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Population Structure of Swamp Eel *Monopterus albus* in East Coast of Peninsular Malaysia Inferred from 16S Mitochondrial DNA

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Abstract: Swamp eel *Monopterus albus* is a highly used commercial and medicinal food fish especially in China, Malaysia and other East and Southeast Asian countries. The population of this vital species is declining due to overfishing and pollution. There is a need for molecular genetics study that could aid management, conservation and sustainable exploitation of this species. To investigate the population structure of swamp eel *Monopterus albus* in East Coast of Peninsular Malaysia (ECPM), a total of sixty-one samples were collected from three states (Kelantan, Terengganu and Pahang) at six different sampling sites for mitochondrial DNA analysis. Four haplotypes were detected among all the samples. Haplotype 1 was the most widespread haplotype among the six sampling sites, comprising about 75.4% of all samples. Both the non-significant values of Tajima's *D* and Fu's *Fs* suggested that all populations were at genetic equilibrium. Analysis of Molecular Variance (AMOVA) revealed a significant differences among sampling sites (F_{ST}= 0.92401, $P < 0.05$). In pairwise comparisons of F_{ST}, the Kuala Terengganu population showed significant values between all populations which suggested that it is genetically different. On the other side, Pasir Puteh, Tanah Merah, Rantau Panjang, Kampung Raja and Pekan populations considered as closely related populations.

Key words: Conservation · Mitochondrial DNA · *Monopterus albus* · Population structure · Seed production

INTRODUCTION

The swamp eel*Monopterusalbus* is widely distributed in East and Southeast Asia [1, 2]. It can be found in rice fields, ponds, marshes and rivers [3]. It is a highly commercial food fish and medicine especially in China and Malaysia [4, 5]. The total production of wild stock of swamp eel in Malaysia within public water bodies (rivers, ex-mining pools, dams, lakes and others) from 2010 to 2013 (4 years) was estimated at 187.6 tonnes with a retail value of about RM2.3 million; excluding those from the hatchery and commercial output. The East Coast of Peninsular Malaysia (ECPM) is the second largest producing region of swamp eel in Malaysia. The ECPM consists of many paddy fields that favoured people in the region to engage in farming of this fish species. The swamp eel is characterised by long elongated snake like body, lacking scales and fins with an ability to breathe air via buccal mucosa, usually covered with mucus and possessed a special feature of hemaphrodism [6]. Department of Fisheries Malaysia reported a significant reduction of swamp eel production from wild, which might result due to overfishing [7, 8]. Insufficient data available for hatchery and commercial production is evidence that fishermen depends highly on wild stock. Therefore, development of mass seed production technology and aquaculture of swamp eel is highly needed, not only to increase the yield for satisfying the high demand of consumers, but also to conserve the wild swamp eel resource and increasing revenue generation. Broodstock selection is one of the important aspects in broodstock management, which is also an essential step in seed production for aquaculture [9]. A better understanding of swamp eel population structure will aid the broodstock selection. However, there is insufficient information about the population structure of swamp eel in ECPM and even Malaysia.

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Using genetic markers allows the accurate dissection of a population structure, which is often unaccomplished with the application of morphological markers alone [10]. A recent progress in molecular biology, principally in the development of the polymerase chain reaction (PCR) for amplifying DNA, sequencing and data analysis, have yielded a strong technique that have been continuously applied for screening, characterization, identification of broodstock and evaluation of genetic diversity of different species [11, 12]. As a result, a good understanding of the fish population structure and genetic variation would help more efficient and sustainable fisheries management [11, 12]. Mitochondrial DNA (mtDNA) is a powerful marker due to its maternal inheritance, lack of recombination, high mutation rate that make it effective for detecting recent population isolation and for providing genealogical relationships among populations within species [13].

The goal of this study was to determine the population structure and genetic diversity of the swamp eel in ECPM using partial sequences of the 16S rRNA of mtDNA gene analysis. Each sampling site is considered as population in the current study.

MATERIALS AND METHODS

Sampling: A total of sixty-one samples of the swamp eel were collected at six sampling sites in ECPM, which consist of Kelantan, Terengganu and Pahang States from January to April, 2015 (Fig. 1; Table 1). Approximately 1 cm of tail tissue was removed with scissors and preserved in a sterile 15 ml blue cap tube containing 95% ethanol.

