

Multiple Introduction and High Genetic Diversity: Drivers for Successful Establishment of Invasive Peacock Bass (*Cichla* spp.) in Peninsular Malaysia

Aliyu Garba Khaleel^{1,2}, Muhammad Syafiq Aiman Mohd Nasir¹,
Mohamad Zulkarnain Mohd Dali¹, Norainsyafikah Madiran¹,
Shahreza Md Sheriff³, Lee Seong Wei⁴, Ha Hou Chew¹,
Norshida Ismail¹ and Ahmad Syazni Kamarudin^{1,*}

¹School of Animal Science, Aquatic Science and Environment, Faculty of Bioresources and Food Industry, Sultan Zainal Abidin University, Terengganu 21300, Malaysia

²Department of Animal Science, Faculty of Agriculture and Agricultural Technology, Kano University of Science and Technology, Kano 713101, Nigeria

³Institute of Tropical Aquaculture, University of Malaysia Terengganu, Terengganu 21300, Malaysia

⁴Faculty of Agro Based Industry, University of Malaysia Kelantan, Kelantan 17600, Malaysia

(*Corresponding author's e-mail: ahmadsyazni@unisza.edu.my)

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Abstract

The introduction of invasive *Cichla* spp. was followed by the several reports regarding the decline of local freshwater native species in Malaysia. The purpose of this research was to generate information concerning the introduction history of *Cichla* spp. in Malaysia. Studies on genetic diversity and structure provides the essential baseline data for monitoring and conservation of invasive species. This will help to clearly understand the propagule pressure and genetic distribution of *Cichla* spp. in the invaded freshwaters of Malaysia. A total of 61 samples of *Cichla* spp. were collected from 9 sampling location in Malaysia for mitochondrial DNA (control region) analysis. Overall samples showed high genetic diversity (Hd , 0.6700; π , 0.0254) and yielded 9 haplotypes resulted from different *Cichla* spp. present. The maximum likelihood tree with strong bootstrap support revealed that *Cichla* spp. forms 3 clusters, and confirmed to be *C. monocoulus*, *C. ocellaris* and *C. temensis*. Except for Malacca populations, other populations were largely dominated by either *C. monocoulus* or *C. ocellaris*. The current study suggesting that more than one introduction event of invasive *Cichla* spp. occurred in Malaysia.

Keywords: Conservation, Genetic diversity, Invasive species, Introduction event, Peacock bass

Introduction

The invasive *Cichla* spp. (peacock bass) is a highly predatory fish originated from the Amazon [1]. The peacock bass was first recorded in Perak, West Coast of Peninsular Malaysia in the early 1990s [2]. Since then, it has been reported and expands to different freshwater bodies across Peninsular Malaysia [3-6]. Although the introduction was well intended by anglers, *Cichla* spp. are not considered as food fish in Malaysia [7]. Hence, the species are now widely regarded as a threat to the native species in Malaysia's freshwater ecosystems. This was resulted from their high predation ability that may cause the drastic decline of the native species' populations [8,9]. The wild populations of *Cichla* spp. have been established in various freshwater bodies of Malaysia such as Lake Telabak and Timah Tasoh Dam [6]. Yet, no record has been found concerning their invasion history, release events and genetic structure in Malaysia. This is vital for a clear understanding of their establishment success and for taking the appropriate management decisions. Genetic factors play a critical role in alien species to occupy a new habitat and expanding their range [10].

Humans are the major vectors responsible for the introduction of non-native fish species into a newly ecological habitat [11]. The need to anticipate which non-native species would turn invasive and which native populations would be invaded is highly important [12]. Because invasive species have continuously exerted ecological impacts and high economic losses toward the ecosystem [13,14].

Consequently, a popular concept for the excellent prediction of the establishment success of invasive species is required. Recently, the concept of introduction effort (propagule pressure) is proved to be a useful tool for the estimation of invasive species establishment [15]. According to the Britton and Gozlan [16], a propagule pressure can be explained as the number of individuals species release into a specific area (propagule size) and their introduction frequency (propagule number). High propagule pressure describes high immigration rates, large sizes of founder population and high genetic variability [17,18]. As such, the invasive species can overcome all dynamical processes and established.

Biological invasions with a documented history are relatively rare [19]. These includes lack of the initial size of propagule, appropriate taxonomic classification and population dynamics which restrict proper management plans and risk assessment of ecological impact [20]. For instance, it was discovered that more than one introduction of a particular species (genetically different population) into the introduced habitat may results to a high genetic diversity compared to single population [21]. The use of molecular tools could be an efficient approach in reconstructing the history of the invasion, such as evaluation of propagule pressure and hybridization with native species [20,22]. Utilisation of DNA markers (mitochondrial and microsatellite) in the Neotropics has made it possible to genetically characterise the invasion of the *Cichla* spp. into multiple watersheds in the South-Eastern Brazil [23-25]. These markers are continuously in use for the determination of propagule pressure and genetic diversity for the invasion history of different non-native species [10,19,20,26-28].

The introduction of invasive *Cichla* spp. in Malaysia happened in the past 30 years. The establishment of wild *Cichla* spp. populations is still progressing within the freshwater bodies of Malaysia [6]. Tendencies for *Cichla* spp. to concur new habitats keeps increasing despite the ecological vices related to them. This remarkable invasion has captivated keen interest from researchers. However, information regarding the genetic diversity, structure and propagule pressure of these species has been scarce. Genetic factors play a critical role for alien species to occupy a new habitat and expand their range. Therefore, this study was aimed to clarify the genetic diversity of invasive *Cichla* spp. in Malaysia through the molecular phylogenetic analysis of partial mitochondrial DNA (control region). The general purpose of the current study was to provide a basic data regarding the introduction history of *Cichla* spp. in Malaysia to aid the monitoring and conservation programs.

Materials and methods

Sampling

A total of 61 feral samples of the *Cichla* spp. were captured from 9 freshwater bodies across Peninsular Malaysia from September 2018 to September 2020 (**Table 1**). All samples were captured by anglers using a fishing rod and artificial bait. Approximately 1 cm of tail tissue was removed using scissors and preserved in a sterile 1.5 mL tube containing 95 % ethanol. **Figure 1** showed photos of some *Cichla* spp. specimens collected from various location in Malaysia.

