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DNA Barcoding and Phylogenetics Relationship of Pangasiid Catfishes in Peninsular Malaysia Revealed the Impacts of Aquaculture on the Native Species Conservation

Baharuddin Haslawati [1,*], Md Ali Amatul-Samahah [1], Mohammed Rizman-Idid [2] and Sekaran Muniandy [3]

[1] Fisheries Research Institute, Glami Lemi, Titi Jelebu 71660, Negeri Sembilan, Malaysia; amatul@dof.gov.my
[2] Institute of Ocean and Earth Sciences, Universiti Malaya, Kuala Lumpur 50603, Malaysia
[3] Faculty of Medicine, MAHSA University, Bandar Saujana Putra, Jenjarom 42610, Selangor, Malaysia
* Correspondence: haslawati@dof.gov.my

Abstract: Pangasiids are an economically significant group of catfish, and many pangasiids are threatened in the wild from anthropogenic pressures, including increases in fishing pressure, habitat degradation, and improperly managed aquaculture practices. This study demonstrates the usage of DNA barcoding of the Cytochrome Oxidase subunit I (COI) gene as an identification tool in detecting potentially threatening invasive pangasiid species by establishing the diversity and phylogenetic relationship of Pangasiidae catfishes in Peninsular Malaysia. A neighbour-joining (NJ) dendrogram (Kimura-2-parameter model) generated five clades to represent distinct genera. *Pangasius* was further subdivided into two clades (Clade A: *Pangasius bocourti-P. djambal* and Clade B: *P. nasutus-P. conchophilus*). Given the marginal genetic divergence, indigenous and non-native species should be treated cautiously in allopatrically distributed species. The analysis used Automatic Barcode Gap Discovery (ABGD) and revealed barcode gaps between the intraspecific and interspecific distances. The sequences were partitioned into five groupings, corresponding with the species delineation based on the distribution of pairwise differences, which could not be differentiated using the NJ dendrogram. ABGD allows the recognition of one or two additional species using the recursive approach, but other taxonomic methods should be considered for a solid conclusion. DNA barcoding demonstrates the identification of closely related species, thus justifying its application towards the conservation of these fish.

Keywords: DNA barcoding; catfish; native species; taxonomy; aquaculture

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1. Introduction

Fish of the family Pangasiidae are medium- to large-sized catfish with diverse morphologies and ecologies [1]. Adults range from 20 to 300 cm in length, but most species are larger than 50 cm. Pangasiids are generally found in freshwater areas; however, some species can be found in brackish and marine environments [2,3]. Pangasiid catfish are widely distributed throughout Asia, ranging from the Indian subcontinent, the Indo-Malayan Archipelago [3], and China [4]. Kottelat (2013) catalogued four valid genera: *Helicophagus* Bleeker, 1858; *Pangasianodon* Chevey, 1930; *Pangasius* Valenciennes, in Cuvier & Valenciennes, 1840; and *Pseudolais*, Vaillant, 1902 [5,6], which comprises 30 species [7]. Several taxonomic ambiguities were encountered with respect to this group within these genera due to the morphological variations between conspecifics found on the Asian Mainland and the Indo-Malayan Archipelago [3]. These included morphologically disparate life stages [8], species complexes [6,9], and local-scale ecological variations in morphology [1,3,6,7,10].

Pangasiids are highly valued in aquaculture, and the demand for these fish is rapidly increasing. The major markets include the European and Asian countries, Mexico, Australia, the USA, the Middle East, and Russia [11,12]. The most commercially farmed Pangasius species are *Pangasius bocourti* and *Pangasianodon hypophthalmus* (also known as 'iridescent

shark' or the striped catfish) [13,14]. The juveniles of *P. hypophthalmus* are also traded as ornamental fish [15,16]. Despite their economic importance, however, there were several other pangasiid species reported to be rare in the wild due to threats of extinction [1,10,17].

In Malaysia, pangasiid catfish are also among the most popularly consumed freshwater fish and are also exported as fillets (Department of Fisheries Malaysia, unpublished). Even though only four species are recorded as being native to Peninsular Malaysia: *Helicophagus waandersii*, *Pangasius nasutus*, *Pangasius polyuranodon*, and *Pseudolais micronemus* [3,10,18], the cultured *P. hypophthalmus, Pangasius nasutus,* and *P. polyuranodon* are currently listed as least concern on the IUCN Red List [19,20]. Additionally, *H. waandersii* and *P. nasutus* were reported as moderately threatened (MT), which is equal to endangered (EN) on the IUCN Red List [21]. On the other hand, *P. micronemus*, which is very abundant in Malaysia, showed a declining population trend in Cambodia and Vietnam [10].

High local demand for the wild, native species has attracted the interest of breeders and aquaculturists in importing and cultivating the species, particularly from the Chao Phraya and Mekong River areas [17]. Some of the introduced species might have established themselves in the new environment and affected the native populations, possibly through hybridisation [22]. Hybridisation between the wild and imported pangasiids is being practised [23] to cater to the high demand and preference for local species (especially *P. nasutus*) (personal observation). However, such activities may increase the threat posed by introduced species escapees if they unintentionally hybridise with natural populations [15,24]. Besides, environmental disturbances and overfishing can lead to bottlenecks that promote genetic variability loss and inbreeding in natural fish populations. This may ultimately reduce species' ability to adapt to their environments [25].

Introgressive hybridisation has confounded catfish species identification in a few Asian countries [24,26]. Thus, to overcome these challenges, DNA barcoding was adopted into the identification process of pangasiid catfish in Peninsular Malaysia. DNA barcoding is a widely used technique using the cytochrome c oxidase I (COI) gene sequence—as a genetic marker for species identification and has been used in many fish biodiversity studies [27–29]. The COI gene is short enough to be sequenced quickly and cheaply, yet long enough to characterise variation among species [30].

