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Genetic structure of the Asian Grass Frog, *Fejervarya limnocharis* (Amphibia: Anura: Dicroglossidae) of Peninsular Malaysia: a preliminary report

Amirah Hurzaid^{1*}, Ibrahim Jaafar¹, Zalina Awang¹ and Siti Azizah Mohd Nor^{2,3}

Abstract

Background: *Fejervarya limnocharis* (Dicroglossidae) is found widespread in Peninsular Malaysia. We analyzed the mitochondrial DNA control region sequence data to evaluate the genetic variability and population structure of this species, based on 106 individuals from 14 populations in the west coast (northwest and central west) and two populations from the east coast of Peninsular Malaysia.

Results: Of 519, 13 variable sites (six parsimony informative sites) were observed, defining 14 unique haplotypes. We found very low levels of genetic variability, with three of the 16 populations exhibiting total absence of genetic variation. Both phylogenetic analyses based on NJ and MP methods failed to detect any geographic structuring between the east and west coast (northwest and central west combined) populations.

Conclusions: Consequently, it can be inferred that genetic structuring *F. limnocharis* populations in the Peninsular Malaysia was largely shaped by natural barriers. However, when this breaks down as between the central west and east peninsular regions, genetic differentiation is reduced. Further studies, with more efficient markers and larger populations, especially from the southern regions, are required to corroborate the findings.

Keywords: *Fejervarya limnocharis*; Peninsular Malaysia; mtDNA; D-loop

Background

The Asian Grass Frog, *Fejervarya limnocharis*, is a common and widespread amphibian in South Asia and Southeast Asia. Its distribution covers a number of islands in Indonesia, the Philippines, Phuket, and Singapore and is also found as far as the northern, central, southern, and southwestern China (including Taiwan, Hong Kong, and Macau), western Japan, western half of Honshu, Shikoku and Kyushu Districts, and Nansei Islands. Recently, this species was introduced into Tsushima and Iki (van Dijk et al. 2009). It has also been observed throughout a wide range of altitudes (2 to 2,000 m) in China (Fei and Ye, 2001). In Malaysia, the species is ubiquitous in disturbed habitats associated with human activities including paddy

fields, roadsides, lawns, agricultural fields, football fields, and lowland forests (Inger and Stuebing, 1997; Ibrahim, 2004; Norhayati et al. 2009). Being widely distributed, *F. limnocharis* is an ideal subject for population genetics and phylogeographic investigations and the mechanisms or forces most likely to have been involved in shaping their population pattern.

Maternally inherited mitochondrial DNA is an efficient genetic marker in genetic-differentiation studies, due to its higher mutation rate of base substitution compared to nuclear DNA (Qiongying et al. 2006). The control region or displacement loop (D-loop) gene is the only noncoding segment in vertebrate mtDNA (Faber and Stepien, 1998) and encompasses the sites of initiation for H-strand replication and both H- and L-strand transcription (Sumida et al. 2000). The D-loop segment evolves much faster than the average mitochondrial gene (Brown, 1985), although it also has short sequence elements conserved among most

* Correspondence: amirahhurzaid@gmail.com

¹Biological Sciences Program, School of Distance Education, Universiti Sains Malaysia, Minden, Penang 11800, Malaysia

Full list of author information is available at the end of the article

vertebrates studied (Wolstenholme, 1992). Because of its ability for rapid change, the D-loop region is an ideal choice for addressing population-level genetic questions (Hoelzel et al. 1991).

The genetic variation of amphibians in Malaysia using mtDNA sequencing methods have been reported by Ramlah (2009) and Ramlah et al. (2010). Their studies mainly involved amphibian populations from Malaysian Borneo (Sabah and Sarawak) with limited samples from Peninsular Malaysia. However, they have provided a foundation for further studies in addressing population subdivision of the Malaysian amphibians. The objective herein was to determine the genetic variation in *F. limnocharis* populations from 16 different populations throughout Peninsular Malaysia, through DNA sequencing of D-loop of mtDNA.

