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## Genetic identification and structure of *Clarias batrachus* (Linnaeus, 1758) from Southeast Asia using a mitochondrial DNA marker

PAOLIN LEE<sup>1</sup> & ZOHRAH SULAIMAN<sup>1,2</sup>

<sup>1</sup> Environment and Life Science Programme, Faculty of Science, Universiti Brunei Darussalam, Tungku BE1410, Brunei Darussalam; E.mail: paolinlee@yahoo.com; zohrah.sulaiman@ubd.edu.bn.

<sup>2</sup> Institut Teknologi Brunei, Tungku BE 1410, Brunei Darussalam; E.mail: zohrah.sulaiman@itb.edu.bn

### Abstract

A phylogenetic tree and median-joining network based on cytochrome b sequence data revealed clades consistent with morphological differences and geographical distribution of *Clarias batrachus* (Linnaeus, 1758) in Southeast Asia. AMOVA analysis for variation was significant among populations ( $P < 0.05$ ) and was in agreement with morphological differences. Pairwise differences were significant between Java and Brunei/Borneo, Brunei/Borneo and west Malaysia, and Java and west Malaysia samples ( $P < 0.05$ ). Closest relationships were found between samples from Brunei/Borneo and Java, and between west Malaysia and Laos-Sumatra. Nine haplotypes were unique to geographical regions. The Java species had high haplotype ( $1.000 \pm 0.126$ ) but low nucleotide ( $0.017$ ) diversities, suggesting a population bottleneck followed by expansion. However, *SSD* and *Hri* ( $P=0.5$ ) did not support demographic expansion. Instead, purifying selection where mutations occur and accumulate at silent sites is a more acceptable explanation.

**Keywords:** Borneo, Java, Clariidae, cytochrome *b*, phylogenetics

### Introduction

In Southeast Asia, 19 species of the genus *Clarias* are currently recognized (Ng *et al.*, 2011) including 13 species in Borneo (Kottelat, 2013). In spite of the relatively recent colonisation of Southeast Asia from central Asia about 15 MYA (Agnese & Teugels, 2005), *Clarias* shows considerable diversity in morphology and habitat.

*Clarias batrachus* is a common species found in South and Southeast Asia and is widely utilised in aquaculture and the aquarium fish trade, and has been the subject of numerous studies (see Begum, 2004; Hora, 1936; Kottelat, 2001; Ng & Kottelat, 2008; Khedkar *et al.* 2010). The species has been introduced to many parts of the world and has the potential to threaten non-native environments (Lever, 1996).

The identity of *C. batrachus* has recently been studied by Ng & Kottelat (2008). They redescribed *C. batrachus* and designated a neotype from Java. Ng & Kottelat (2008) designated the non-Javanese *C. batrachus* as: *Clarias* aff. *batrachus* ‘Indochina’ from the Mekong River drainage, and *Clarias* aff. *batrachus* ‘Sundaland’ from west Malaysia and Sarawak. They noted morphological differences in these forms in the shape of the supraoccipital process and width of the frontal fontanelles. The morphological difference between *C. aff. batrachus* ‘Sundaland’ and *C. aff. Indochina* was in the shape of the supraoccipital process. The latter was more triangular. The shape of supraoccipital process also is a useful diagnostic character in Asian *Clarias* (Teugels *et al.* 1999). The frontal fontanelle of *C. batrachus* from the Malay Peninsula and Sarawak was wider and referred to as ‘sole shaped’ by Teugels (1986) than that on specimens from Java and mainland Southeast Asia, referred to as ‘knife-shaped’ by Teugels (1986). The morphological differences seemed to correlate with geographical areas of Southeast Asia.

Several molecular genetics studies had been conducted on Asian *Clarias* (Agnese & Teugels, 2005; Pouyaud *et al.* 2009), Asian *C. batrachus* (Islam *et al.* 2007; Khedkar *et al.* 2010) and Southeast Asian *C. batrachus* (Mohindra *et al.* 2007). However, no molecular study has investigated variation in sequence data from the cytochrome *b* gene in *C. batrachus* from Southeast Asia. The objective of the present study is to determine whether genetic data from the cytochrome *b* gene is in agreement with morphological variation in *C. batrachus* from Southeast Asia.