The present study aimed to understand the genetic diversity and assess the genetic variation in the Pangasiid family in Peninsular Malaysia for further fisheries conservation management. Here, we investigate the utility of COI-based DNA barcoding as a tool for the rapid and accurate identification of invasive pangasiid catfishes by evaluating its ability to distinguish between native and potentially invasive species. Implications for conservation will be discussed for fisheries management and native pangasiid conservation.

2. Materials and Methods

2.1. Collection of Samples and DNA Extraction

Pangasiids were collected from two rivers in Peninsular Malaysia in which the species are known to be abundant, the Pahang River and the Perak River (Figure 1). There were four sampling collection points for the Pahang River compared to only one for the Perak River, as the Pahang River is well known for its native pangasiid population. Local fishing gear such as gillnets, drift gillnets, longlines, hooks, and lines, and other traditional methods were used for specimen collection. Specimens were identified on-site based on their external morphology [3,6]. Doubtful specimens were further examined in the laboratory. Voucher specimens were fixed in 10% formalin for at least seven days; rinsed with tap water and soaked for another seven days before being transferred to 70% ethanol. They were then deposited at the Fisheries Research Institute (FRI) in Glami Lemi, Negeri Sembilan, Malaysia. Muscle tissues were taken from the caudal region on the right side of the fish. Photographs were taken on the left side of the specimens. Tissue samples were preserved in 90% ethanol and stored at 4 °C until further analysis.

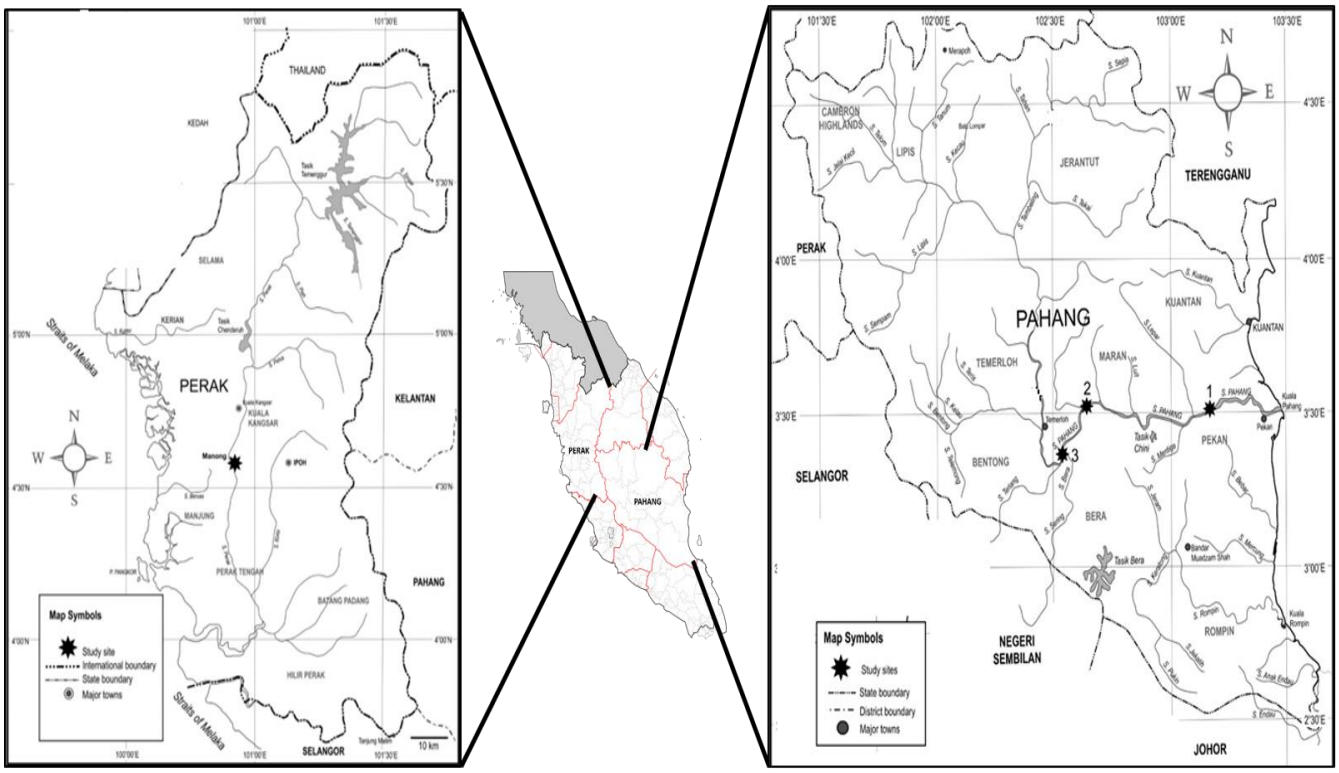


Figure 1. Map of Peninsular Malaysia showing Pahang and Perak states where the samples were collected, from the Pahang River and the Perak River. Numbers refer to the following locations: (1) Paloh Hinai, Pekan, Pahang; (2) Lubok Paku, Maran, Pahang; (3) Chenor, Maran, Pahang (Pahang River); and (4) Manong, Kuala Kangsar, Perak of the Perak River.

DNA was extracted using the Promega Wizard Genomic Animal Tissue Extraction Kit (Mouse Tail Procedure) (Promega, Madison, WI, USA), following the manufacturer's guidelines. Cytochrome c Oxidase I (COI) was amplified using primers from Ward et al. (2005) [29] with slight modifications. Polymerase Chain Reaction (PCR) was performed in a total volume of 25 µL that contained 1.5 µL DNA template, 2.5 µL 10× PCR buffer, 3.3 µL 25 mM $MgCl_2$, 0.5 µL 10 mM dNTPs, 1.5 µL of each primer, and 5U Taq DNA polymerase. Thermocycling conditions were conducted with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 40 s at 58 °C, extension at 72 °C for 1 min, and a final extension for 10 min at 72 °C. Three µL of successful amplified PCR product was checked by electrophoresis with 1% agarose that contained ethidium bromide staining, and the results were visualised under UV illumination. Both strands of PCR fragments were sequenced to obtain a consensus sequence.