Methods

Sampling location and collection

A total of 106 individuals of *F. limnocharis* representing 16 populations throughout Peninsular Malaysia were sampled during the period of 2010 to 2011. These populations were provisionally divided into three regions; northwest, central west, and east (Table 1). The northwest was defined as the region in west Peninsular Malaysia restricted by the Titiwangsa mountain range in the east and Bintang mountain range in the south. Populations to the south of the Bintang mountain range and west of the southernmost tip of the Titiwangsa mountain range were categorized as central west Peninsular, whereas those east of the Titiwangsa mountain range were classified as east Peninsular (Figure 1)

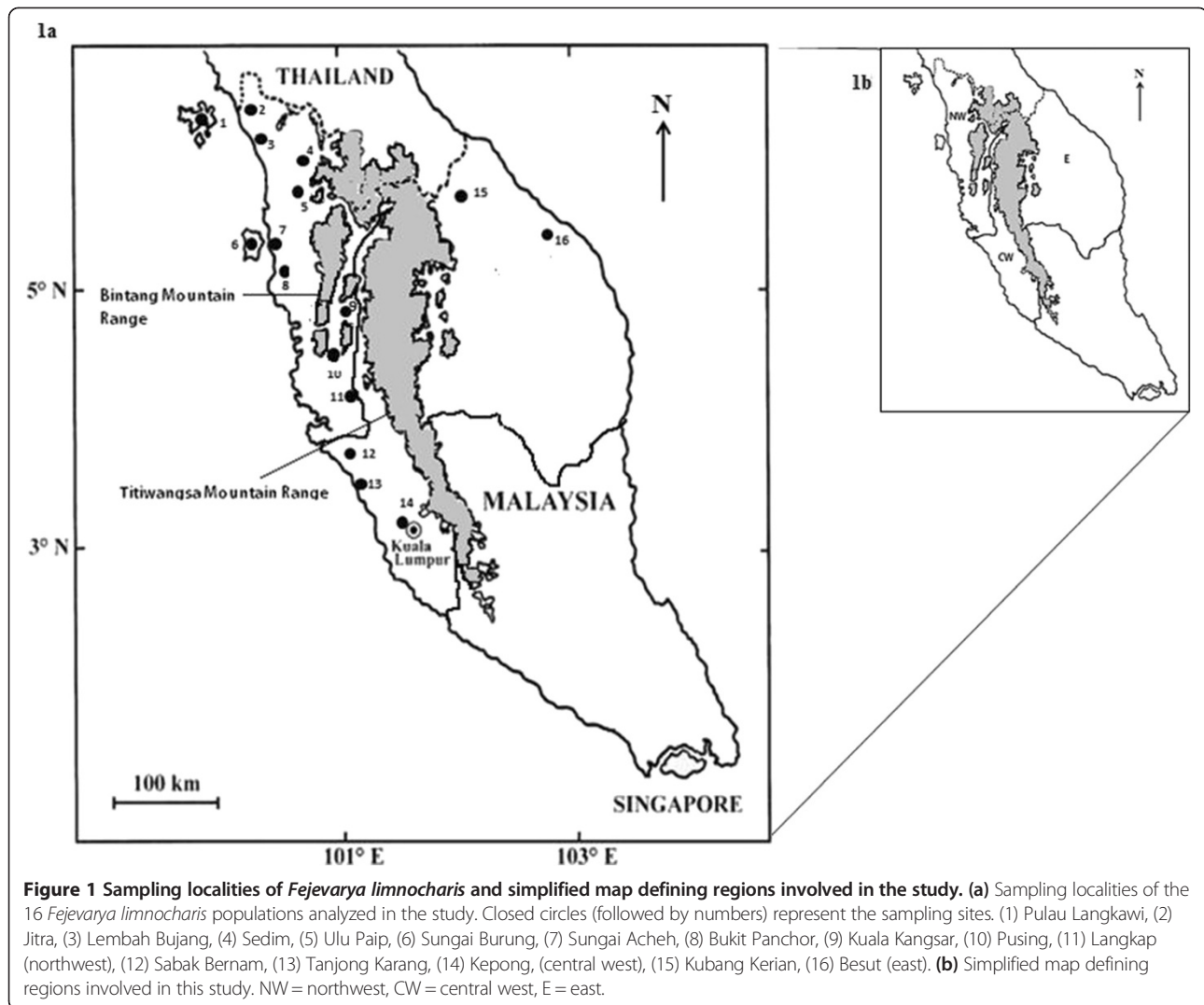
(Tan et al. 2012). Freshly captured specimens were immediately identified by referring to Berry (1975) and Norhayati et al. (2009). All specimens were injected with benzocaine solution into its dorsal lymph sacs to humanely kill them before dissection. Each liver tissue (approximately 10 mg) was preserved in 95% ethanol and stored at room temperature (approximately 25°C) until use. This study has been approved by the USM Ethics Committee. All practical steps to ameliorate suffering by specimens were taken throughout this study. Voucher specimens were then deposited at the Herpetological Collections, Universiti Sains Malaysia for future reference.