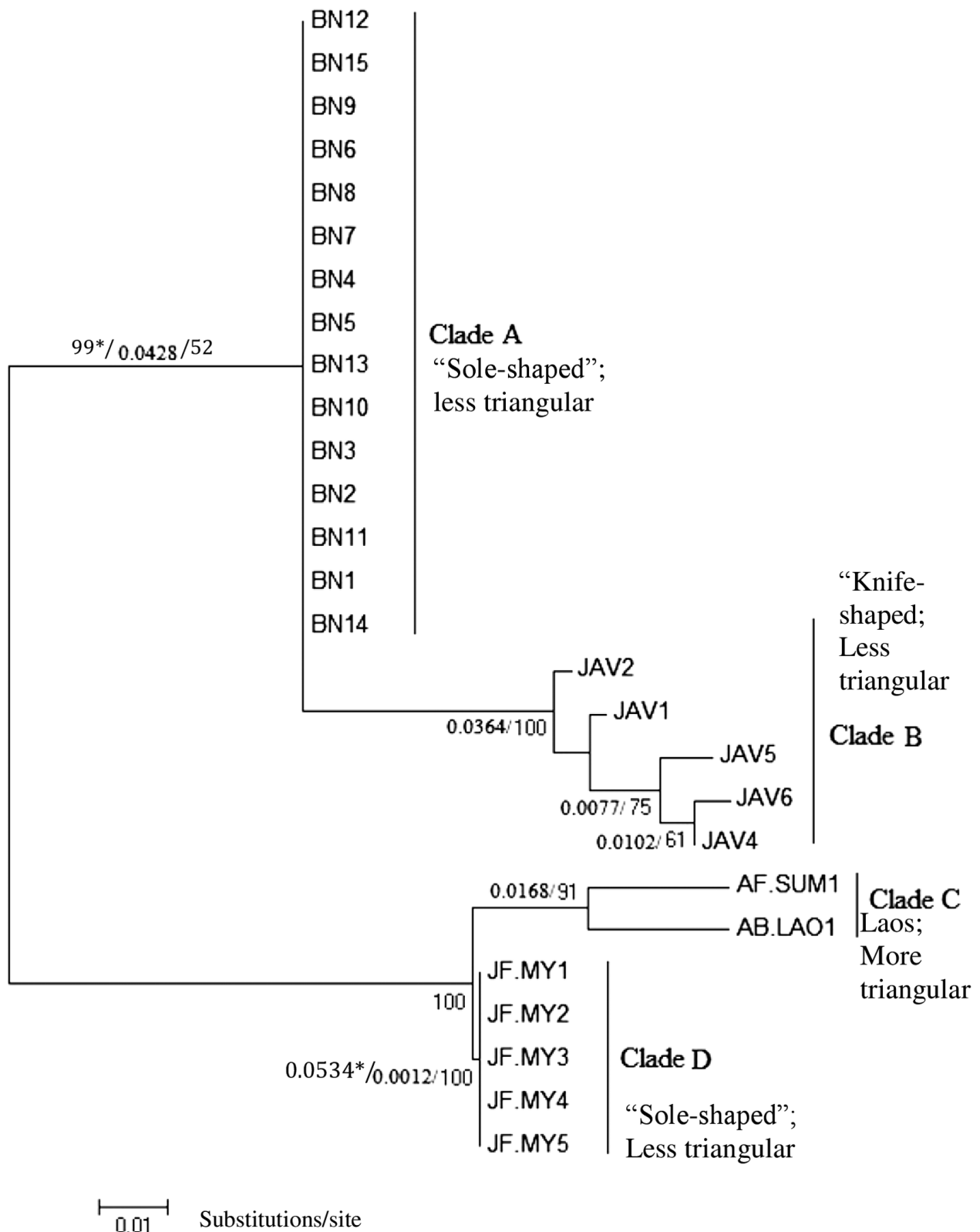
A



B



**FIGURE 1.** Dorsal views of heads of: A, *Clarias batrachus* from Brunei/Borneo, UBD 0412, 202.6 mm SL; B, *C. batrachus* from Java, UBD 0814, 221.8 mm SL. Frontal fontanelles ‘sole-shaped’ in A and ‘knife-shaped’ in B; less triangular shape of the supraoccipital process in both A and B.