2.2. PCR Amplification and Sequencing

PCR products were purified with the Promega PCR Purification Kit following the manufacturer's protocol. Purified samples were then sent for sequencing at First Base Laboratories, Sri Kembangan, Selangor, Malaysia. To control the sequence accuracy and resolve any ambiguous bases, the same primer pairs were used in both directions for cycle sequencing using the ABI PRISM Dye-Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and electrophoresis (on an Applied Biosystems Automated Sequencer). Sequences were viewed and edited manually using Chromas version 1.45 [31], whereas contiguous sequence (contig) assembly and multiple sequence alignment were performed using ClustalW [32]. All of the sequences generated were deposited in the NCBI GenBank, together with the specimen voucher numbers, with the nomenclature suggested by Chakrabarty et al. (2013) (Table 1) [33]. Other published Pangasiid COI sequences from the GenBank database were also included in the analysis. The final alignment was

screened for stop codons and insertion-deletion mutations using the same software to ensure that there was no amplification of non-target fragments [34]. Additionally, the determinations of base compositional frequencies and nucleotide substitutions between pairwise comparisons were performed using MEGA 6.0 [35]. Aligned sequences were compared with existing data and submitted to GenBank and BOLD (Table 1). Specimen data such as images, collection information, museum accession numbers, and sequence trace files were assembled in BOLD.

Table 1. List of specimens used for barcoding analysis.

Species	Sampling Location	Country	Accession Number[@]	BOLD Systems		Specimen Voucher	Reference
				Sequence ID	BIN ID		
Helicophagus waandersii	Lubok Paku, Pahang	Malaysia	KP036415 ** KP036416 **	GBMIN93860-17 GBMIN128296-17	BOLD:AAE7042 BOLD:ACI8082	Hewa03LP Hewa07LP	This study
H. waandersii	Paloh Hinai, Pahang	Malaysia	KP036417 **	ANGBF43735-19	BOLD:ACI8082	Hewa24PH	This study
Pangasianodon hypophthalmus	Kuala Kangsar, Perak	Malaysia	KP036425 **	ANGBF43738-19	BOLD:AAE3237	Pahy0901KK	This study
P. hypophthalmus	Paloh Hinai, Pahang	Malaysia	KP036426 *	ANGBF43739-19	BOLD:AAE3237	Pahy60PH	This study
P. hypophthalmus	Laguna, Calabarzon	Philippines	HQ682713-16	NA	NA	Phyp1-Phyp4-LdB	[36]
P. hypophthalmus	NA	NA	NC021752	NA	NA	NA	[37]
P. hypophthalmus	Nakhon Ratchasima	Thailand	JF292393 JF292394 JF292395 JF292396 JF292397 JF292398 JF292399 JF292400 JF292401 JF292402 JF292403 JF292404 JF292405 JF292406 JF292407 JF292408 JF292409 JF292410	ANGBF8216-12 ANGBF8282-12 ANGBF8215-12 ANGBF8281-12 ANGBF8214-12 ANGBF8280-12 ANGBF8213-12 ANGBF8279-12 ANGBF8212-12 ANGBF43753-19 ANGBF43753-19 ANGBF43753-19 ANGBF43753-19 ANGBF43753-19 ANGBF8209-12 ANGBF43753-19 ANGBF43753-19 ANGBF43753-19	BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:ADW5681 BOLD:AAE3237 BOLD:ADW5681 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237 BOLD:AAE3237	AUPH1 AUPH2 AUPH3 AUPH4 AUPH5 AUPH7 AUPH8 AUPH10 AUPH11 AUPH12 AUPH13 AUPH14 AUPH15 AUPH16 AUPH17 AUPH18 AUPH19 AUPH20	[38]
P. hypophthalmus	NA	NA	EU752151	NA	NA	PANGHYPO-J01-009	[39]
P. hypophthalmus	An Giang	Vietnam	EF609427	NA	NA	BW-1778	[40]
Pangasianodon bocourti	Paloh Hinai, Pahang	Malaysia	KP036428 *	GBMIN118560-17	BOLD:AAB7484	Pabo55PH	This study
P. bocourti	NA	NA	EU752149	NA	NA	PANGBOCO-J01-003	[40]
P. bocourti	An Giang	Viet Nam	EF 609425	NA	NA	BW-1791	[40]
P. bocourti	Yasothon Province	Thailand	JF292411	ANGBF8207-12	BOLD:AAB7484	AUPH19	[38]
			JF292412 JF292413 JF292414 JF292415 JF292416 JF292417 JF292418 JF292419 JF292420 JF292421 JF292422	ANGBF8273-12 ANGBF8206-12 ANGBF8272-12 ANGBF8205-12 ANGBF8271-12 ANGBF8204-12 ANGBF8270-12 ANGBF8203-12 ANGBF8269-12 ANGBF8202-12 ANGBF8268-12	BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484 BOLD:AAB7484	AUPH20 AUPB2 AUPB4 AUPB5 AUPB6 AUPB7 AUPB9 AUPB10 AUPB11 AUPB12 AUPB13	