Table 1 Sampling area, habitat, code, coordinates, and number of individuals (N) on *Cichla* spp. collected across Peninsular Malaysia.

Sampling area	Freshwater body	Habitat	Code	Coordinates	N
Terengganu†	Tasik Telabak	Lake	TTT	5°37'56.9"N 102°28'24.5"E	10
Pahang†	Lombong Chendering	Lake	PLC	3°46'44.9"N 103°14'48.8"E	02
Perlis*	Empangan Timah Tasoh	Dam	PET	6°33'57.5"N 100°13'02.7"E	11
Perak‡	Tasik Raban	Lake	PTR	4°59'53.2"N 100°56'56.4"E	10
Selangor∞	Tasik Prima	Lake	STP	2°59'42.6"N 101°36'08.5"E	10
Malacca∞	Tasik Chinchin	Lake	MTC	2°16'32.2"N 102°28'50.5"E	03
Malacca∞	Tasik UiTM Jasin	Lake	MUJ	2°13'35.4"N 102°27'13.2"E	04
Johor∞	Tasik Tanjung Laboh	Lake	JTL	1°44'47.2"N 102°59'24.9"E	04
Johor∞	Tasik Merdeka	Lake	JTM	1°52'47.7"N 102°56'41.8"E	07

†East Coast Peninsular Malaysia (ECPM); ‡West Coast Peninsular Malaysia (WCPM); ∞Southern Peninsular Malaysia (SPM); *Northern Peninsular Malaysia; Tasik (Lake); Empangan (Dam)

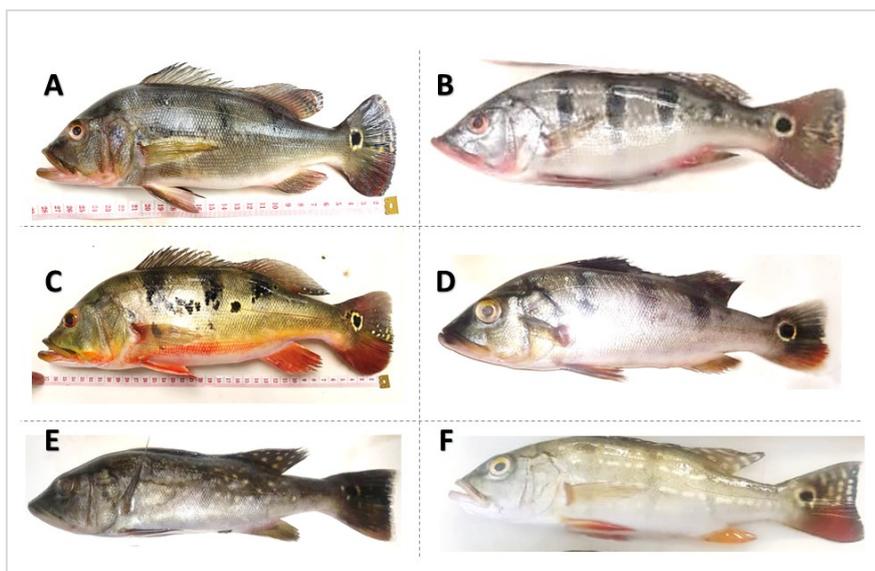


Figure 1 Photos of *Cichla* spp. specimens collected from sampling location of Peninsular Malaysia. A Tasik Raban; B Tasik Prima; C Tasik Telabak; D Empangan Timah Tasoh; E Tasik Chinchin, and F Tasik UiTM Jasin.

DNA isolation, amplification, and sequencing

The total genomic DNA of *Cichla* spp. was isolated using Favorgen DNA extraction Kit (Favorgen Biotech Corp., Ping-Tung 908, Taiwan) by following the manufacturer's instructions. The partial control region of mitochondrial DNA (mtDNA) was amplified using the forward primer tPro2 (5'ACC CTA ACT CCC AAA GC3') [29], and reverse primer HN-20 (3'GTG TTA TGC TTT AGT TAA GC5') [30]. The PCR was performed using Veriti 96 Well Thermal Cycler (Applied Biosystem, California, USA) in 25 μ L reaction volume containing 18.2 μ L sterile distilled water, 2.5 μ L Taq buffer, 2.0 μ L dNTP Mix (2.5 mM), 0.5 μ L of each primer (10 μ M), 0.3 μ L of 5 unit/ μ L Taq polymerase (TaKaRa) and 1 μ L template DNA (1 - 50 ng/ μ L). The PCR started with an initial denaturation step at 94 $^{\circ}$ C for 3 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 52 $^{\circ}$ C for 45 s, elongation at 72 $^{\circ}$ C for 90 s and then a final extension at 72 $^{\circ}$ C for 5 min.

The agarose gel electrophoresis separated the samples through 2 % of agarose gel in 1.0 X TBE using gel red. Electrophoresis was run at 100 volts for 40 min. The gel was photographed and visualized using Luminescent Image Analyzer LAS-1000plus v.2.0 (Fuji Photo Film Co. Ltd., Tokyo, Japan). Immediate sequencing of the amplified fragments was done by Apical Scientific Sdn Bhd. It was carried out using BigDyeTM Terminator Cycle Sequencing ready reaction Kit 3.1 (Applied Biosystems) following manufacturer's instructions, using primers tPro2 and HN-20 to sequence both strands. The sequencing products were analysed using an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems).

Data analysis

The sequences were aligned and edited using GENETYX v9.1.3 in multiple sequence alignment programs. The nucleotide composition and number of variable sites were determined using DnaSP v6 [31]. Population structure and genetic variation were analysed using ARLEQUIN v3.5 (CMPG, University of Berne [32]. Genetic diversity in each population was measured as haplotypic diversity [33], and nucleotide diversity [34]. The level of genetic population differentiation was tested using analysis of molecular variance (AMOVA) as implemented in ARLEQUIN v3.5 [32], using the genetic distance matrix to estimate the components of variance that are attributable to differences among groups, among populations within group and within populations. we considered sampling location as population, while the populations were grouped into 4 geopolitical zones of Peninsular Malaysia (west, east, north, and south). Two neutrality tests were examined: Tajima's D [35], and Fu's F_s [36], as implemented in ARLEQUIN v3.5 [32]. These tests were used to evaluate the neutrality of the investigated sequences to find out if populations are deviated from genetic equilibrium, population expansion or bottleneck.