Molecular analysis

DNA extraction was with a DNeasy Tissue Kit (QIAGEN, Venlo, Limburg, Netherlands) according to manufacturer's instructions. A segment of the D-loop mtDNA gene was amplified using a pair of primers - forward primer 327-L (5'-CTG TCC ATA TCA TGA CTA CTT G-3') and reverse primer 885-H (5'-GGT CTT AGC TTG TAG AGA GGT C-3') (Zhong et al. 2008). PCR was done in a Peltier thermal cycler (MJ Research Waltham, MA, USA), with the following profile: pre-denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C, annealing at 50°C, and extension at 70°C for 1 min each, followed by final extension at 72°C for 5 min. The PCR products were then purified using Wizard® SV Gel and a PCR Clean-Up System by Promega (Promega Madison, WI, USA) and sequenced on an ABI3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Table 1 Region, sampling localities, locality numbers, site abbreviations (abbrev.), and coordinates (latitude and longitude)

Region	Population	Locality number	Abbrev.	Latitude (N)	Longitude (E)
Northwest Peninsular	Pulau Langkawi, Kedah	1	LKW	6.31346°	99.77410°
	Jitra, Kedah	2	JIT	6.26008°	100.41717°
	Lembah Bujang, Kedah	3	LBG	5.73709°	100.41437°
	Sedim, Kedah	4	SED	5.49962°	100.62954°
	Ulu Paip, Kedah	5	ULP	5.39229°	100.66593°
	Sungai Burung, Penang	6	SBG	5.33592°	100.22379°
	Sungai Acheh, Penang	7	SGA	5.12341°	100.42055°
	Bukit Panchor, Penang	8	BPR	5.15224°	100.53479°
	Kuala Kangsar, Perak	9	KK	4.76851°	100.93929°
	Pusing, Perak	10	PUS	4.49405°	101.01008°
	Langkap, Perak	11	LKP	4.06534°	101.14691°
Central west Peninsular	Sabak Bernam, Selangor	12	SBK	3.67576°	100.98998°
	Tanjong Karang, Selangor	13	TJG	3.42258°	101.17671°
	Kepong, Kuala Lumpur	14	KEP	3.23599°	101.63449°
East Peninsular	Kubang Kerian, Kelantan	15	KBG	6.10020°	102.28502°
	Besut, Terengganu	16	BST	5.61088°	102.51926°



Data analysis

Sequences were edited using MEGA 6.0 (Tamura et al., 2013) and aligned with Clustal W 1.6, implemented in the same software. The aligned sequences were then referenced to the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/blast>) to confirm

the identity of the samples, where possible. The aligned sequences were then exported to Collapse version 1.2 (Vigo, Spain) (Posada 2004) to compute the haplotype datasheet. Haplotype sequences were then generated using DNA Sequences Polymorphism (DnaSP) program version 5.10.01 (Librado and Rozas, 2009). Arlequin