DNA Extraction, Amplification and Sequencing: Total genomic DNA was isolated from 30 g of dried tail tissue using the Genomic DNA Tissue Mini Kit (Genaid). The partial 16S rRNA gene of mtDNA was amplified by PCR using the universal primers L1567 (5'-AAG GGG AGG CAA GTC GTA-3') [3] and H2196 (5'-GTC TGA GCT TTA ACG CTT TCT-3') [14]. PCR was carried out in a 10 µl reaction volume containing 5.15 µl steriledistilled H_2O, 1 µl buffer (TaKaRa), 0.8 µl dNTP Mix (2.5 mM), 1 µl of each primer (10 µM), 0.05 µl of 5 unit/µl Taq DNApolymerase (Ex-Taq; TaKaRa) and 1 µl template (50 ng/µl) on athermal cycler (GeneAmp PCR System 9902, Applied Biosystems), under the following thermal cycling conditions: predenaturation at 96°C for 4 min; 35 cycles of denaturation at 94°C for10 s, annealing at 50°C for 10 s and elongation at 72°C for30 s; followed by a final extension for 7 min at 72°C. Sequencing was succeeded using BigDye Terminator v3.1 cycle sequencing kit sequencing reaction (Applied Biosystems) by following the manufacturer's instructions, performed on an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems).

Data Analysis: The sequences were aligned and edited using GENETYX v9.1.3 multiple sequence alignment programs.The nucleotide composition and number of variable sites were determined using DnaSP v5 [15]. Genetic diversity in each population was measured as haplotypic diversity [16] and nucleotide diversity [17]. Population structure and genetic variation were analyzed using ARLEQUIN v3.5.1.2 (CMPG, University of Berne; [18]. The level of genetic population differentiation was tested using analysis of molecular variance (AMOVA)

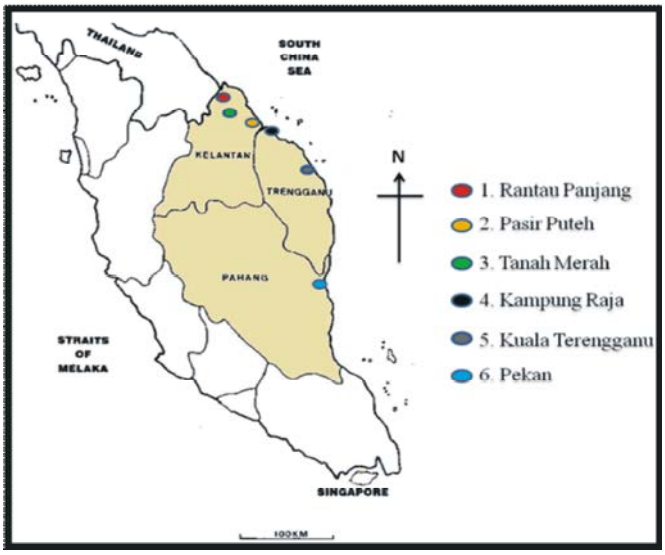

Fig. 1: Location map of swamp eel *Monopterusalbus* from six sampling sites in East Coast of Peninsular Malaysia (ECPM)

Table 1: List of sample sizes and sample abbreviations for mtDNA analysis according to the sampling sites with geographical location from East Coast of Peninsular Malaysia

No.	Sampling sites	Longitude/latitude	Abbreviation	Sample size
1	Pasir Puteh, Kelantan	5 50' 0"N, 102 24' 0"E	KEN	10
2	Rantau Panjang, Kelantan	6 1' 0"N, 101 59' 0"E	RPK	10
3	Tanah Merah, Kelantan	5 48' 0"N, 102 9' 0"E	TMK	10
4	Kampung Raja, Terengganu	5 48' 0"N, 102 35' 0"E	BET	11
5	Kuala Terengganu, Terengganu	5 20' 0"N, 103 8' 0"E	KTT	10
6	Pekan, Pahang	3 37' 0"N, 103 27' 0"E	PEK	10
			Total	61

as implemented in ARLEQUIN v3.5.1.2 [18], using the genetic distance matrix to estimate the components of variance that are attributable to differences among sampling sites and within sampling sites. Genetic differentiation between populations was tested by pairwise comparison F_{ST} with Slatekin's and Reynold's distances with 1, 000 permutations as implemented in ARLEQUIN v3.5.1.2 [18]. 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.