Genetic differentiation between populations were tested by pairwise comparison F_{ST} with Slatekin's and Reynold's distances with 1,000 permutations as implemented in ARLEQUIN v3.5 [32]. The F_{ST} provide view of both variance structure of populations, and overall comparison of the degree at which

populations are structure. Zero value of F_{ST} shows lack of structure and differentiation in population while, when $F_{ST}=1$ meaning that population is completely differentiated. Phylogenetic and molecular evolutionary analyses were conducted using MEGA v.7 [37]. A neighbour-joining tree of the haplotypes was constructed on the model of the Kimura 2-parameter and evaluated with 1,000 bootstrap replicates to obtain the evolutionary history of peacock bass haplotypes [37]. All positions with gaps and missing data were deleted from the dataset. The sequence of *Oreochromis niloticus* (MF385002) was included in the construction of phylogenetic tree as an outgroup. Additional sequences of *Cichla* spp. (from native populations) were retrieved from NCBI for phylogenetic comparisons.

Results and discussion

Sequence polymorphism

Electrophoresis gel showed a good quality and successful amplicons extracted using control region of mitochondrial DNA region (**Figure 2**). A total of 61 sequences (875 base pair) of control region fragments of *Cichla* spp. mitochondrial DNA were obtained after deleting both primer ends. Among 875 sites, 755 were invariable (monomorphic) sites and 105 were variable or polymorphic sites (4 singleton variable sites and 101 parsimony informative sites) and 15 gaps (**Table 2**). The sequences from all samples yielded 9 haplotypes (**Table 3**). Haplotype 1 was the most common haplotype (54.10 %) found in all sampling location except for Tasik Chinchin and Tasik UiTM Jasin in Malacca. In addition, 5 distinct haplotypes (*h) were obtained from Tasik Prima (3*h), Tasik UiTM Jasin (1*h) and Tasik Merdeka (1*h). No mutations observed in the sequences of Tasik Telabak, Lombong Chendering, Tasik Chinchin and Tanjung Laboh sampling sites.

The overall haplotype diversity (H_d) and nucleotide diversity (π) of 61 sequences tested were 0.6700 and 0.0254 respectively for genetic diversity of peacock bass. The highest haplotype and nucleotide diversities were detected in Tasik Prima (H_d , 0.6667; π , 0.0037), followed by Empangan Timah Tasoh and Tasik Raban (**Table 4**). The zero values for H_d and π in Tasik Telabak, Lombong Chendering, Tasik Chinchin and Tanjung Laboh occurred from lack of variable sites observed in their sequences. Both neutrality tests of Tajima' D and Fu's F_s shows no significance deviation from neutrality in all sampling locations ($p < 0.05$).

Sampling site differentiation

AMOVA results based on the sampling locations indicated that genetic variation clearly appeared among populations (96.2 % of variance attributed to $F_{ST} = 0.966$, $p < 0.05$), which can support the strong genetic differentiation in our samples (**Table 5**). The pairwise comparison F_{ST} value of population differentiation further showed significant difference on Malacca population (Tasik Chinchin and Tasik UiTM Jasin, ($p < 0.05$, **Table 6**). A phylogenetic tree provided further confirmation of existence of the 3 clusters among the haplotypes (**Figure 3**). The separation of the 3 genetic clusters is also apparent on the Neighbor-Joining Tree.

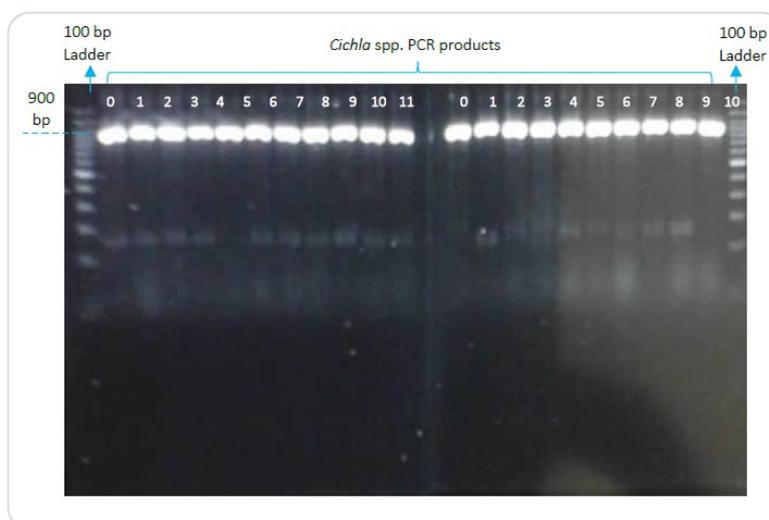


Figure 2 Agarose gel electrophoresis (2 % agarose) showing PCR products of 20 *Cichla* spp.

Table 2 Sequence variations of 9 haplotype in mitochondrial DNA control region of 61 samples of *Cichla* spp., the numbers represent the nucleotide position.

Haplotype									Haplotype										
VS	1	2	3	4	5	6	7	8	9	VS	1	2	3	4	5	6	7	8	9
12	C	A	A	.	289	T	C	C	.
19	T	C	C	.	293	A	.	.	G
33	A	G	G	.	325	C	T	T	.
40	A	C	C	.	335	A	T	T	.
43	A	T	T	.	348	C	G	G	.
58	A	T	T	.	356	G	A	A	.
60	T	C	C	.	361	C	T	T	.
61	A	G	G	.	375	T	C	C	.
77	G	A	A	.	376	T	A	A	.
79	T	A	A	.	378	G	A	A	.
91	A	C	C	.	383	C	T	T	.
94	G	A	A	.	430	A	C	C	.
99	C	T	T	.	442	C	.	.	T	T	T	.	.	.
100	T	C	C	.	467	C	T	T	.
101	C	.	.	T	T	T	T	T	.	469	A	G	G	.
113	G	.	.	.	A	509	G	T	T	.
124	*	C	C	.	515	G	.	.	A	A	A	A	A	.
125	*	T	T	.	539	*	T	T	.
126	*	A	A	.	542	C	T	T	.
130	G	A	A	.	544	T	C	C	.
131	A	C	C	.	550	*	C	C	.
138	C	A	A	.	575	A	G	G	.
142	C	T	T	.	576	G	.	A
144	C	T	T	.	585	T	C	C	.
151	T	C	C	.	598	A	T	T	.
152	A	C	C	.	599	T	C	C	.
167	A	T	T	.	600	T	C	C	.
170	A	T	T	.	606	T	C	C	.
172	T	C	C	.	607	T	C	C	.
174	C	T	T	.	608	C	T	T	.
177	T	G	G	.	611	C	G	G	.
180	C	T	T	.	613	T	*	*	.
183	A	*	*	.	617	A	G	G	.
187	A	G	G	.	618	A	G	G	.
189	*	.	T	.	T	T	C	C	.	622	A	*	*	.