Table 2 Number of samples (N), nucleotide diversity, number of haplotypes, haplotype diversity, and number of polymorphic sites, data generated by ARLEQUIN version 3.0 software

	Northwest								Central west				East			
	LKW	JIT	LBG	SED	ULP	SBG	BPR	SGA	PUS	LKP	KK	SBK	TJG	KEP	KBG	BST
	N = 4	N = 4	N = 3	N = 7	N = 3	N = 4	N = 8	N = 6	N = 7	N = 8	N = 5	N = 7	N = 16	N = 5	N = 6	N = 13
Nucleotide diversity (π)	0.001	0.002	0.000	0.003	0.001	0.000	0.001	0.001	0.000	0.003	0.001	0.004	0.004	0.004	0.001	0.006
Number of haplotypes	2	3	1	3	2	1	2	2	1	3	2	4	3	2	2	5
Haplotype diversity (h)	0.500	0.833	0.000	0.667	0.667	0.000	0.536	0.600	0.000	0.464	0.400	0.714	0.675	0.400	0.333	0.744
Number of polymorphic sites	1	2	0	5	1	0	1	1	0	6	1	6	5	4	1	8

LKW Langkawi, JIT Jitra, LBG Lembah Bujang, SED Sedim, ULP Ulu Paip, SBG Sungai Burung, BPR Bukit Panchor, SGA Sungai Acheh, PUS Pusing, LKP Langkap, KK Kuala Kangsar, SBK Sabak Bernam, TJG Tanjong Karang, KEP Kepong, KBG Kubang Kerian, BST Besut.

version 3.0 (Excoffier et al. 2005) was used for calculating nucleotide and haplotype diversities and F_{ST} values. Bonferroni correction was applied, with global significance level at 0.05 to correct for multiple comparisons. A bootstrap (10,000 replicates) neighbor-joining (NJ) and maximum parsimony (MP) tree was constructed based on the Kimura-2-parameter model (Kimura 1980) to depict haplotype relationships using the MEGA 6.0 software. A minimum-spanning network (MSN) connecting all haplotypes was generated using Network (Bandelt et al. 1999) program to illustrate this relationship.

Results

Final aligned sequences of 519 bp in the mtDNA D-loop gene were obtained. Fourteen unique haplotypes were identified from the 16 populations of 106 individuals. The sequences have been submitted to the GenBank (accession numbers KF051071 to KF051084). Low within population nucleotide diversity, π (0.000 to 0.006) was observed in all populations while haplotype diversity, h , was in the range of very low to moderate in each locality ($h = 0.000$ to 0.833). Number of haplotypes and polymorphic sites per population ranged from 1 to 8 (Table 2). Trend of low intrapopulation variability was also supported by the genetic distances observed based on Kimura-2 parameter (Table 3).

The minimum spanning network relationships among all the haplotypes are presented in Figure 2. The two common haplotypes, namely Haplotype 1 and Haplotype 5, were observed in almost equal frequency (35% and 38%, respectively). Haplotype 1 was common to all three regions although was missing in one population each in central west and east (Kepong and Kubang Kerian) while Haplotype 5 was observed in all central west and the eastern regions except a few populations namely from Sedim, Pusing, Langkap, and Kuala Kangsar (northwest). Eleven of the 14 haplotypes (except 1, 3, and 5) were unique to either the western or eastern regions.

Low genetic differentiation among populations in the northwest region was indicated by the low pairwise F_{ST} (Table 3) values. Populations from central west and eastern regions were highly differentiated from the northwest populations with the exception of Sabak Bernam (central west), which showed low genetic differentiation with the northwest population. This was strongly supported by the NJ (data not shown) and MP phylogenetic trees, which divided the haplotypes into two major groups with high bootstrap values (100%) (Figure 3).

Discussion

The present study has provided initial insights into the genetic distribution of *F. limnocharis* in Peninsular Malaysia. In general, mean nucleotide and haplotype diversities were low over all samples ($\pi = 0.004 \pm 0.00005$; $h = 0.471 \pm 0.27$).

