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Different Population of Malaysia Swamp Eel in East and Southeast Asia Inferred from Partial 16S Mitochondrial DNA

Aliyu Garba Khaleel, Ha Hou Chew, and Ahmad-Syazni Kamarudin*

School of Animal Science, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin (UniSZA) Besut Campus 22000, Terengganu, **Malaysia**

***Correspondence:** ahmadsyazni@unisza.edu.my **Revised: 0**6 Aug. 2019, **Accepted: 10** Oct. 2019, e-**Published:** 06 Nov. 2019
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Swamp eel, *Monopterus albus* is highly used medicinal and commercial food especially in Malaysia, China, Taiwan and other Asia pacific countries. There is a drastic decrease in percentage number of this important species that could be due to pollution and overfishing. Molecular genetics research approach could be a useful tool in conservation and management of swamp eel species to reach their maximum production potential. This could help to discover and resolve more information regarding the populations, phylogeny and genetic diversity of these species across this region. To discover the population structure of swamp eel in Southeast and East Asia; a total of sixty-one samples were obtained from three states (Kelantan, Terengganu, and Pahang) of East Coast Peninsular Malaysia at six variable sampling sites for partial 16S mitochondrial DNA analysis. Among all the samples, four (4) haplotypes were detected. Additionally, thirty haplotypes were obtained from NCBI that originated from China, Japan, Indonesia and Taiwan. The phylogenetic result shows three different clades; China and Japan (A), Taiwan and Indonesia (B) and East Coast Peninsular Malaysia (C) across East and Southeast Asia. The East Coast Peninsular Malaysia haplotypes (Clade C) did not belonging to clade B that was previously reported to represent the entire Southeast Asia population. The current result show high genetic diversity of swamp eel across this region. In pairwise comparisons of F_{ST}, the genetic distances between the three clades were significant ($P < 0.05$), which suggested that all clades were genetically different. Newly clade C discovered in this study is evidence that swamp eel species have a wide geographical distribution across East and Southeast Asia.

Keywords: *Monopterus albus*, Mitochondrial DNA, Population structure, Phylogeny, Conservation

INTRODUCTION

Swamp eel *Monopterus albus* belongs to the suborder *Synbranchoidei,* in *Synbranchiformes* order that consisted about three genera and nearly 13 species (NCBI, 2015). Swamp eel is a native to sub-tropical and tropical Asia, and it is widely distributed in various countries including India, China, Japan, Malaysia, Indonesia and Bangladesh (Froese and Pauly 2012; Guan et al., 1996). However, Nelson (1994) added that it is also found in West Africa and South, North and Central America as an introduced species. Swamp eel is a primary freshwater fish found in marshes, rice fields, rivers and ponds (Matsumoto et al., 2010). It is a highly used as commercial food fish and medicine across East and Southeast Asian countries (Najiah et al., 2006; Sow et al., 2012; Nor et al., 2013). The co-existence of large markets of swamp eel present in China, Hong Kong, Japan and United States are a clear evidence that shows how swamp eel play a

significant advantage in the economic development of the society (Guan et al., 1996; Chu et al., 2011). Amirah (2009) explained the nutritional value of swamp eel as greater than that of Spanish mackerel and crevalle jack with a significant amount of vitamin and minerals. Sow et al*.,* (2012) highlighted swamp eel as a popular and important fish species in Malaysia, which serve as food for many people. Hence, it is considered as nutritious and divine species for esteemed cure in East Asian countries (Najiah et al., 2006).

Due to several reports showing a significant reduction of swamp eel production in the wild which might result from pollution and overfishing (He et al., 2003; Yin et al., 2005) derived the attention of many researchers for solving the phylogenetic and genetic studies for better management and conservation of swamp eel (Cai et al., 2008; Matsumoto et al., 2010; Cai and Zhang, 2011; Sun et al., 2015; Devi et al., 2014). Swamp eel was initially thought to be a single species (Fuller et al. 2015). It is later cleared that four identified populations of these species within Southeastern United States might reflect three distinct species or texa, each originating from a different area in Asia (Collins et al., 2002). Population structure of swamp eel was studied previously by Matsumoto et al., (2010) using 16S mitochondrial DNA revealed the phylogeny among swamp eel populations in East and Southeast Asia including China, Japan, Taiwan and Indonesia. Swamp eel origin, phylogeny and genetic diversity of swamp eel species of many Asian countries are still unknown. Therefore, more sampling sites, especially from large swamp eel producing countries like Malaysia, will aid to discover new populations and add the additional information to the previous studies. In addition, results obtained from East Coast of Peninsular Malaysia and previous studies to investigate the genetic diversity and phylogenetic relationships among the geographical populations in East and Southeast Asia will enhance and provide further information regarding the phylogeny and genetic diversity of this species (Syazni et al., 2017).