Table 1. *Cont.*

Species	Sampling Location	Country	Accession Number[@]	BOLD Systems		Specimen Voucher	Reference
				Sequence ID	BIN ID		
			JF292423	ANGBF8201-12	BOLD:AAB7484	AUPB14	
			JF292424	ANGBF8267-12	BOLD:AAB7484	AUPB15	
			JF292425	ANGBF8200-12	BOLD:AAB7484	AUPB16	
			JF292426	ANGBF8266-12	BOLD:AAB7484	AUPB17	
			JF292427	ANGBF8199-12	BOLD:AAB7484	AUPB18	
			JF292428	ANGBF8265-12	BOLD:AAB7484	AUPB19	
			JF292429	ANGBF8198-12	BOLD:AAB7484	AUPB20	
Pangasianodon conchophilus	Paloh Hinai, Pahang	Malaysia	KP036413 *	GBMIN128295-17	BOLD:AAE7042	Paco23PH	This study
P. conchophilus	Chenor, Pahang	Malaysia	KP036414 **	GBMIN118559-17	BOLD:AAE7042	Paco03CR	This study
P. conchophilus	NA	Vietnam	EF609426	NA	NA	BW-1796	[40]
Pangasianodon djambal	Paloh Hinai, Pahang	Malaysia	KP036427 *	GBMIN128301-17	BOLD:AAB7484	Padj53PH	This study
P. nasutus	Paloh Hinai, Pahang	Malaysia	KP036410 *	GBMIN123337-17	BOLD:AAE7042	Pana57PH	This study
			KP036411 *	GBMIN93860-17	BOLD:AAE7042	Pana61PH	
			KP036412 *	GBMIN123338-17	BOLD:AAE7042	Pana93PH	
P. nasutus	Pahang	Malaysia	JF781172	NA	NA	SLM-PN(PH)-01	[34]
			JF781173	NA	NA	SLM-PN(PH)-02	
			JF781174	NA	NA	SLM-PN(PH)-03	
			JF781175	NA	NA	SLM-PN(PH)-04	
Pangasianodon micronemus	Kuala Kangsar, Perak	Malaysia	KP036418 **	GBMIN123340-17	BOLD:AAU2068	Pami0801KK	This study
P. micronemus	Paloh Hinai, Pahang	Malaysia	KP036419 *	GBMIN128297-17	BOLD:AAU2068	Pami50PH	This study
			KP036420 *	GBMIN128298-17	BOLD:AAU2068	Pami51PH	
			KP036421 *	GBMIN128299-17	BOLD:AAU2068	Pami52PH	
			KP036422 *	GBMIN128300-17	BOLD:AAU2068	Pami59PH	
			KP036423 *	GBMIN123341-17	BOLD:AAU2068	Pami85PH	
P. micronemus	Kuala Kangsar, Perak	Malaysia	KP036424 **	GBMIN123342-17	BOLD:AAU2068	Pami0902KK	This study
P. micronemus	Pahang	Malaysia	HM156360	NA	NA	SLM-PM(PH)-01	[34]
			HM156361	NA	NA	SLM-PM(PH)-02	
			HM156362	NA	NA	SLM-PM(PH)-03	
			HM156363	NA	NA	SLM-PM(PH)-04	
			HM156364	NA	NA	SLM-PM(PH)-05	
H. macropterus	Hechuan, Chongqing	China	NC019592	NA	NA	NA	[41]
Clarias macrocephalus	Kasetsart University	Thailand	JF292337	ANGBF8244-12	BOLD:AAE8721	AUCM19	[38]
C. batrachus	Nakhon Ratchasima	Thailand	JF292297	ANGBF8264-12	BOLD:ACB6804	AUCB1	[38]
C. batrachus	Laguna, Calabarzon	Philippines	HQ682679	NA	NA	Cbat3-LdB	[36]

@GenSeq nomenclature from Chakrabarty et al. (2013) [33]: * genseq-4: collection-vouchered non-types; ** genseq-5: non-types that have photo vouchers but lack a specimen voucher; NA = not available.

2.3. Phylogenetic Analysis

A phylogenetic analysis was performed to illustrate the divergence and relationships among the taxa using the same software. Other Siluriformes sequences, *Hemibagrus macropterus* (family Bagridae), *Clarias batrachus,* and *C. macrocephalus* (family Clariidae), were chosen as outgroups to root the tree. The mean pairwise genetic distance matrix was calculated using the Kimura two-parameter (K2P) model with the pairwise deletion of

gaps [42]. A neighbour-joining tree was constructed while assuming uniform rates across the sites. The tree's robustness was assessed by bootstrapping analysis with 1000 replicates.

2.4. Automatic Barcoding Gap Discovery (ABGD)

The online version of the Automated Barcoding Gap Discovery (ABGD) web tool (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) [43] (accessed on 23 January 2023) was used to determine the barcode gap occurrence, and it partitioned the sequences into putative groups or species by first implementing the default parameters [38,39]. (Pmin = 0.001, Pmax = 0.1, Steps = 10, X (relative gap width) = 1.5, Nb bins = 20). Kimura 2-parameter (K2P) distances were used to correct the transition rate bias in the substitutions. The default for the minimum relative gap width was set to values between 0 and 1.2 [37].

3. Results

3.1. Genetic Variation

The analyses involved 75 nucleotide sequences of Pangasiids with four outgroup catfishes, which produced a final trimmed alignment of 620 base pairs. The alignment contains 492 (79.4%) conserved sites and 128 (20.7%) variable sites, of which 120 sites (19.4%) are parsimony informative. No indels or stop codons were observed, which suggests that there were no pseudogenes. The average base composition was 30.4% (T), 26.0% (C), 26.2% (A), and 17.2% (G). The estimated transition/transversion (si/sv) ratio, R, ranged from 0.10 to 18.25. The mean corrected pairwise distances (K2P) among the eight species of Pangasiids are shown in Table 2. Interspecific divergence between the species ranged from 3 to 19.7%, which is sufficiently sensitive to delineate species. A barcode gap is detected where the minimum interspecific divergence (3.0%) is lower than the maximum intraspecific divergence (5.1%).

Table 2. Mean corrected (K2P) genetic distance (pairwise intraspecific, *p* divergence) and interspecific divergence value, *d*, of Pangasiid cytochrome c oxidase I (COI) sequences from this study. The percentage of divergence is shown in parentheses.