Haplotype									Haplotype										
190	*	•	T	•	T	T	C	C	•	629	G	•	•	•	•	•	A	A	•
193	G	•	•	•	•	•	T	T	•	631	A	•	•	•	•	•	G	G	•
195	T	•	•	•	•	•	C	C	•	632	C	•	•	•	•	•	T	T	•
200	C	•	•	•	•	•	T	T	•	633	A	•	•	•	G	G	G	G	•
203	T	•	•	•	•	•	C	C	•	641	G	•	•	•	•	•	A	A	•
206	T	•	•	•	•	•	G	G	•	642	G	•	•	•	•	•	A	A	•
207	*	•	•	•	•	•	A	A	•	643	A	•	•	•	•	•	G	G	•
212	G	•	•	•	•	•	A	A	•	651	G	A	A	A	•	•	•	•	•
215	C	•	•	•	•	•	G	G	•	687	G	•	•	•	•	•	A	A	•
232	A	•	•	•	•	•	G	G	•	700	A	•	•	•	•	•	G	G	•
235	*	•	•	•	•	•	C	C	•	706	C	•	•	•	•	•	T	T	•
237	C	•	•	•	•	•	T	T	•	766	T	•	•	•	•	•	C	C	•
242	C	•	•	•	•	•	T	T	•	771	T	•	•	•	•	•	C	C	•
243	T	•	•	•	•	•	A	A	•	777	C	•	•	•	•	•	T	T	•
247	T	•	•	C	C	C	C	C	•	813	C	•	•	•	•	•	T	*	•
260	A	•	•	•	•	•	G	G	•	820	C	•	•	•	•	•	T	T	•
261	C	•	•	•	•	•	T	T	•	823	G	•	•	•	•	•	A	A	•
264	T	•	•	•	•	•	C	C	•	825	*	•	•	•	•	•	A	A	•
274	A	•	•	G	G	G	G	G	•	827	G	•	•	•	•	•	A	A	•
280	C	•	•	•	•	•	A	A	•	835	G	•	•	•	•	•	A	A	•
283	G	•	•	•	•	•	A	A	•	841	T	•	•	•	•	•	•	A	•
286	T	•	•	•	•	•	A	A	•										

VR, variable site; Dot (•) Represents the identical nucleotide with the reference haplotype (Hap-1), *Gap

Table 3 Detected haplotypes of *Cichla* spp. and their percentage distribution from 9 sampling locations in Malaysia.

Haplotype	Sampling location										N	%N
	TTT	PLC	PET	PTR	STP	MTC	MUJ	JTL	JTM			
Hap-1	10	2	5	5	1			5	5	33	54.10	
Hap-2			4	6						10	16.39	
Hap-3			1		1					2	3.28	
Hap-4*					1					1	1.64	
Hap-5*					1					1	1.64	
Hap-6*					6					6	9.84	
Hap-7						3	3			6	9.84	
Hap-8*							1			1	1.64	
Hap-9*									1	1	1.64	
Total	10	2	10	11	10	3	4	5	6	61	100	

*Distinct haplotype; %N, percentage number of samples in a haplotype; TTT, Tasik Telabak; PLC, Lombong Chendering; PET, Empangan Timah Tasoh; PTR, Tasik Raban; STR, Tasik Prima; MTC, Tasik Chinchin; MUJ, Tasik UiTM Jasin; JTL, Tasik Tanjung Laboh; JTM, Tasik Merdeka

Table 4 Nucleotide sequence data of 9 sampling sites based on partial fragments of the mitochondrial DNA (control region) haplotype diversity, nucleotide diversity (mean \pm SD) and neutrality test.

SL	Noh	π	<i>Hd</i>	Tajima' <i>D</i>	Tajima' <i>D</i> <i>p</i> -value	Fu's <i>F_s</i>	Fu's <i>F_s</i> <i>p</i> -value
TTT	1	0.0000	0.0000	0.000	1.000	0.000	NA
PLC	1	0.0000	0.0000	0.000	1.000	0.000	NA
PET	3	0.0009	0.6444	0.272	0.755	-0.046	0.408
PTR	2	0.0006	0.5455	1.442	0.933	1.137	0.653
STP	5	0.0037	0.6667	-0.420	0.3560	0.950	0.693
MTC	1	0.0000	0.0000	0.000	1.000	0.000	NA
MUJ	2	0.0006	0.5000	-0.612	0.380	0.172	0.373
JTL	1	0.0000	0.0000	0.000	1.000	0.000	NA
JTM	2	0.0004	0.3333	-0.933	0.355	-0.003	0.259

No significant Tajima's *D* *p*-value and Fu's *F_s* *p*-value observed in the neutrality test, showing that all sampling sites were in genetic equilibrium, SL, sampling location. See **Table 1** for the abbreviation of sampling location

Table 5 Analysis of molecular variance (AMOVA) of mitochondrial DNA (control region) nucleotide data of 9 sampling location in Peninsular Malaysia.

Source of variation	df	Sum of squares	Variance component	Percentage of variation	<i>F_{ST}</i> Value
Among groups	2	216.7	0.05759	0.40	0.96619*
Among population within group	6	518.3	13.85375	96.2	
Within population	52	25.3	0.48674	3.4	
Total	60	760.4	14.39808	100%	

*Significant level ($p < 0.05$)

Table 6 Pairwise comparison *F_{ST}* value of population differentiation.