**FIGURE 2.** Maximum likelihood (GTR) tree of *Clarias batrachus* cytochrome *b* gene. Numbers on the nodes correspond to branch length/ML bootstrap probability except \* represents NJ bootstrap value/ branch length. Only values >50% are shown. Brunei/Borneo (BN1-15), Java (JAV1, 2, 4, 5, 6), west Malaysia (JF.MY1-5), Laos (AB.LAO1), and Sumatra (AF.SUM1). Morphological characters are labeled on each Clade.

## Materials and Methods

### Fish samples and morphology

Specimens of *C. batrachus* (Fig.1) were collected from the Tutong District in Brunei, Borneo (n=15) and east Java, Indonesia (n=5). The identity of *C. batrachus* from Java was confirmed by the recent designation of the type locality by Ng & Kottelat (2008). Fish sampling in Brunei was done using gill nets and fishing rods. Samples from Indonesia were obtained from a local fish market at Bekasi, Java. The fishes were stored at -20°C until subjected to DNA extraction. Material examined in this study is deposited at the Universiti Brunei Darussalam, Brunei (UBD).

### DNA extraction and sequencing

Total genomic DNA was extracted from 30–100 mg tissue using DNeasy™ tissue kits (Qiagen GmbH, Germany). Amplifications were carried out in 100 µl containing 1x reaction Buffer, 3.5mM MgCl<sub>2</sub>, 0.2mM dNTP, 1 µM of forward and reverse primers, 2.5 units of *Taq* DNA polymerase (Invitrogen, San Diego, CA) and about 50–100 ng of genomic DNA. The 435 bp partial sequences of mitochondrial cytochrome *b* gene were amplified with primers NEW-FOR 5' AGC CTA CGA AAA ACC CAC CC 3' (Chang *et al.* 1994) and H15149 5' AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A 3' (Kocher *et al.* 1989).

Thermal cyclings were performed using GeneAmp PCR system 9700 (Applied Biosystem) with an initial denaturation of 94°C for 5 minutes, followed by 30 cycles of 94°C at 30 seconds, annealing temperature of 55°C for 30 seconds, 72°C for 60 seconds and the final extension of 5 minutes at 72°C. PCR products were purified using a Qiagen purification kit according to manufacturer's instructions and subsequently sequenced at First Base ([www.base-asia.com](http://www.base-asia.com)).

### Data analysis

All 27 sequences were edited using Clustal X version 2.0 (Larkin *et al.* 2007). Missing nucleotide data were excluded on a pair-wise basis. Haplotypes (*H*), haplotype diversity (*Hd*) (Nei, 1987) and nucleotide diversity (*p*) (Nei, 1987) were calculated for each geographic population using DnaSP 5 (Librado & Rozas, 2009). Nucleotide composition, parsimony informative and variable sites were estimated using MEGA 6.06 (Tamura *et al.* 2013).

A maximum-likelihood (ML) tree based on General Time Reversible (GTR) model (Nei & Kumar, 2000) and a neighbour-joining (NJ) tree based the Tamura 3-parameter (Tamura, 1992) were generated using MEGA 6.06 (Tamura *et al.* 2013). Trees were unrooted to specify only relationships among *C. batrachus*. The robustness of statistical support for branches on the ML and NJ trees was determined by 1000 bootstrap replicates (Felsenstein, 1985). The branch length measured the number of substitutions per site. The evolutionary relationship among cytochrome *b* haplotypes was resolved for *C. batrachus* with unrooted networks constructed with the program Network 4.6 (<http://www.fluxus-engineering.com>) using a median-joining algorithm and default settings (Bandelt *et al.* 1999). The partial cytochrome *b* gene sequences from west Malaysia (GenBank Accession Nos. JF280847, JF280849, JF280854, JF280855 and JF280859), Laos (GenBank Accession No. AB8222528), and Sumatra (GenBank Accession No. AF 235932) were included in the trees and the MJ network.

An analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was conducted in Arlequin 3.0 (Excoffier *et al.*, 2005) to test the spatial genetic variation of *C. batrachus* species within the five areas. Additionally, the fixation index  $F_{ST}$  value was calculated from haplotype frequencies, to determine the level of genetic differentiation (Weir & Cockerham, 1984). Tajima's *D* (Tajima, 1989a; 1989b) and Fu's *F* (Fu, 1997) statistics were also conducted through Arlequin 3.0 (Excoffier *et al.*, 2005) as well to understand the degree of population expansion and to test the deviation from equilibrium expectations. The goodness of fit test between the observed and expected distributions was estimated based on the Harpending's raggedness index (*Hri*) and the sum of squared deviations (*SSD*) for the expected stepwise expansion model (Harpending, 1994). The sudden expansion model  $\theta$  and  $\tau$  statistics parameters were used to measure changes in population size (whereas  $\theta$  was being expressed in a mutational and general scale) and the age of populations in mutational time was calculated to test whether the observed mismatch distributions in each population better fit the model of constant population size or the one model with population size changes (sudden expansion model) (Rogers, 1995).

