysia *B. schwanenfeldii* suggested the occurrence of nucleotide substitutions resulted from the isolation phenomenon of Borneo. Accordingly, we also suggest that the direction of gene flow was from Peninsular Malaysia to Borneo through the Sunda River during the last Pleistocene. The reconstructed phylogenetic trees also showed population structure within Peninsular Malaysia but the findings were not fully supported by the zoogeographic reconstruction from Dodson *et al*. (1995) and not entirely in congruence with the main divisions suggested by Mohsin & Ambak (1983, 1991). More detailed studies will be required to clarify such population structure.

Phylogeography

According to Mohsin & Ambak (1983), during the middle Pleistocene, maximum lowering of sea level had occurred and the eastern slopes of

Peninsular Malaysia, West Borneo and Sumatera were drained by Sunda River. Sunda River was rich in freshwater fish and has been dominated by cyprinids. Meanwhile, the faunas of Sunda shelf may have mingled in these lowlands. The formation of Pleistocene land bridges had permitted free migration of fauna and flora between mainland and islands. The interconnection between some rivers in Peninsular Malaysia and Sarawak during the last Pleistocene (Figure 4) may have led to the intermixing of *cytochrome b* gene of cyprinids between the two regions, by which low genetic differentiation was calculated among samples of *H. macrolepidota* from southern Peninsular Malaysia, and southern and central Sarawak (Ryan & Esa, 2006). The sharing of same haplotype between *Tor tambroides* from Batang Ai, Sarawak and Perak, northern Peninsular Malaysia (Esa *et al*., 2008) further supported the historical connection of drainages. When this great river system submerged and the sea level increased, Borneo was formed and separated from Peninsular Malaysia by the South China Sea (Mohsin & Ambak, 1983; Dodson *et al*., 1995).

Two major groupings consisted of a widespread basal group of Peninsular Malaysia and another distinct group of Sarawak region, as revealed by the phylogenetic trees (Figure 3), correspond to the low level of gene flow observed between the two geographical regions. In this case, the South China Sea is considered as the geographic barrier, which leads to the restriction of gene flow between the two isolated regions. Likewise, Dodson *et al*. (1995) has suggested a genetically distinct 'Sarawak' group of *Hemibagrus nemurus* Valenciennes (1839) in West Borneo, based on mtDNA test and morphological analysis. Clearly, the identical mtDNA lineage observed among *B. schwanenfeldii* from Sarawak as well as Kalimantan indicated as if Borneo was one big population instead of a region with multiple populations of *B. schwanenfeldii*. Within the Peninsular Malaysia, our current data support the validation of two main divisions central and southern divisions: CS and NWE. The presence of a single haplotype representing NWE and four different haplotypes for CS further support the division validation.

Based on our current findings, we suggest that there was no colonization of *B. schwanenfeldii* in Borneo, particularly in Sarawak, before the period of the Pleistocene land bridge. When the islands of the Sunda shelf and the mainland were connected by lowlands traversed by the Sunda River, the faunas of Peninsular Malaysia, Sumatera, Borneo and Java may have mingled (Dodson *et al.*, 1995. In the meantime, it was believed that founders of *B. schwanenfeldii* migrated into Borneo and colonized the rivers during the last Pleistocene. Consequently, after the recent isolation period, genetic drift has slowly caused allopatric speciation between Peninsular Malaysia and Borneo *B. schwanenfeldii*, in this case *B. schwanenfeldii* from Sarawak and Kalimantan. In an isolated population, gradual adaptation to a new condition could allow a genome to evolve gradually. The small number of transitions between *B. schwanenfeldii* from Peninsular Malaysia and Sarawak (Ti= 3) correspond with the recent isolation of Borneo from the mainland of Peninsular Malaysia.

The proposition of the Kalimantan type *B. schwanenfeldii* as a distinct species from any other *B. schwanenfeldii* from Sarawak populations was not discerned by the phylogenetic trees (Figure 3). All individuals of Indonesian type *B. schwanenfeldii* (BSFI) mixed with local individuals of *B. schwanenfeldii* from Sarawak. Morphologically, both types of *B. schwanenfeldii* shared similar physical appearance. Furthermore, the identical mtDNA lineage between local type and pure Indonesian type *B. schwanenfeldii* from Kapit, suggested similar genetic relationship between the two types of *B. schwanenfeldii*. These corresponding data from the morphological characteristics and mtDNA information strongly rejected the

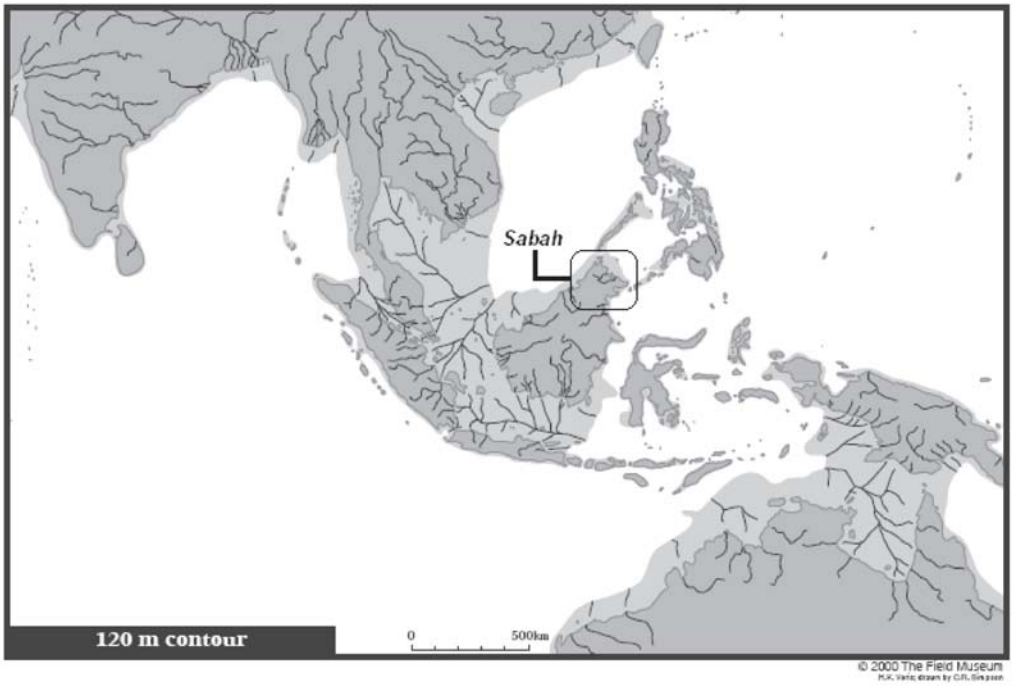

Figure 4. Map of Pleistocene sea level for tropical Southeast Asia and Austral-Asia based on depth contour of 120 m below present level. Modified from Voris (2000).

proposition of Indonesian type *B. schwanenfeldii* as a distinct species from any other *B. schwanenfeldii* from Sarawak populations. In other words, both *B. schwanenfeldii* from Sarawak and Kalimantan are morphologically and genetically identical.

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