Grant and Bowen (1998) hypothesized that populations with low values of both parameters ($\pi \leq 0.5\%$, $\hat{h} < 0.5$) is a feature of recent population bottleneck or founder event by a single or a few mtDNA lineages. Farjallah et al. (2012), through Cytb gene analysis, reported that low levels of both nucleotide and haplotype diversities ($\pi = 0.002$; $\hat{h} = 0.7$) in the North African Green Frog *Pelophylax saharicus* in Tunisia may be due to a recent population expansion from a small founder population. Nevertheless, as a consequence of marker characteristics, an underestimation of the true levels of genetic variability is possible. Hence, other markers for population studies, such as microsatellites, should also be investigated.

Regional genetic differentiation among *F. limnocharis* populations were relatively high between the northwest with central west (excluding Sabak Bernam population) populations and each pairwise population comparison between the two regions showed significant genetic differentiation, the Bintang range being an effective divider between the two regions. However, remarkably high intra-regional gene flow was observed within the northwest region, perhaps a result of connectivity between populations of this region.

In contrast, the central west and east coast region showed unexpected genetic homogeneity. Some frog species use roads as activity corridors, including the Cane Toad, *Bufo marinus* (Seabrook and Dettmann, 1996), and Natterjack Toad, *Bufo calamita* (Stevens et al. 2006). *Fejervarya limnocharis* is generally abundant in human habitation and this may increase the opportunity for the frog to be transported accidentally or deliberately into new areas (Toda et al. 1997). Thus, it seems plausible that migration had occurred between the central west and east coast of Peninsular Malaysia via the East-West Highway and colonized new populations, thus reducing their genetic differentiation. This is in tandem with the high capability of dispersal possessed by this species.

It is not possible to compare the pattern of population structuring with other Malaysian amphibians as to date only limited molecular data (Ramlah et al. 2010) is available on the amphibians in Peninsular Malaysia. More intensive sampling on the investigated sites or sampling of increased number of sites particularly in the east coast and southern regions could confirm this.

Conclusions

Based on this preliminary analysis, it can be inferred that the genetic structuring of the Peninsular Malaysia *F. limnocharis* population was largely shaped by natural barriers. However, when this breaks down as between the central west and east Peninsular regions, genetic differentiation is reduced. Further studies, with more efficient markers and larger populations, especially from the southern regions, are required to corroborate the findings.

Table 3 Pairwise F_{ST} values (below diagonal) and mean pairwise genetic distance within (on diagonal) and between (above diagonal) the 16 populations of *Fejervarya limnocharis*