## Results

### Molecular characteristics

Alignment of 27 sequences of the cytochrome *b* gene resulted in 395 sites, of which 74 were variable (18.7%), and 61 were parsimony informative (15.4%). Nine haplotypes were identified. The base composition was 32.4% (A), 29% (G), 21.8% (C) and 16.8% (T). The Java specimens were the most diverse (Haplotype diversity, *Hd* was 1.000 ± 0.126), and nucleotide diversity ( $\pi$ ) was 0.01722 (Table 1). The Brunei/Borneo and west Malaysia populations had 1 haplotype each and 0.00 for haplotype and nucleotide diversities. Tajima's *D* and Fu's *F*s for *C. batrachus* from Java were -0.525 and -0.145, respectively, and not statistically significant.

**TABLE 1.** Sampling regions, number of samples (*N*), number of haplotypes (*H*), haplotype diversity (*Hd*), nucleotide diversity (*p*), Tajima's *D* and Fu's *F*s for cytochrome *b* gene sequences for each population of *Clarias batrachus*.

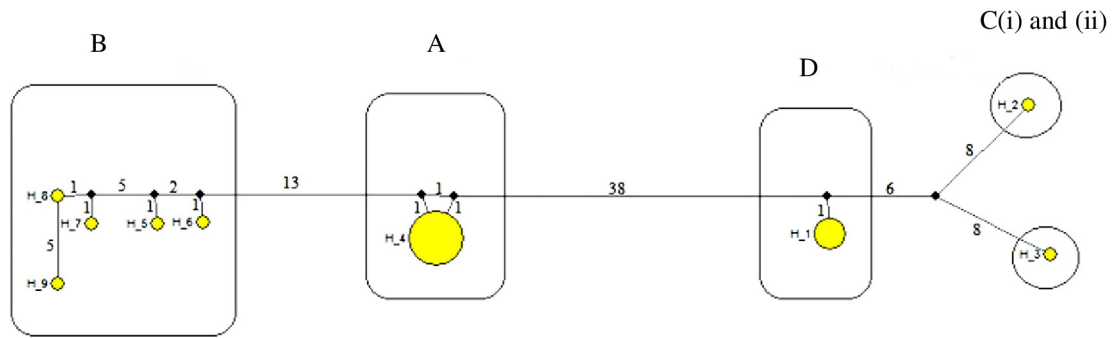
Sampling region	<i>N</i>	<i>H</i>	<i>Hd</i>	<i>p</i>	Tajima's <i>D</i>	Fu's <i>F</i> s
East Java/Bekasi	5	5	1.00±0.126	0.01722	0.08677	-0.875
Malaysia	5	1	0.000	0.000	0.000	0.000
Brunei	15	1	0.000	0.000	0.000	0.000
Laos PDR	1	1	-	-	-	-
Sumatra	1	1	-	-	-	-
Total	27	9				