	TTT	PLC	PET	PTR	STP	MTC	MUJ	JTL	JTM
TTT	-								
PLC	0.00000	-							
PET	0.37037*	0.07609	-						
PTR	0.48478*	0.23345	-0.08313	-					
STP	0.71242*	0.53092	0.67502*	0.69670*	-				
MTC	1.00000*	1.00000	0.99457*	0.99600*	0.97121*	-			
MUJ	0.99890*	0.99669	0.99393*	0.99530*	0.97250*	-0.09091	-		
JTL	0.00000	0.00000	0.25764	0.38202	0.63118*	1.00000*	0.99811*	-	
JTM	0.09091	-0.30435	0.25513	0.35750*	0.64172*	0.99790*	0.99651*	-0.03448	-

*Significant level ($p < 0.05$) With the Sequential Bonferroni Correction; See **Table 1** for the Abbreviation of Sampling Location

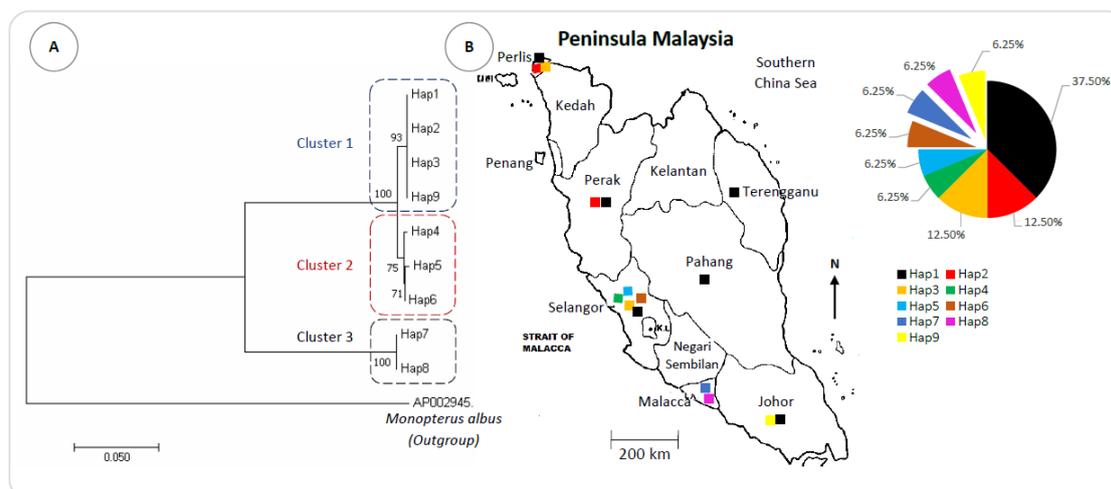


Figure 3 (A) Neighbor-Joining tree showing phylogenetic relationships among 9 haplotypes of *Cichla* spp. inferred from partial mitochondrial DNA sequences (control region). A distance matrix was calculated using the Kimura's 2-Parameter model in MEGA v7. The data set was bootstrapped 1,000 times, and the appropriate bootstrap values were placed on each branch. (B) Distribution of the 9 haplotypes of *Cichla* spp. in accordance with sampling sites, and percentage distribution of each haplotype

Genetic diversity of *Cichla* spp.

The genetic constitution of an invasive species mutates from its native to non-native ranges which in turn can lead to the differences in genetic diversity distribution within and between populations [38]. Nucleotide variations in the mitochondrial DNA sequences in the current study describe the possibility of multiple introductions of invasive species. The number of polymorphisms (120) and haplotypes (9) were high among the invasive *Cichla* spp. studied, which suggests high propagule pressure. Our results disagree with other studies where low genetic diversity was observed among the introduced *Cichla* spp. populations [1,25]. In these studies, the *Cichla* spp. were mainly introduced and expand through river canals routes. In contrast, anglers are the main agents that promote the introduction and increase releases of *Cichla* spp. for quenching their catching desire in Malaysia [7]. Therefore, more variations are expected to occur among the species introduced artificially compared to those arrived accidentally.

Although a single introductory event involving many individuals can lead to the emergence of multiple haplotypes in the population [39]. Hence, too much angling pressure could be a reason for the number of haplotypes found in Tasik Prima (Selangor), Empangan Timah Tasoh (Perlis) and Tasik Raban (Perak). In Malaysia, Perak and Selangor are hotspot areas for tourist and anglers [7], which may probably increase the chances of *Cichla* spp. recurrent event of new introduction. A widely distributed Haplotype 1 might be initially begun with population from Perak (first introduction location), where they certainly expand (transmitted via anglers) and established into the various freshwater bodies across the country. For instance, capturing many juveniles and matured *Cichla* spp. in Tasik Telabak revealed the high reproductive success of *Cichla* spp. [9]. The high haplotypic diversity in the Tasik Prima, which included 2 clusters suggest that the lake may have served as an aquatic refuge for *Cichla* species in Peninsular Malaysia.

Genetic structure

Studies on population structure provides the essential baseline data for monitoring and conservation. In fact, understanding population genetic structure and diversity within invasive species is crucial for determining their capacity to respond to local selection pressures in non-native ranges [40]. The 3 clusters earlier identified in **Figure 2** are representing 3 species of genus *Cichla*. Phylogenetic tree constructed with the native haplotypes showed that cluster-3 belong to the *C. temensis*, whereas both cluster-1 and cluster-2 have fallen within the range of similar species and therefore considered as *C. monocoulus* (**Figure 4(A)**). However, cluster-2 appeared to be closer to the 3 complete mitochondrial DNA sequences of *C. ocellaris* added to the phylogenetic tree (**Figure 4(B)**). Interestingly, the whole Cluster-2 haplotypes (*C. ocellaris*) and 3 added sequences were also interwoven inside a *C. monocoulus* clade. This

ambiguity might possibly be resulted from high sequence resemblance or hybridization between the *Cichla* spp. present in Malaysia (see **Figure 1**).