Region	Northwest								Centralwest				East			
Population	LKW	JIT	LBG	SED	ULP	SBG	BPR	SGA	PUS	LKP	KK	SBK	TJG	KEP	KBG	BST
LKW	0.001	0.001	0.000	0.002	0.001	0.000	0.002	0.001	0.010	0.009	0.011	0.002	0.008	0.009	0.011	0.006
JIT	0.000	0.002	0.001	0.002	0.001	0.001	0.002	0.001	0.009	0.008	0.009	0.003	0.007	0.007	0.009	0.006
LBG	-0.091	-0.091	0.000	(0.002)	0.001	0.000	0.001	0.001	0.010	0.009	0.010	0.002	0.007	0.008	0.010	0.006
SED	0.051	-0.144	-0.033	0.003	0.002	0.002	0.002	0.002	0.008	0.007	0.008	0.003	0.007	0.007	0.008	0.006
ULP	(0.403)	0.102	0.415	-0.023	0.001	0.001	0.001	0.001	0.009	0.008	0.009	0.002	0.007	0.008	0.009	0.006
SBG	0.258	-0.043	0.250	-0.090	-0.134	0.000	0.001	0.001	0.010	0.009	0.010	0.002	0.007	0.008	0.010	0.006
BPR	0.000	0.000	0.000	0.034	0.461	0.314	0.001	0.001	0.009	0.008	0.009	0.003	0.007	0.007	0.009	0.006
SGA	0.014	-0.268	0.000	-0.213	-0.085	-0.258	0.111	0.001	0.009	0.008	0.009	0.003	0.007	0.007	0.009	0.006
PUS	(0.968)*	(0.924)*	(1.000)*	(0.786)	(0.934)*	(0.939)*	(1.000)*	(0.964)	0.000	0.001	0.000	0.008	0.003	0.002	0.000	0.005
LKP	(0.754)	(0.678)*	(0.754)	(0.573)	(0.748)*	(0.731)	(0.776)	(0.700)	-0.018	0.003	0.002	0.008	0.003	0.003	0.002	0.005
KK	(0.919)	(0.859)*	(0.949)	(0.723)	(0.894)*	(0.891)*	(0.956)	(0.899)	0.073	-0.004	0.001	0.009	0.003	0.002	0.001	0.005
SBK	-0.026	-0.103	-0.123	-0.081	(0.170)	0.063	-0.055	-0.127	(0.778)*	(0.577)	(0.716)*	0.004	0.007	0.007	0.009	0.006
TJG	(0.622)*	(0.551)*	0.610	(0.473)	(0.643)*	(0.619)*	(0.630)	(0.578)	(0.199)	0.034	0.163	(0.455)*	0.004	0.004	0.003	0.005
KEP	(0.749)	0.649	(0.754)*	(0.514)	(0.734)	(0.715)	(0.786)	(0.674)	0.073	-0.146	0.000	(0.530)	0.045	0.003	0.002	0.005
KBG	(0.927)	(0.874)*	(0.954)	(0.742)*	(0.901)*	(0.900)*	(0.960)*	(0.911)	0.028	-0.027	0.003	(0.736)*	(0.180)	0.026	0.001	0.005
BST	(0.288)	0.178	0.244	0.118	(0.300)	(0.264)	0.287	0.181	(0.282)	0.118	0.235	0.134	0.066	0.042	0.257	0.006

Significant probabilities ($P < 0.05$) based on 1,000 permutations of haplotype frequencies among samples are indicated in parentheses.

*Significant population differentiation via exact test (after Bonferroni corrections), $P < 0.05$.

LKW Langkawi, JIT Jitra, LBG Lembah Bujang, SED Sedim, ULP Ulu Paip, SBG Sungai Burung, BPR Bukit Panchor, SGA Sungai Acheh, PUS Pusing, LKP Langkap, KK Kuala Kangsar, SBK Sabak Bernam, TJG Tanjong Karang, KEP Kepong, KBG Kubang Kerian, BST Terengganu.