### Patterns of population structure

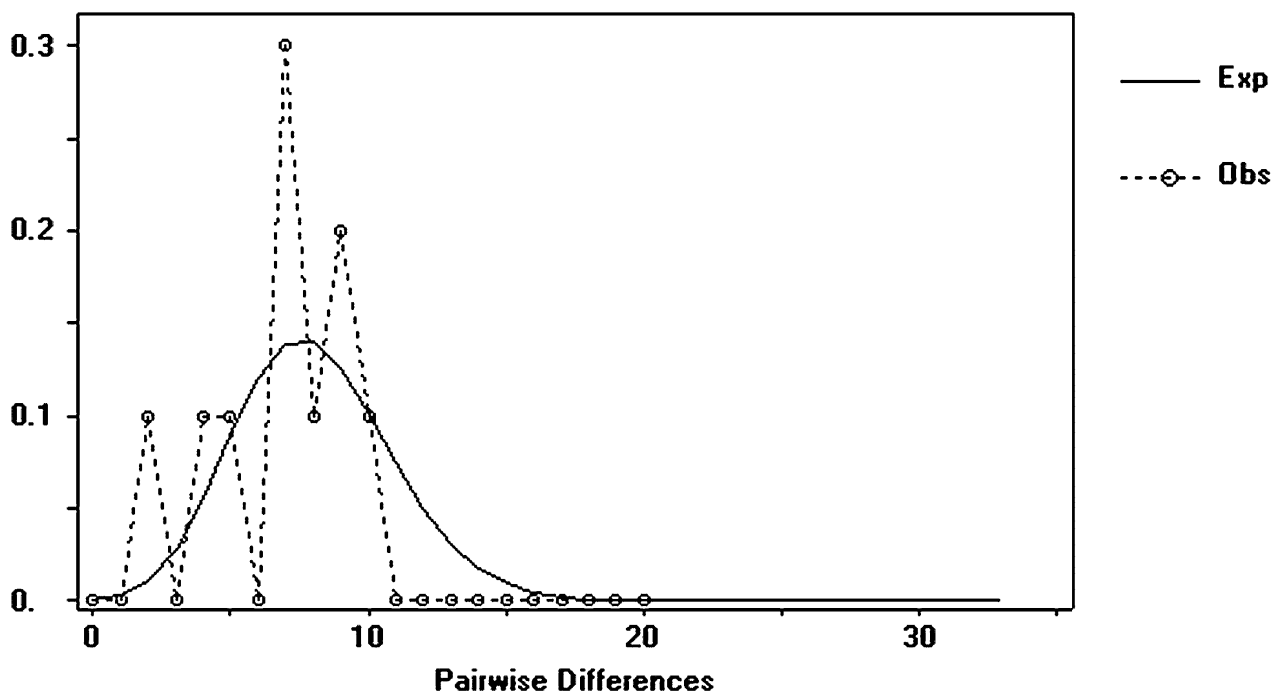
The phylogenetic analyses revealed four geographically exclusive clades (Fig. 2). The topology of ML and NJ were similar. The bootstrap probabilities and branch lengths from ML and NJ were in agreement except where it was marked as \* on the ML tree. Clade A contained 100% of *C. batrachus* from Brunei/Borneo and was supported by 99% (NJ)/ 52% (ML). Clade B contained 100% of *C. batrachus* from Java and supported by 100% bootstrap. Clade C contained specimens from Laos and Sumatra, and was supported by 100% bootstrap. Clade D contained 100% of the *C. batrachus* from west Malaysia and was supported by 100% bootstrap. Clades A–B and C–D were resolved as sister taxa with 100% bootstrap. Evolutionary relationships using a median-joining network were further analysed between haplotypes of *C. batrachus*. The haplotype networks (Fig. 3) supported the topology of the phylogenetic tree in Figure 2. It formed four haplotype groups (A, B, C and D). Haplotype groups A (Brunei/Borneo) and B (Java) were separated by 13 mutational steps. Specimens from Java showed five haplotypes and could represent various populations. A and D (west Malaysia) haplotype groups were separated by 38 mutational steps. Haplotype groups C split into (i) Sumatra and (ii) Laos, and each was separated from west Malaysia by 6 mutational steps.

The pairwise  $F_{ST}$  values ranged from 0–1.0 and agreed with the phylogenetic tree. It was significant only between samples from Java and Brunei/Borneo, *C. batrachus* from Java and west Malaysia, and *C. batrachus* from Brunei/Borneo and west Malaysia (Table 2). Genetic differentiation was high, indicating low gene flow and connectivity among populations, except pairwise differences between Java–Sumatra and Java–Laos were 0.0.

AMOVA analysis showed that genetic variation observed in *C. batrachus* was significant ( $P < 0.05$ ) among populations (Table 3). The tests for *SSD* and *Hri* were performed on *C. batrachus* from Java only as mismatch variances for other samples were too small. It had a multimodal and rough mismatch distribution (Fig. 4), consistent with old stationary populations. *Hri* was higher (0.2) and *SSD* was lower (0.06), but both tests were insignificant ( $P = 0.5$ ), suggesting a poor fit to demographic expansion.