Species	1	2	3	4	5	6	d
Pangasius nasutus	-						0.011
P. conchophilus	0.030 (3.0)	-					0.051
P. bocourti	0.120 (12.0)	0.111 (11.1)	-				0.012
P. djambal	0.065 (6.5)	0.083 (8.3)	0.071 (7.1)	-			NC
Pangasianodon hypophthalmus	0.157 (15.7)	0.144 (14.4)	0.117 (11.7)	0.163 (16.3)	-		0.014
Pseudolais micronemus	0.077 (7.7)	0.097 (9.7)	0.133 (13.3)	0.074 (7.4)	0.166 (16.6)	-	0.008
Helicophagus waandersii	0.113 (11.3)	0.129 (12.9)	0.168 (16.8)	0.097 (9.7)	0.197 (19.7)	0.090 (9.0)	0.001

NC = not calculated.

The lowest interspecific divergence value (3%) was found between *P. nasutus* and *P. conchophilus*, which indicates that there is a close genetic distance. However, the intraspecific value of 0.051 for *P. conchophilus* is higher than the interspecific pairwise value (0.030) between *P. conchophilus* and *P. nasutus*, which suggests a possible sign of species complexity or evidence of hybridisation following unclear species delineation for *P. conchophilus*. *P. djambal* and *P. bocourti* had the second -lowest divergence value (7.1%). Within *Pangasius*, the genus that has the most significant number of species in the family, the highest divergence value (11.1%) was observed between *P. bocourti* and *P. conchophilus*.

As expected, the highest pairwise divergence value (19.7% divergence) was found to be between the species of two genera, *H. waandersii* (*Helicophagus*) and *P. hypophthalmus* (*Pangasianodon*). Apart from *Pangasius*, other genera in the Pangasiidae family (*Pangasianodon*, *Pseudolais*, and *Helicophagus*) were represented by only a single species each and do not have any comparisons of COI sequences either from Genbank or from this work. For the interspe-

cific divergence, the lowest value was observed in *H. waandersii* (1.0% divergence), while the highest (5.1% divergence) was observed in *P. conchophilus* (Table 2).

3.2. Phylogenetic Analysis—Neighbour-Joining (NJ)

Relationships between Pangasiid COI sequences were reconstructed using the K2P model and shown in the NJ tree (Figure 2). The branches are monophyletic according to their respective genera (*Pangasius*, *Helicophagus*, *Pseudolais*, and *Pangasianodon*) relative to the outgroup. Using Siluriformes as outgroups, the value of 85% bootstrap separated the Pangasiidae family into five clades. Five significant clades (A–E) were identified based on high bootstrap values (>90%) of distinctive lineages. Clades A (*P. bocourti* and *P. djambal*) and B (*P. conchophilus* and *P. nasutus*) represent the genus *Pangasius*. In contrast, Clades C, D, and E identify the monotypic genera of *Helicophagus* (*H. waandersii*), *Pseudolais* (*P. micronemus*), and *Pangasianodon* (*P. hypophthalmus*), respectively. Although the five clades were well defined, their branching order was not resolved (polytomy) due to the low bootstrap values (<70%), which suggest simultaneous radiation, except for Clade E.

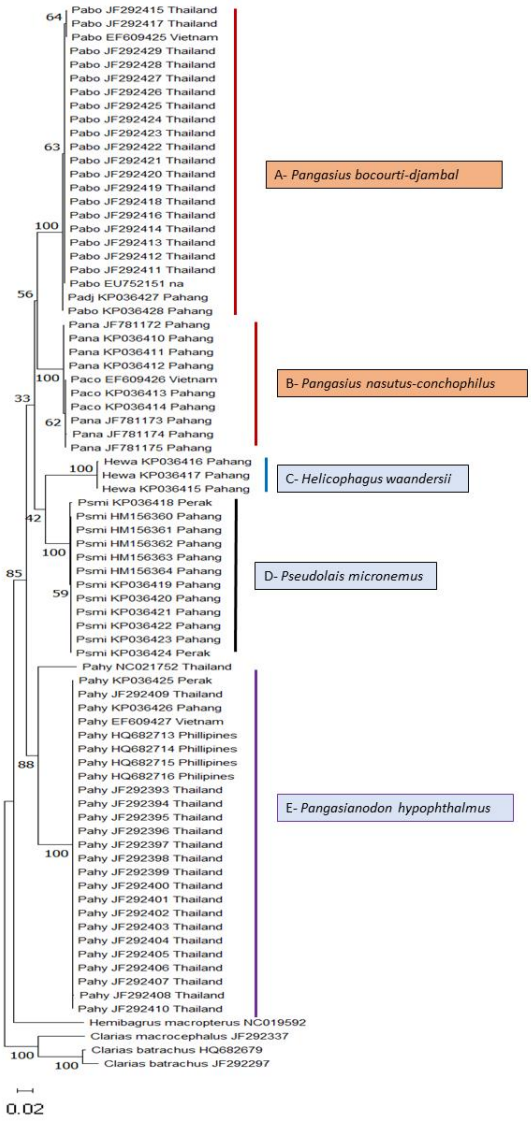


Figure 2. Neighbour-joining tree of Kimura-2-parameter distances for Peninsular Malaysian pangasiids combined with pangasiid COI sequences from GenBank and outgroups from the Siluriformes family. Bootstrap values are given near the branches. The Pangasius clades (A and B) are problematic groups. The scale below indicates the branch length.