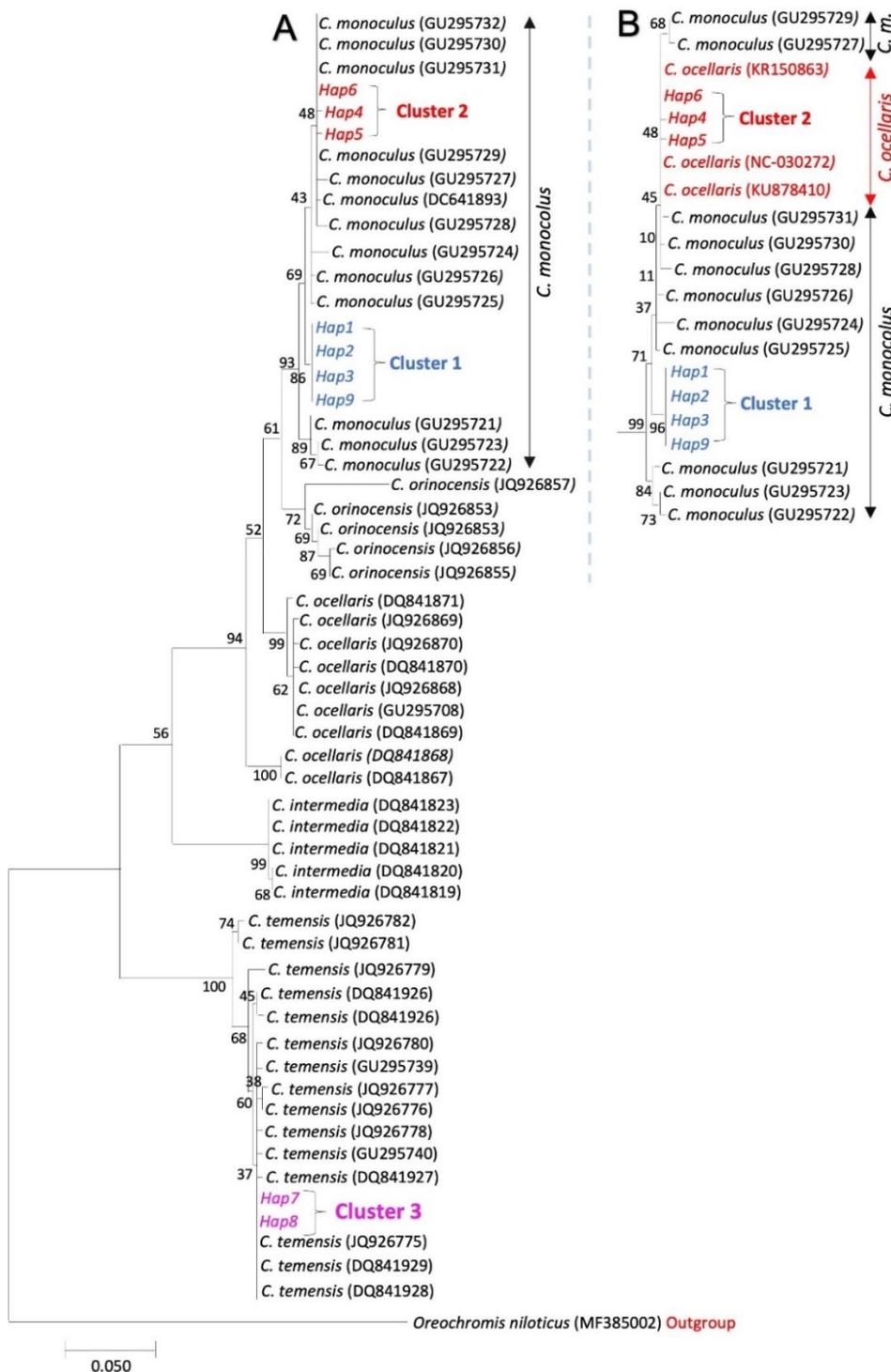


Figure 4 Molecular phylogenetic analysis by Maximum Likelihood method showing phylogenetic relationships among 9 haplotypes of *Cichla* spp. (Malaysia) and those obtained from GenBank. A distance matrix was calculated using the Kimura’s 2-Parameter Model in MEGA v7. The data set bootstrapped 1,000 times and the appropriate bootstrap values were placed on each branch. (A) Displaying Malaysia’s haplotypes matching to only *C. monoculus* and *C. temensis*. (B) Tree constructed in **Figure 4(A)** with 3 additional complete mitochondrial DNA sequences of *C. ocellaris*.

Even though, cluster-1 haplotypes exhibited more closeness to the *C. monocoulus* however, previous identification of *Cichla* spp. in Tasik Telabak through mitochondrial DNA (COI) barcoding marked the specimens as *C. ocellaris* with 99.72 % similarity index [6]. At the same lake also, Sastraprawira *et al.* [41], claimed that *Cichla* spp. specimens captured during their experiment were *C. kelberi* with 92 % similarity index (using COI). Though the latter authors concluded the argument as less conclusive due to the small sample size obtained in their study [41]. In fact, the number of *Cichla* spp. samples (40 specimens) used in by the former study were strongly enough to conclude peacock bass identity [6]. Perhaps, species identification using DNA barcoding method is possible and proven to be successful even with only single specimen [42].

For instance, Willis *et al.* [43], in Brazil discovered that *C. ocellaris* from the Cuyuni River displayed haplotypes are having more characteristic of *C. temensis*, whilst *C. temensis* from the Guri Reservoir on the Caroni River displayed haplotypes are having characteristic of *C. orinocensis*. The authors explained that these alleged hybrids were either sympatric with members of the donor species or lived near the locations where they were discovered. However, the mitochondrial DNA (maternal only) analysis could not detect the incidences of hybridization in the present study. Hence, our priority focuses more on the genetic diversity and introduction events of these species. Similarly, earlier report showed that *C. monocoulus* *sensu stricto* for being a sister group with *C. ocellaris* sampled from the Maroni in French Guiana [44]. Based on the present finding, *C. monocoulus* and *C. ocellaris* are the main *Cichla* spp. that have invaded the entire freshwater bodies examined except for Malacca state where only *C. temensis* existed. Deliberate introductions by local anglers seem to be the main explanation for the expansion of these species in Malaysia [7].

Implication of high genetic diversity of *Cichla* spp.