Two different neutrality tests were examined to for the combined DNA sequence alignment:Tajima's*D* [19] and Fu's *Fs* [20] tests, as implemented in ARLEQUIN v3.5.1.2 [18]. 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.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [21]. 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 swamp eel haplotypes [21]. All positions with gaps and missing data were deleted from the dataset. The sequence of *Synbranchusmarmoratus* was included in the construction of phylogenetic tree as an out-group.

RESULTS

Genetic Diversity and Population Structure: A total of 624 bp fragments from the 16S rRNA of mtDNA genes were successfully sequenced and aligned for 61 samples from six sampling sites. Among the 624 sites, 618 were invariable (monomorphic) sites and 6 were variable sites. All variable sites were parsimony informative sites with no singleton variable site (Table 2). The A/T base contents were significantly higher than the C/G contents (Mean: A=36. 85%, T=21. 29%, C=24. 06% and G=17. 80%). The 16S rRNA gene sequences of the present study showed higher transition frequency with no transverse frequency, insertion or deletion. The sequences from Rantau Panjang and Tanah Merah have single mutation sites. No variable sites were detected in the Pasir Puteh, Kampung Raja, Kuala Terengganu and Pekan sampling sites. Kuala Terengganu population was observed to contain many mutation sites compared with the other sampling sites.

Among all the samples, four haplotypes were detected. Haplotype 1 (Hap-1) is shared among the total population except for Kuala Terengganu population. This common haplotype represented 75.4% of the total samples. Three distinct Hap-2, Hap-3 and Hap-4 represents 2 samples of Rantau Panjang, 3 samples of Tanah Merah and entire samples of Kuala Terengganu respectively (Table 3).

The genetic diversity of all populations' sequences is shown in Table 4. Tanah Merah has the highest haplotype diversity (0.4667) and nucleotide diversity (0.00075), followed by Rantau Panjang with (0.3556) and (0.00046) respectively. The zero values for haplotype and nucleotide diversity in Pasir Puteh, Kampung Raja, Kuala Terengganu and Pekan occurred from lack of variable sites detected in their sequences.Neutrality tests of Tajima's, *D* and Fu's *F*s shows no significant deviation of neutrality in all population (P<0.05).

AMOVA produces an estimate of variance component of haplotype diversity at different levels of hierarchical divisions. Results of AMOVA show a significant difference among the populations (92.40%) of variance attributed to F_{ST}= 0.92401 ($P<0.05$). It is indicated that there is more variation among population than within the population (Table 5). The mtDNA data in this study revealed a strong pattern of population subdivision among population at this level (Table 5). The pairwise comparison F_{ST} value of population differentiation showed significant difference on Kuala Terengganu population *(P<0.05)* in Table 6.

Table 2: Sequence variations of four haplotype in 16S rRNA mtDNA of 61 samples and the numbers represent the nucleotide position

	Nucleotide position					
Haplotype number	118	179	295	524	589	590
Hap-1	A	T	C	G	G	A
Hap-2	.	.	T	.	.	.
Hap-3	.	.	.	.	.	G
Hap-4	G	C	.	A	A	G

Dot (.) represents the identical nucleotide with the Hap-1

Table 3: Number of fish from six sampling sites of swamp eel according to the haplotype distribution in ECPM

Haplotype	Pekan	Pasir Puteh	Rantau Panjang	Tanah Merah	Kampung Raja	Kuala Terengganu	Sample % in haplotype
Hap-1	10	10	8	7	11	-	75.4%
Hap-2*	-	-	2	-	-	-	3.2%
Hap-3*	-	-	-	3	-	-	4.9%
Hap-4*	-	-	-	-	-	10	16.4%

*Distinct haplotype

Table 4: Nucleotide sequence data of six sampling sites based on partial fragments of the mtDNA 16S rRNA region haplotype diversity, nucleotide diversity (mean ± SD) and Neutrality test

Sampling site	Sample size	Number of polymorphic sites	Number of haplotype	Nucleotide diversity	Haplotype diversity	Tajima's *D*	Tajima's *D p-value*	*Fu's* FS	Fu's *p*-value
Rantau Panjang	10	1	2	0.0006	0.3556	0.015	0.750	0.417	0.381
Tanah Merah	10	1	2	0.0008	0.4667	0.819	0.872	0.818	0.511
Pasir Puteh	10	0	1	0.0000	0.0000	0.000	1.000	0.000	NA
Kampung Raja	11	0	1	0.0000	0.0000	0.000	1.000	0.000	NA
Kuala Terengganu	10	0	1	0.0000	0.0000	0.000	1.000	0.000	NA
Pekan	10	0	1	0.0000	0.0000	0.000	1.000	0.000	NA

No significant Tajima's *D P*-value and Fu's *Fs P-value* observed in the neutrality test, showing that all sampling sites were in genetic equilibrium. NA = Not applicable.