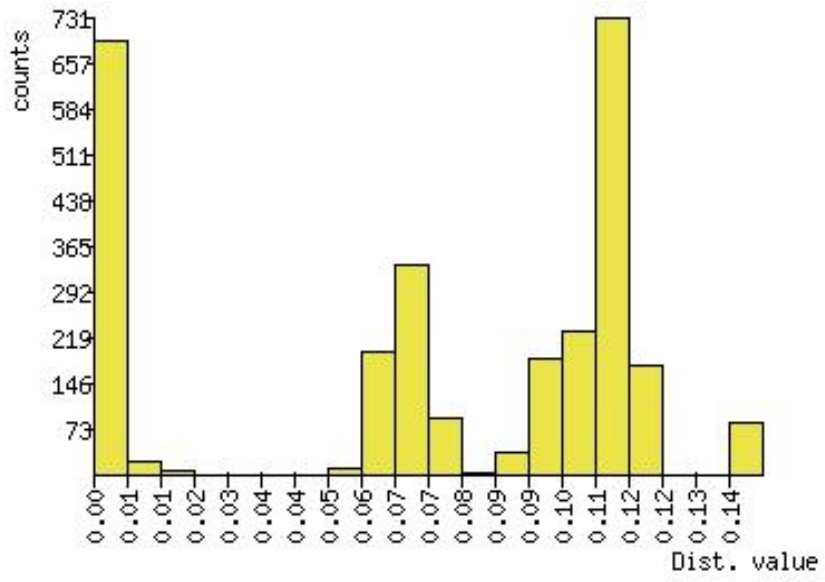
Clade E (*Pangasianodon*) is well distinguished from the other four clades with a high confidence level (88%). A medium bootstrap value (56%) separated the genus *Pangasius* (Clades A and B) from Clade C (*Helicophagus*) and Clade D (*Pseudolais*). The two clades within *Pangasius* were divided by a 56% bootstrap value and further differentiated into their respective clades by strong bootstrap confidence (100%) (Figure 2).

Within clade A, the sequences show some differences in the evolutionary distance from other sequences obtained from Genbank. *Pangasius bocourti* KP036428 is very close to *P. djambal* KP036427 (both derived from this study) but has a small divergence from different *P. bocourti* sequences, with a 63% bootstrap value. Other *P. bocourti* sequences are clustered together with 64% bootstrap confidence.

A similar result was also observed in Clade B, where our sequences of *P. conchophilus* (KP036413 and KP036414) matched with EF609426 from Vietnam [38]. In this clade, *P. nasutus* and *P. conchophilus* should have been separated into two groups with a bootstrap value of 100%. Clearly, *P. nasutus* sequences from this study (KP036410-12) showed a consistent grouping, together with one sequence (JF781172) from Song et al. (2013) [34]. However, the other three sequences of *P. nasutus* from their study (JF781173-75) were clustered into the *P. conchophilus* group. One of the possible explanations was most likely due to misidentification during sampling, either by a wrongly identified sample or samples taken from hybrid specimens.

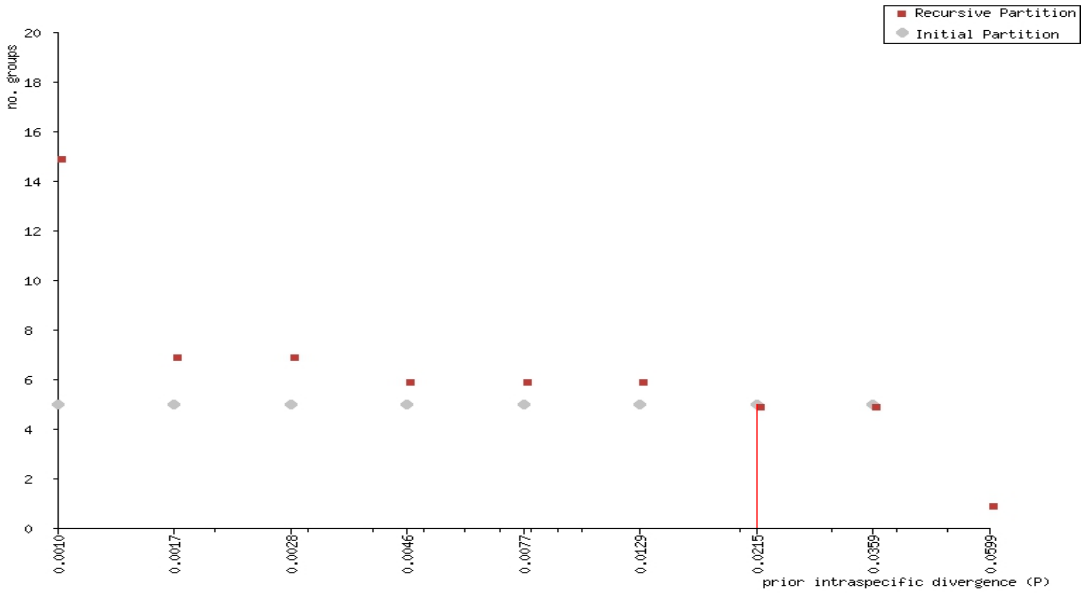
3.3. Automatic Barcoding Gap Discovery (ABGD)

The ABGD analysis indicates that by using the default standard settings, a barcode gap is detected between the intraspecific and interspecific distances. These gaps are found when the divergence among organisms from the same species (intraspecific) is smaller than the divergence between species (interspecific) [43]. In this analysis, the sequences were partitioned in an initial approach into a very stable five species, as shown in Figure 3b. In the recursive method, two additional species can be recognised. This finding is congruent with the primary species concept; the threshold value (p = 0.021544), which defines the species boundary of pangasiid COI sequences analysed in this study, followed the observations on the Indian Mahseers [44] and the Narmada River fishes [45].



(**a**)

Figure 3. *Cont.*



(**b**)

Figure 3. Barcode gap analysis of pangasiid species generated by Automatic Barcode Discovery Gap Discovery. Distributions of K2P distances and between each pair of specimens for the COI gene (**a**) histogram of the distance and (**b**) number of species obtained for each prior intraspecific divergence. The red line is the threshold value, $p = 0.0215$, above line shows stable five species, while below are false positives.