Invaders have a superior ability to exploit local resources when compared with native residents [25]. Considering the invaded environments (such as lakes and dam) which can rapidly change received an invasive species, preliminary features and adaptations of native species may no longer be favourable. In such cases, native organisms may become “locked” by evolutionary responses, unable to quickly adapt and compete with invasive species, leading to decreased survival and eventual extinction of native species [45,46]. Despite the small genetic data set generated in the current study, our findings suggest that populations of *Cichla* spp. in Malaysia have a reasonably high diversity and admixture of individuals with different haplotypes. This is favourable for increasing the individual fitness and may contribute to the invasion success. In contrast, *Cichla*'s invasive ability appeared to be unaffected by the low genetic diversity in non-Amazonian rivers of Brazil [25]. The authors suggested that the prey naiveté and super competitive ability could be the possible factors making the *Cichla* spp. to be highly invasive. Since there is a lack of information regarding the invasive *Cichla*'s diversity, for future there is need for meta-analysis method. Meta-analysis is well-known as the quantitative method to critically evaluate the importance of relatively study [47].

Conclusions

Even though the pathways of *Cichla* spp. introduction into Malaysia are still not fully clear, the current study's findings provide new and more reliable information on genetic diversity and stocking events of this species. This is particularly true when the haplotype distribution patterns are examined alongside the first *Cichla* spp. records in the Perak area. Considering the high dominance of haplotype 1 and the earliest record of *Cichla* spp. introduction (early 1990s) among the invasive populations studied, there is clear evidence that the first translocation of *Cichla* spp. occurred in Perak location. In addition, 3 genetically distinct clusters of *Cichla* clearly indicates that more than one *Cichla* introduction event occurred in Malaysia). The *C. monocoulus* or *C. ocellaris* and *C. temensis* are the main *Cichla* spp. discovered in this study. However, further study on nuclear DNA such as microsatellite and minisatellite is required to access the possible hybridization of *Cichla* spp. in Malaysia.

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References

- [1] ACPB Marques, ACS Franco, F Salgueiro, E García-Berthou and LN Santos. Genetic divergence among invasive and native populations of the yellow peacock cichlid *Cichla kelberi*. *J. Fish Biol.* 2016; **89**, 2595-606.
- [2] KAA Rahim, Y Esa and A Arshad. The influence of alien fish species on native fish community structure in Malaysian waters. *Kuroshio Sci.* 2013; **7**, 81-93.
- [3] SK Yap, I Muneera, MI Syakir, HH Zarul and F Widad. Stable isotopes approach to infer the feeding habit and trophic position of freshwater fishes in tropical lakes. *Iranian J. Energ. Environ.* 2016; **7**, 177-83.
- [4] KW Tan and KZ Sze. Determination of heavy metal concentration in fish species in Kampar mining lake, Perak state of Malaysia. *Fresen. Environ. Bull.* 2017; **26**, 4202-7.
- [5] CKC Ng, PA Ooi, W Wong and G Khoo. Ichthyofauna checklist (Chordata: Actinopterygii) for indicating water quality in Kampar River catchment, Malaysia. *Biodiversitas* 2018; **19**, 2252-74.
- [6] AG Khaleel, SAM Nasir, I Norshida and K Ahmad-Syazni. Origin of invasive fish species, peacock bass *Cichla* species in Lake Telabak Malaysia revealed by mitochondrial DNA barcoding. *Egypt. J. Aquat. Biol. Fish.* 2020; **24**, 311-22.
- [7] AG Khaleel, N Ismail and K Ahmad-Syazni. Introduction of invasive peacock bass (*Cichla* spp.), its rapid distribution and future impact on freshwater ecosystem in Malaysia. *Ribarstvo* 2021; **79**, 33-46.
- [8] ACS Franco, LN dos Santos, AC Petry and E García-Berthou. Abundance of invasive peacock bass increases with water residence time of reservoirs in south-eastern Brazil. *Hydrobiologia* 2017; **817**, 155-66.
- [9] AG Khaleel, NN Rusli, NIM Radzif, SAM Nasir, MZM Dali, N Ismail and K Ahmad-Syazni. Prey identification of invasive peacock bass from Telabak Lake Malaysia using DNA barcoding technique. *Int. J. Aquat. Biol.* 2020; **8**, 272-80.
- [10] I Pervukhina-Smith, RF Sforza, M Cristofaro, JF Smith and SJ Novak. Genetic analysis of invasive populations of *Venttenata dubia* (Poaceae): An assessment of propagule pressure and pattern of range expansion in the Western United States. *Biol. Invasions* 2020; **22**, 3575-92.
- [11] S Eagderi, M Nasri and E Çiçek. First record of the Amur goby *Rhinogobius lindbergi* Berg 1933 (Gobiidae) from the Tigris River drainage, Iran. *Int. J. Aquat. Biol.* 2018; **6**, 202-7.
- [12] T Heger and L Trepl. Predicting biological invasions. *Biol. Invasions* 2003; **5**, 313-21.
- [13] C Bellard, P Cassey and TM Blackburn. Alien species as a driver of recent extinctions. *Biol. Lett.* 2016; **12**, 20150623.
- [14] AJ Reid, AK Carlson, IF Creed, EJ Eliason, PA Gell, PT Johnson and SJ Cooke. Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biol. Rev.* 2019; **94**, 849-73.
- [15] TM Blackburn, JL Lockwood and P Cassey. The influence of numbers on invasion success. *Mol. Ecol.* 2015; **24**, 1942-53.
- [16] JR Britton and RE Gozlan. How many founders for a biological invasion? Predicting introduction outcomes from propagule pressure. *Ecology* 2013; **94**, 2558-66.
- [17] D Simberloff. The role of propagule pressure in biological invasions. *Annu. Rev. Ecol. Evol. Systemat.* 2009; **40**, 81-102.
- [18] JT Wootton and CA Pfister. Experimental separation of genetic and demographic factors on extinction risk in wild populations. *Ecology* 2013; **94**, 2117-23.
- [19] MR Gaither, RJ Toonen and BW Bowen. Coming out of the starting blocks: extended lag time rearranges genetic diversity in introduced marine fishes of Hawaii. *Proc. Biol. Sci.* 2012; **279**, 3948-57.
- [20] NG Sales, TC Pessali, FRA Neto and DC Carvalho. Introgression from non-native species unveils a hidden threat to the migratory Neotropical fish *Prochilodus hartii*. *Biol. Invasions* 2018; **20**, 555-66.
- [21] DM Rosenthal, AP Ramakrishnan and MB Cruzan. Evidence for multiple sources of invasion and intraspecific hybridization in *Brachypodium sylvaticum* (Hudson) Beauv. in North America. *Mol. Ecol.* 2008; **17**, 4657-69.
- [22] ME Cristescu. Genetic reconstructions of invasion history. *Mol. Ecol.* 2015; **24**, 2212-25.
- [23] AV Oliveira, AJ Prioli, SMAP Prioli, TS Bignotto, HF Júlio, H Carrerck, CS Agostinho and LM Prioli. Genetic diversity of invasive and native *Cichla* (Pisces: Perciformes) populations in Brazil with evidence of interspecific hybridization. *J. Fish Biol.* 2006; **69**, 260-77.