Table 5: Analysis of molecular variance (AMOVA) of 16S mtDNA nucleotide data of six sampling sites in East Coast of Peninsular Malaysia

Source of variations	df	Sum of squares	Variance components	Percentage of variation	F_{ST} value
Among sampling sites	5	41.907	0.81799	92.40	0.92401*
Within sampling sites	55	3.700	0.67270	7.60	
Total	60	45.607	0.88527		

* Significant level *(P<0.05)*

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

	Pasir Puteh	Rantau Panjang	Tanah Merah	Kampung Raja	Kuala Terengganu	Pekan
Pasir Puteh	-					
Rantau Panjang	0.11111	-				
Tanah Merah	0.22222	0.17778	-			
Kampung Raja	0.00000	0.12438	0.23762	-		
Kuala Terengganu	1.00000*	0.96548*	0.95035*	1.00000*	-	
Pekan	0.00000	0.11111	0.22222	0.00000	1.00000*	-

* Significant level *(p<0.05)*with the sequential Bonferroni correction

Phylogenetic Relationships: The neighbor-joining tree revealed that Hap-1 of swamp eel is widespread in ECPM (Fig. 2). Hap-1, loosely supported by a bootstrap value less than 50%, was the major haplotype, which contained most specimens in all sampling areas except Kuala Terengganu. In contrast, Hap-2 and Hap-3 were weakly supported by a bootstrap value of 67% and 52% respectively a nd were restricted exclusively to Rantau Panjang and Tanah Merah respectively. Lastly, Hap-4 was strongly supported by a bootstrap value of 97% and was exclusively restricted to the Kuala Terengganu. The neighbor-joining tree showed all haplotypes clustered into a single clade.