The value above, which partitioned the sequences into five groups, produced the same groupings as in the phylogenetic tree. The values below the threshold are treated as false positives because real species would split into two or more partitions. No barcode gap is shown in the values above the threshold, which are thus treated as false negatives. Conversely, the ABGD method, which is based on pairwise distances, clearly defined the groupings of sequences to be congruent with the pairwise intraspecific divergence in Table 2, which could not be differentiated into distinctive clusters according to species using the phylogenetic concept.

4. Discussion

4.1. Species Relationship and Taxonomic Accounts

The reconstructed neighbour-joining tree demonstrates the relationships between the native and introduced pangasiid species. The clades were monophyletic according to their genera, except for *Pangasius*. In this analysis, *Pangasianodon* is separated as the basal lineage, which is similar to the observations of Karinthanyakit & Jondeung (2012) [46]. The difference in the swim bladder chamber (a single chamber vs two to four in other Pangasiids) and pelvic fin rays (eight to nine pelvic fin rays vs six in other genera) might have contributed to this character [46]. Sharing six pelvic fin rays, the genus *Helicophagus* is characterised as having a combination of a slender anterior snout (<16.5% HL) and a predorsal length of 34.5–40.5% SL. While *Pseudolais* can be differentiated from other genera by its large eye diameter, minute maxillary barbel, and minute adipose fin, *Helicophagus* and *Pseudolais*, are defined as sister groups. The final genus, *Pangasius*, can be differentiated by these characters: relatively long maxillary barbel, robust dorsal and pectoral fins, as well as a robust adipose fin [6].

In this study, COI showed the ability to separate the four genera, according to their taxonomic accounts but could not resolve the relationship between species within the genus *Pangasius*. Even with many more genetic markers in previous studies [9,46,47], no clear relationship can be defined. In the present study, the COI analysis shows the clustering of *P. bocourti* and *P. djambal* in a common group, although both have a 7.1%

genetic distance. In fact, they are two allopatric species that have a very close morphological appearance [3,48], and therefore two taxonomic keys were created to distinguish *P. bocourti* (described from the Asian mainland) from *P. djambal* (a species from the Indo-Malayan Archipelago) [3]. *Pangasius bocourti* is widely cultivated in the Mekong Delta, mostly in southern Vietnam [49,50]. The taxonomy of this species is still problematic, and the species currently described as *P. bocourti* could be an undescribed species [51]. However, from the sequences analysed in this study, there is no evidence for this confusion.

The morphologically closest species to *P. bocourti*, *P. djambal*, has the same features of palatal dentition but with a wider median vomerine tooth plate and a larger palatine juxtaposed to it. Additionally, the head shapes are indistinguishable. The two species differed from each other only by the higher number of gills raker counts in *P. bocourti* (36–46 vs. 24–35 [3], 35–47 vs. 27–39 [8]). Roberts & Vidthayanon (1991) [3] observed some well-marked colour patterns on the fins, but these were not found in this study. Some of the distinguishing characteristics may only develop upon maturation (such as the gill raker counts and dentition characters). Therefore, it is challenging to differentiate the fish at a younger age. Moreover, it is somewhat difficult to distinguish between these two species only by using the partial COI gene; other characters that are expressed only by proteins must be incorporated if the DNA method is to be used. The NJ tree also revealed another allopatric species, *P. nasutus* and *P. conchophilus*, to be in the same clade, discriminated by a genetic divergence of 3%, which is a threshold value for species delimitation [52,53]. These two species differ morphologically by only two main distinguishing features; the more pointed snout (in large adults of *P. nasutus*) and the larger eye diameter (only observed in specimens of more than 300 mm of *P. conchophilus*).

In ABGD analysis, a barcode gap is detected between the intraspecific and interspecific distances. A barcode gap is the difference between the maximum intraspecific and minimum interspecific distances, which is well defined in this analysis [43]. Five stable species were observed and partitioned initially, and by using a recursive approach, another two additional species could be recognised. However, this approach requires confirmation by integration with other methods, including initial morphological identification, additional genetic loci, and specimens [43,45]. The ABGD method is solely based on pairwise genetic differences; it does not rely on the genealogical tree or the properties of the internal nodes. As a result, it works well on speciation radiations, bifurcating events, or both speciations mixed [43].

However, for recently diverged speciation, a barcoding gap might not be present [54], and it is not possible to use genetic data inference [43]. This hindrance could be attributed to the unclear separation in the *Pangasius* genus, which diverged more recently (in the late Pleistocene period—3.99 million years before present, MBp) compared to *Helicophagus* and *Pseudolais* (4.26 MBp) and *Pangasianodon* (late Miocene, 6.75 MBp) [46]. *P. nasutus* and *P. conchophilus* diverged as recently as 1.74 MBp in the mid-Pleistocene period. There is no report on the divergence of *P. bocourti* and *P. djambal* when calibrated using the *Pseudotropius* basis [46]. However, a similar level of divergence was predicted to occur in the late Pleistocene (0.3–1.0 MBp) [55], at nearly the same time as another allopatric species in the Pangasiid family (*H. leptorhynchus* vs. *H. typus*—0.29 MBp) [46].