- [24] DC Carvalho, DAA Oliveira, JE Santos, P Teske, LB Beheregaray, H Schneider and I Sampaio. Genetic characterization of native and introduced populations of the neotropical cichlid genus *Cichla* in Brazil. *Genet. Mol. Biol.* 2009; **32**, 601-7.
- [25] DC Carvalho, DA Oliveira, I Sampaio and LB Beheregaray. Analysis of propagule pressure and genetic diversity in the invasibility of a freshwater apex predator: the peacock bass (genus *Cichla*). *Neotropical Ichthyol.* 2014; **12**, 105-16.
- [26] ML Vahsen, K Shea, CL Hovis, BJ Teller and RA Hufbauer. Prior adaptation, diversity, and introduction frequency mediate the positive relationship between propagule pressure and the initial success of founding populations. *Biol. Invasions* 2018; **20**, 2451-9.
- [27] SM Hovick and KD Whitney. Propagule pressure and genetic diversity enhance colonization by a ruderal species: A multi-generation field experiment. *Ecol. Monogr.* 2019; **89**, e01368.
- [28] DF Teixeira, FRA Neto, LC Gomes, LB Beheregaray and DC Carvalho. Invasion dynamics of the white piranha (*Serrasalmus brandtii*) in a Neotropical River Basin. *Biol. Invasions* 2020; **22**, 983-95.
- [29] WJ Lee, J Conroy, WH Howell and TD Kocher. Structure and evolution of teleost mitochondrial control regions. *J. Mol. Evol.* 1995; **41**, 54-66.
- [30] SR Palumbi. *Nucleic acid II: The polymerase chain reaction*. In: DM Hillis, G Moritz and BK Mable (Eds.). *Molecular Systematics*. Sinauer Associates, Massachusetts, 1996, p. 205-47.
- [31] P Librado and J Rozas. DnaSP v5: Software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 2009; **25**, 1451-2.
- [32] L Excoffier and HE Lischer. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Res.* 2010; **10**, 564-7.
- [33] M Nei. *Molecular evolutionary genetics*. New York Columbia University Press, New York, 1987.
- [34] F Tajima. Evolutionary relationship of DNA sequences in finite populations. *Genetics* 1983; **105**, 437-60.
- [35] F Tajima. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 1989; **123**, 585-95.
- [36] FX Fu. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* 1997; **147**, 915-25.
- [37] S Kumar, G Stecher and K Tamura. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 2016; **33**, 1870-4.
- [38] S Ueno, JF Rodrigues, A Alves-Pereira, ER Pansarin and EA Veasey. Genetic variability within and among populations of an invasive, exotic orchid. *AoB Plants* 2015; **7**, plv077.
- [39] NA Diamante, AVD Oliveira, AC Petry, PA Catelani, FM Pelicice, SMAP Prioli and AJ Prioli. Molecular analysis of invasive *Cichla* (Perciformes: Cichlidae) populations from neotropical ecosystems. *Biochem. Systemat. Ecol.* 2017; **72**, 15-22.
- [40] JC Stout, KJ Duffy, PA Egan, M Harbourne and TR Hodgkinson. Genetic diversity and floral width variation in introduced and native populations of a long-lived woody perennial. *AoB Plants* 2015; **7**, plu087.
- [41] SM Sastraprawira, IHA Razak, S Shahimi, S Pati, HA Edinur, AB John and BR Nelson. A review on introduced *Cichla* spp. and emerging concerns. *Heliyon* 2020; **6**, e05370.
- [42] D Golani, O Sonin, G Snovsky, L David and R Tadmor-Levi. The occurrence of the peacock bass (*Cichla kelberi* Kullander and Ferreira 2006) in Lake Kinneret (Sea of Galilee), Israel. *BiolInvasions Record.* 2019; **8**, 706-11.
- [43] SC Willis, J Macrander, IP Farias and G Ortí. Simultaneous delimitation of species and quantification of interspecific hybridization in Amazonian peacock cichlids (genus *Cichla*) using multi-locus data. *BMC Evol. Biol.* 2012; **12**, 96.
- [44] JF Renno, N Hubert, JP Torrico, F Duponchelle, JN Rodriguez, CG Davila and E Desmarais. Phylogeography of *Cichla* (Cichlidae) in the upper Madera basin (Bolivian Amazon). *Mol. Phylogenet. Evol.* 2006; **41**, 503-10.
- [45] MA Schlaepfer, PW Sherman, B Blossey and MC Runge. Introduced species as evolutionary traps. *Ecol. Lett.* 2005; **8**, 241-6.
- [46] B Gallardo, M Clavero, MI Sánchez and M Vilà. Global ecological impacts of invasive species in aquatic ecosystems. *Global Change Biol.* 2015; **22**, 151-63.
- [47] DN Adli, O Sjöfjan, A Irawan, DT Utama, MM Sholikin, RR Nurdianti, RA Nurfitriani, C Hidayat, A Jayanegara and S Sadarman. Effects of fibre-rich ingredient levels on goose growth performance, blood profile, foie gras quality and its fatty acid profile: A meta-analysis. *J. Anim. Feed Sci.* 2022; **31**, 301-9.