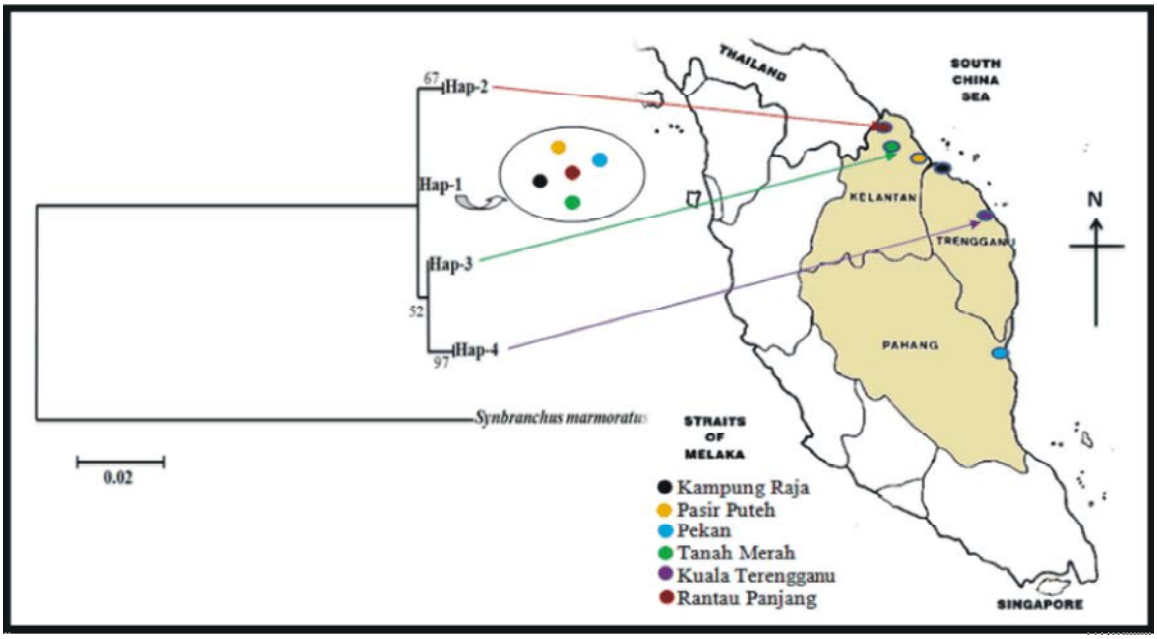

Fig. 2: Left Neighbor-joining tree showing phylogenetic relationships among four haplotypes of swamp eel samples inferred from partial DNA sequences of the mitochondrial 16S rRNA gene. A distance matrix was calculated using the Kimura's 2-parameter model in MEGA v6. The data set bootstrapped 1000 times and the appropriate bootstrap values were placed on each branch. *Right*: Distribution ranges of the four haplotypes of swamp eel and sampling sites

DISCUSSIONS

Genetic Diversity and Population Structure: According to Miah *et al*. [22], genetic diversity is an excellent tool for assessing biological qualities of an organism. In the current research, the genetic diversity was expressed based on haplotype and nucleotide diversity. Hap-1 was considered as the most common and widespread haplotype among the populations in ECPM (Table 3). The wide distribution of this common haplotype may be due to the geographical nature ECPM with no geographical boundaries and regular flooding that happened around the states every year [23]. The three distinct haplotypes could be used as a DNA markers/ indicator to identify the native population at their respective sampling locations.

The relationship between levels of population size and genetic diversity was observed in this study. Lower levels of nucleotide diversity and haplotype diversity might coincide with a small number of samples and sampling sites used in the present study. Contrarily, genetic studies involving swamp eel populations from other studies have revealed comparable high levels of genetic variation using 16S rRNA markers [3, 24]. Similarly, the low level of genetic diversity is also consistents with the study of Cai *et al*. [25] and Sun *et al*. [26], which suggested low genetic heterogeneity within and among the populations. Miah *et al*. [22] described a high level of genetic divergence within population as essential in dealing with environmental changes such as fluctuations in water temperature and epidemics. It is further explained that to ensure adaptation, expansion and reestablishment in natural populations, maintaining genetic variation in species is very essential [22]. Despite the few numbers of haplotypes derived from 16S rRNA in the present study, it is enough to provide the phylogenetic history of swamp eel in ECPM.

Avise [13] describes the populations that possess a low level of genetic diversity could have recently experienced a severe or prolonged demographic bottleneck. The low levels of genetic variation within swamp eelpopulations also suggest that they could be recovered from catastrophic or stochastic events during their recent history. Meanwhile, climatic change and habitat loss may also contribute to reductions in genetic variability of the populations [27]. Chan *et al*. [27] found that during the Holocene, rodent species lost genetic variability caused by major habitat changes and climatic changes. These phenomenons may also reduce the species population size and range [27, 28]. Avise [29]

explanation of 'a single female origin' due to the maternally inherited feature of mtDNA might be a reason for the total absence of genetic diversity in all populations except Rantau Panjang and Tanah Merah (Table 4). Besides, the samples were collected from the wild (rice fields, canals and swamps) and chemical pollution or overfishing could also be the factors responsible for the selective disappearance of most favourable haplotypes in the populations [7-8, 23]. Neutrality tests (Table 4) revealed that all populations are in genetic equilibrium [30]. Ha *et al*. [11] explained that lack of genetic introgression from exotic populations and demographic contraction or expansion leads to equilibrium of gene frequencies.

Li *et al*. [31] described both gene differentiation and gene flow as an indispensable index of evaluating the population genetic structure of a species. The AMOVA results in the current study revealed a strong pattern of population subdivision among sampling site (Table 5). Geographic factors played a vital role in defining the patterns of genetic structure of a given population. Analyzing how environmental variables affect population isolation and demographics is critical in understanding the processes producing genetic variation in the wild [32]. The effects of landscape composition on population connectivity can have important consequences for the maintenance of genetic diversity, local adaptation and population persistence, especially in geographically complex regions like hilly or mountainous areas where ecological variables can vary widely over short distances and where there are many potential physical barriers [33]. Numerous studies have documented genetic isolation resulting from geographic distance including straight-line distances that account for landscape differences [34]. Previous research also shown that, slopes contribute to genetic isolation in closely related species [35]. However, there is no presence of geographical barriers around sampling sites in the current study. Thus, genetic subdivision among sampling site might result from the effects of overfishing and flooding that may cause the selective disappearance of many haplotypes in ECPM.