4.2. Pangasiid Species Diversity and Impacts on the Current Aquaculture Practices

From the results, three species known as native (*H. waandersii*, *P. nasutus,* and *P. micronemus*) are potentially threatened by the current aquaculture practises, with introduction of new pangasiid species from Indochina and Thailand, namely *P. conchophilus*, *P. hypophthalmus,* and *P. bocourti* [1]. DNA barcoding revealed their close identity through phylogenetic relationships and further genetic analyses (ABGD method and K2P genetic distance). These genetic analyses have facilitated the identification of morphologically close pangasiid species, thus evaluating their impact on conservation strategies. Many factors can affect the survival of the natives; among them is the genetic impact of the introduced species [56]. As shown in Figure 2, *P. conchophilus*, shows a close genetic relationship with the native *P. nasutus* due to its position

in the same group (Clade B). Close genetic distance (3% divergence) indicates that they are two different species but very closely related [43]. These two morphologically similar species were once known as one until Roberts & Vidthayanon (1991) [3] revealed *P. conchophilus* as a new, distinct species. Due to the close morphological characters, in the field, they can be easily misidentified, like the samples of *P. nasutus* JF781173–75 from Song et al. (2013) [34] which clustered with *P. conchophilus*. One possible explanation is misidentification during sampling by a wrongly identified sample or samples taken from hybrid specimens. In this case, further confirmation must include other markers, such as the nuclear gene, since COI as a maternally inherited marker could not detect hybrids.

Pangasius conchophilus was introduced in the 1990s by immigrants from Cambodia [52]. The reason for the introduction was mainly to fulfil the high demand for the native *P. nasutus.* The close morphological appearance could create confusion the non-expert, which would make it marketable as *P. nasutus*. They are cultured by Cambodian immigrants near Pekan, Pahang (near location 1 in Figure 1), in the lower reaches of the basin. Ironically, one specimen in this analysis (KP036414) was collected from Chenor, Pahang, which is located in the middle stretch of the river (location 3—Figure 1). Local fishermen reported that they often found both species together in their nets, which meant that they are now occupying the same habitat and living in harmony. Their close genetic and morphological relationship might produce natural crossbreeds or hybrids soon [57].

Competition for habitat (space) and food is one of the factors that affect biodiversity [58,59]. The Pahang River is home to many freshwater fish species; among those that are popularly known are the Jullien's River Carp, *Probarbus jullienni*, and the Pangasiids; many of them share a preference for a particular feeding item, the moluscivoruos bivalve, *Corbicula* sp. This unique preference for molluscs, which was not observed in other *Pangasius* spp., derived the name *P. conchophilus* (etymology: concho = mollusc, philus/philic = like) [3], which is likely to harm or compete with the local fish, not only the pangasiids.

Pangasius bocourti is a commonly cultured pangasiid species. However, there is no record of the introduction of this species into Malaysia. It is thought that aquaculturists imported the fry from Thailand in bulk and misidentified the species as *P. nasutus* (a species that has high local demand). It is a natural practise to culture pangasiids without knowing what species they are, knowing that they look similar to each other. The close morphological appearance to *P. djambal* caused the locals to believe that this species was a native species, even though both were not recorded previously [3,18]. *Pangasianodon hypophthalmus*, a popular worldwide cultural species, was introduced in the 1980s by the Department of Fisheries, Malaysia. Ever since successful induced breeding, the culture of this species has gained popularity among farmers because of its ideal cultural characteristics. The species has a fast growth rate and can breed artificially with low culture maintenance (in floating cages) [60]. It can be cultured using many different practises, including pond cultures and, more specifically, floating cages along the Pahang River basin.

In many Asian countries, where the emphasis is more on aquaculture development [59] to increase production, the impacts of introduced species on biodiversity are rarely evaluated [61]. There are many examples of the effects of introduced species on biodiversity conservation: through predation, genetic interactions (hybridisation, introgression, and other indirect genetic effects), habitat use and modification, and the transmission of a novel disease [58,61,62]. Chong et al. (2010) [21] considered that the establishment of non-native *P. hypophthalmus* in Malaysia is not as invasive as Tilapia (*Oreochromis* spp.). However, there are reports in many other countries that show that such establishments in the wild have led to harmful ecological impacts [63–65]. Singh & Lakra (2012) [15] discussed many possible impacts due to the introduction of *P. hypophthalmus* into India. Escapees from nearby cultured ponds and hatchery sites were detected in the open waters of West Bengal, Andhra Pradesh (Kolleru Lake), Kerala, and Uttar Pradesh. These escapees could hybridise in the wild and, thus, could be a concern in the future [59,61,65]. Likewise, Więcaszek et al. (2009) [16] found that hybrid refugees of *Pangasius* are commonly found in Polish waters and that it is also difficult to obtain pure species in the aquarium trade.

Fish health and management are also considered issues that affect *Pangasius* introduction. There are reports on the risk of disease and parasites associated with catfish culture [66]. The disease, bacillary necrosis of *Pangasius*, is caused by *Edwardsiella ictaluri* (a bacteria native to North America, from ictalurid catfish), and was identified in farmed *P. hypophthalmus* cultures in the Mekong River [64]. Another disease that is related to the Pangasiid culture is aeromonad septicaemia, which is caused by *Aeromonas hydrophila* [67]. Occurrences of pangasiid disease were reported in Bangladesh [65] and New Zealand [68]. Various ecto- and endo-parasites have also been found [68], including *Ichthyophthirius*, *Myxobolus* spp., and *Trichodina* sp., among others. Siti-Zahrah et al. (2014) reported the occurrence of a viral disease in farmed cages in Malaysia caused by the channel catfish virus (CCV). This virus could have also spread to the native species, considering that the commercial pangasiids are now well established in the river.

5. Conclusions

Understanding the genetic status of species with ambiguous taxonomy is critical, and there is a sense of urgency to better control the emergence of exotic species. This study shows that the barcoding gene can identify the pangasiid catfish species found in Malaysia and can detect the genetic variation between taxonomically problematic groups. Potentially invasive species that could threaten the survival of native species have also been determined. The results are beneficial fior developing guidelines on sustainable fisheries and aquaculture practises. Further work should focus on understanding the status of the genetic diversity of the established pangasiid population by assessing the potential impacts on the native population, either ecologically or sociologically. Efforts should be concentrated on the reduction of the adverse effects caused by the introduced species by practising sustainable aquaculture and fishing. Management decisions for the protection and conservation of native species may be facilitated by the findings of this study.

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