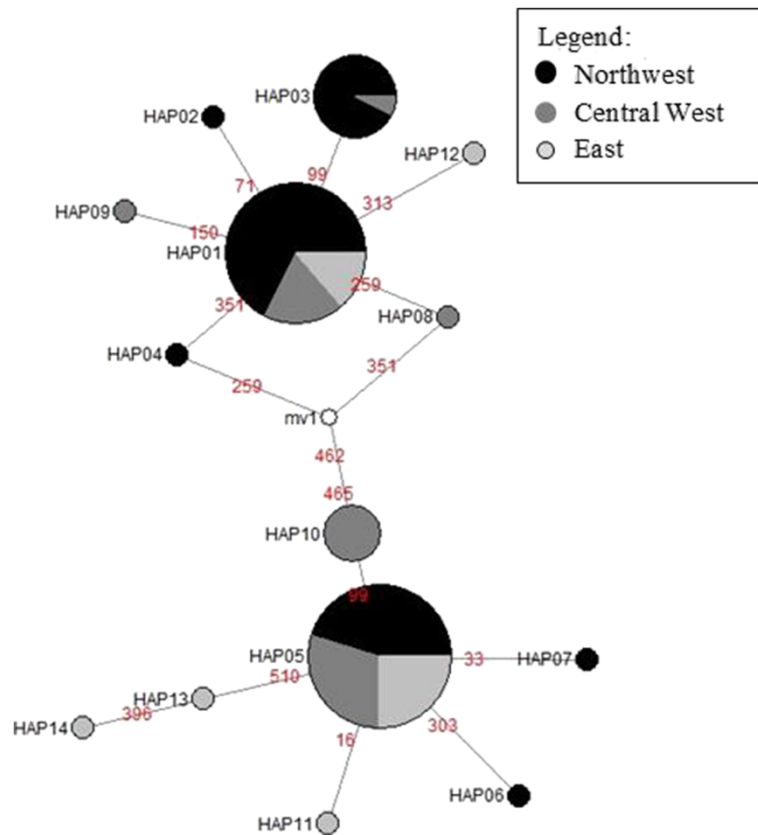


Figure 2 Minimum spanning network showing relationships among 14 haplotypes of mtDNA D-loop gene of *F. limnocharis*. Minimum spanning network showing relationships among 14 haplotypes of mtDNA D-loop gene of *F. limnocharis*, classified according to regions. The nucleotide mutation sites joining two haplotypes are showed in numbers. The size of each circle is an approximate indication of haplotype frequency [black circle found in northern region populations; dark gray circle found in northwest region populations; light gray circle found in east populations; open circle is the intermediate (mv)].

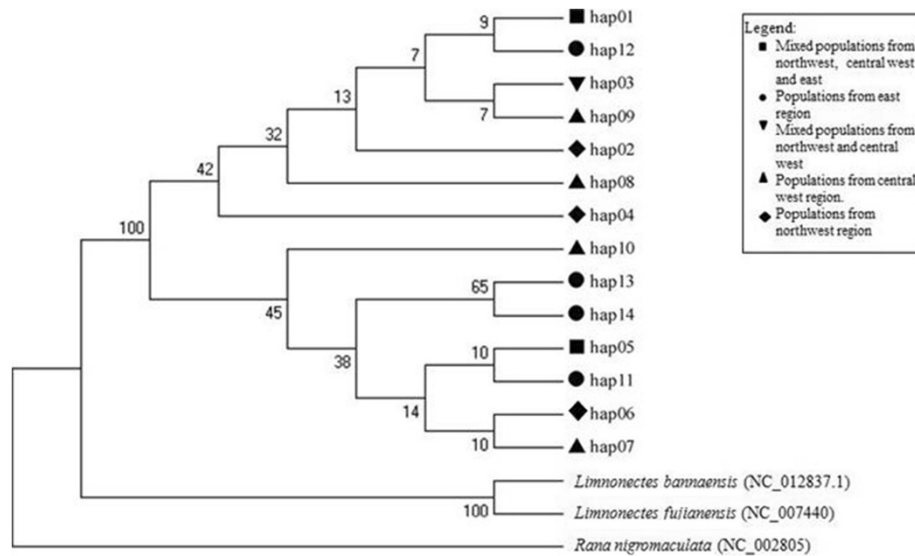


Figure 3 Maximum parsimony tree based on the Kimura-2-parameter distance model. Maximum parsimony tree based on the Kimura-2-parameter distance model showing the control region haplotype relationships among 16 populations of *Fejervarya limnocharis*. The numbers on branches indicate the bootstrap values based on 10,000 replications.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The work presented here was carried out in collaboration among all authors. AH, IJ, and SAMN defined the research theme. AH carried out the molecular genetic studies, participated in the sequence alignment, and drafted the manuscript. AH, IJ, and ZA collected most of the samples in Peninsular Malaysia and wrote the paper. ZA participated in the sequence alignment. IJ participated in the final ID of the samples. SAMN conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Author details

¹Biological Sciences Program, School of Distance Education, Universiti Sains Malaysia, Minden, Penang 11800, Malaysia. ²School of Biological Sciences, Universiti Sains Malaysia, Minden, Penang 11800, Malaysia. ³Centre for Marine and Coastal Studies, Universiti Sains Malaysia, Minden, Penang 11800, Malaysia.

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