**FIGURE 3.** Median-joining network derived from *Clarias batrachus* cytochrome *b* sequences. Each circle represents a haplotype and is scaled to the number of individuals with that haplotype. Small dark spots represent mutational steps between haplotypes.



**FIGURE 4.** Mismatch distribution based on cytochrome *b* gene sequences of *C. batrachus* from Java. Expected distribution (Exp) and Observed distribution (Obs) under the model of population expansion.

**TABLE 2.** Population pairwise  $F_{ST}$  values for cytochrome *b* data of *C. batrachus* are given below, and  $F_{ST}$  *p* values are given above the diagonal. Java (JAV), Brunei (BN), west Malaysia (MY), Sumatra (SUM), and Laos (LAO). Significant *p* values are indicated by an asterisk.

	JAV	BN	MY	SUM	LAO
JAV	0.0000	<0.05*	<0.05*	0.999	0.999
BN	0.71963	0.0000	<0.05*	0.999	0.999
MY	0.50000	1.0000	0.0000	0.999	0.999
SUM	0.00000	1.0000	1.0000	0.0000	0.999
LAO	0.00000	1.0000	1.0000	1.00000	0.0000

**TABLE 3.** AMOVA analysis for *C. batrachus* in Southeast Asia.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	4	6.741	0.381Va (p<0.05)	80.73
Within populations	22	2.000	0.091Vb	19.27
Total	26	8.741	0.472	

Fixation Index  $F_{ST}$ : 0807

## Discussion

In this study, base compositions were similar to those found in Asian *Clarias* (Pouyaud *et al.* 2009) and in agreement with the pattern described for numerous fishes (Cantatore *et al.* 1994).

The Brunei/Borneo-west Malaysia pair had the highest number of distinct mutations, followed by the Brunei/Borneo-Java pair. This was supported by significant pairwise  $F_{ST}$  values between these populations ( $P < 0.05$ ). The high genetic differentiation was expected as freshwater fishes are confined to specific basins (Ward *et al.* 1994).

The genetic variation in this study was consistent with morphological variation in the supraoccipital process (less triangular in Java), frontal fontanelle ('knife-shaped' in Java and 'sole shaped' in Brunei/Borneo).

The haplotypes from the cytochrome *b* gene data identified distinct groups of *C. batrachus* from Java, Brunei/Borneo, west Malaysia and Lao-Sumatra. The single haplotype for Brunei/Borneo was the result of two mutations (Fig. 3). The Laos and Sumatra specimens split from the Malaysian population to form sister branches. However, *C. batrachus* was represented from Laos and Sumatra by one specimen each, and results obtained in this study might not be representative of the populations. More samples will be required to confirm this relationship.

The current study showed a lack of genetic differentiation between the Java-Laos pair and the Java-Sumatra pair. The latter may be caused by the close proximity of the North and East Sunda paleo-drainages (Rainboth, 1991). The results for lack of genetic differentiation between Java and Laos pair was consistent with a "radiation by paleo-drainage" model of diversification observed for halfbeaks across Southeast Asia (De Bruyn *et al.* 2013).

High haplotype and low nucleotide diversities of *C. batrachus* from Java suggested a rapid population growth from an ancestral population with small  $N_e$  after a bottleneck event (Avice, 2000). The results supported the hypothesis of a pre-Pleistocene origin in Asian *Clarias* diversity, with colonization of Southeast Asia from Central Asia about 15 MYA (Agnese & Teugel, 2005) and appeared on the Sunda Shelf (Sumatra, Borneo and Java) about 20 MYA (Pouyaud *et al.* 2009). The negative  $D < 0.1$  may be interpreted as due to increasing population size or as purifying selection at a locus (Tajima 1989a). Negative  $F$  means a recent population expansion or purifying selection. However, population expansion of the Java population was not supported by a mismatch distribution, *Hri* and *SSD* ( $P > 0.1$ ). Purifying selection represents the likely alternative, as shown by the negative  $D$  and  $F$  values. Purifying selection occurs when mutations accumulate at silent sites but with little heterozygosity (Tajima, 1989a).

Our study revealed significant molecular diversification within *C. batrachus*. These results are consistent with morphological disparity and reproductive isolation of the Southeast Asian *C. batrachus* populations. Greater taxon and character sampling throughout the range (especially Indo-China and Sumatra) of *C. batrachus* is necessary to better determine the variation and species boundaries of *C. batrachus*.

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