A study by Song *et al*. [36] shows that organism acquires different genetic structures due to physical separation of an inhabited geographical range. All populations shared common geographical factors with a possible migration movement of fishes from a larger water body (such as Lake Kenyir) towards the Kuala Terengganu basin and river estuary especially the flooding period during monsoon season in the current research. Therefore, it is difficult at this point of time to provide clear geographical evidence that make Kuala Terengganu population genetically different from the other populations. Although, phylogenetic analysis in Fig.2 confirmed that Hap-1 (common haplotype) is closer to the ancestors with Hap-4 (Kuala Terengganu) a little bit far away from the ancestors.In fact, to justify Kuala Terengganu population is genetically different in East Coast of Peninsular Malaysia; sampling around closely areas of Kuala Terengganu (such as Kuala Berang and Marang) should be considered in the future study to further analyze the population structure of swamp eel in Terengganu state.

Geographical distance used for population isolation was also a poor explanatory variable to explain Pekan population that is far away population if related to other sampling sites but yet; retain the same genetic relationship with the rest of the populations of Kampung Raja and Kelantan sampling sites. Flooding could be a possible homogenizing factor making these sampling sites closely related with Pekan sampling site. Therefore, present study was successfully provided preliminary information on genetic relatedness of swamp eel in the three states at East Coast of Peninsular Malaysia.

The swamp eels were reported to have the ability to move at considerable distances over dry land to reach water sources and to migrate in flood-plain areas [1]. This phenomenon could act as a homogenizing agent of different gene pools whenever migration is possible. It is consistent with the study of Jamsari *et al*. [23] study on climbing perch suggesting that translocation might be common because, swamp eel are economically sound and reportedly cultured across East Coast of Peninsular Malaysia; resulted in a single genetic structural pattern between Rantau Panjang, Tanah Merah, Pasir Puteh, Kampung Raja and Pekan. Genetic identity was higher between populations in closest geographic proximity regardless of whether or not they occurred on the same river system [37]. Another possibility is that translocation between two closely populations of Kuala Terengganu and Kampung Raja had not occurred based on genetic distance between them.

Phylogenetic Relationship: The phylogenetic analyses of sequences of 16S rRNA of mtDNA using neighbor-joining method with Tamura–Nei distances revealed that, samples of swamp eel collected from six sampling sites in ECPM are clustered into a single clade distributed across the sampling sites (Fig. 2). The distribution of each sampling sites showed the presence of apparent geographic structuring between haplotypes in this clade.

It also revealed that Hap-1 is closer to the ancestors with Hap-4 as far distance. Single clade appearance in this study suggested that all samples might originate from the same single ancestor in maternal lineage.

In this study, it is concluded that swamp eels collected from ECPM was genetically identified as single species consist of two populations. The Kuala Terengganu population which is genetically different and Pasir Puteh, Tanah Merah, Rantau Panjang, Kampung Raja and Pekan as closely related populations. However, low genetic diversity was observed both within and between sampling sites in this study.

The current study emphasized the small sample size and few sampling sites that were used to represent entire East Coast of Peninsular Malaysia may restrict the analyses to provide conclusive results. However, the data suggested that the detection of population divergence is possible with a minimum sample size of 10 individuals per sampling site using 16S rRNA mtDNA marker.

Further study using larger sample sizes and longer mtDNA fragment is recommended to reveal more genetic variation among these populations. Conservation initiatives on Kuala Terengganu population should be carried out to prevent the loss of these natural gene resources for future selection and cross-breeding program. Distinct haplotypes could be used as DNA markers for their respective sampling sites. Low genetic diversity of swamp eel observed in this research need an attention and initiative from the Department of Fisheries Malaysia, such as introducing a newly population that will increase the genetic variation of swamp eel in East Coast of Peninsular Malaysia.

The current study would aid in identification of potential population for commercial cultivation of this fish in East Coast of Peninsular Malaysia. It also provides a preliminary baseline genetic data of wild swamp eel population that is important in setting up suitable guideline for selective breeding program and consequently producing good and more reliable quality seed for aquaculture.

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