Many researchers reported mitochondrial DNA polymorphism in various animal populations used to clarify the ancestral lineages or to compare populations (Kawabe et al., 2014). The 16S rRNA genes are non-coding and conserved in nature that play an important role in checking the reliability of earlier established phyletic classification, and in the discovery of a newly phylogenetic relationships (Sharma et al., 2014). The 16S rRNA genes evolve slowly in eukaryotic mitochondria, and these genes existed in the fishes of the current study. Mitochondrial 16S rRNA analysis is a rapid and cost-effective established molecular marker standard for identification of eel species (Miah et al., 2013). Similarly Yang et al., (2014) described 16S rRNA markers as a reliable method for taxonomic classification of vertebrate species including fish.

The main objective of the current study was to provide additional information on the phylogenetic relationship and genetic diversity using 16S mitochondrial DNA genes of *Monopterus albus* to discover more maternal ancestral origin of these species populations across East and Southeast Asia.

MATERIALS AND METHODS

Sampling

A total of sixty-one (61) samples of swamp eel were collected at six sampling sites from East Coast of Peninsular Malaysia which consist of 30 samples (Kelantan), 21 samples (Terengganu) and 10 samples (Pahang) states from January to April 2015 *(current study).* Other haplotypes were obtained from NCBI for comparison *(previous studies)* Table 1. Approximately 1 cm of tail tissue was removed with scissors and preserved in 95% ethanol for DNA extraction.

DNA Extraction, Amplification and Sequencing

Total genomic DNA was isolated from dried tail tissue using the Genomic DNA Tissue Mini Kit (Genaid Biotech Ltd., New Taipei City, Taiwan) following manufacturer's instructions. The partial 16S rRNA gene of mitochondrial DNA was amplified by PCR using the universal primers L1567 (5'-AAG GGG AGG CAA GTC GTA-3') (Matsumoto et al. 2010) and H2196 (5'-GTC TGA GCT TTA ACG CTT TCT-3') (Yamaguchi et al. 2000). PCR was carried out in a 10 µL reaction volume containing 5.15 µL sterile distilled H_2O, 1 µL buffer (TaKaRa, Kusatsu, Japan), 0.8 µL dNTP Mix (2.5 mM), 1 µL of each primer (10 µM), 0.05 µL of 5 unit/µL Taq DNA polymerase (Ex-Taq; TaKaRa), and 1 µL template (50 ng/µL) on a thermal cycler (GeneAmp PCR System 9902, Applied Biosystems California, USA), under the following thermal cycling conditions: predenaturation at 96°C for 4 min; 35 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 10 s, and elongation at 72°C for 30 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

Table 1. Haplotype names, sampling location, accession number and references of swamp eel mtDNA used in this study

Haplotype	Location	GenBank Accession No.	Reference
Hap-1 to Hap-4	ECPM		This study
Monopterus albus	Unspecified location	AP002945	Miya et al., (2001)
Ma01 - Ma17	China and Japan	AB494967-494983	Matsumoto et al., (2010)
Ma18 - Ma29	Taiwan and Indonesia	AB494984-494995	Matsumoto et al. ,(2010)

(Applied Biosystems, California, USA) by following the manufacturer's instructions, performed on an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems California, USA).

Data Analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura 2013). All positions with missing data and gaps were deleted from the dataset. The list of haplotypes used with their corresponding GenBank accession numbers are given in Table 1 above.

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 (Librado and Rozas, 2009). Genetic diversity in each population was measured as haplotypic diversity (Nei, 1987) and nucleotide diversity (Tajima, 1983) using ARLEQUIN v3.5.1.2 (Excoffier and Lischer, 2010). Two different neutrality tests were examined for the combined haplotype sequences alignment: Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) tests, implemented in ARLEQUIN v3.5.1.2 (Excoffier and Lischer, 2010). These tests were used to evaluate the neutrality of the investigated sequences to find out if populations are deviate from genetic equilibrium, population expansion or bottleneck. Genetic differentiations between populations were tested by pairwise comparison F_{ST} with Slatkin's and Reynold's distances with 1,000 permutations as implemented in ARLEQUIN v3.5.1.2 (Excoffier and Lischer, 2010). The F_{ST} provide a view of both variance structure of populations, and overall comparison of the degree at which populations are structure. A zero value of F_{ST} shows the lack of structure and differentiation in population while when F_{ST}= 1 meaning population is completely differentiated.

et al., 2013). A neighbor-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 (Tamura et al., animal

RESULTS

Population Differentiation

The phylogenetic analysis of 16S rRNA sequences using neighbor-joining tree revealed that, haplotypes of swamp eel from East and Southeast Asia are clustered into three different clades: Clade A (A1 and A2), B and C (Figure 1). The mtDNA data in this study revealed a strong pattern of population subdivision among the population at this level of analysis. In addition, pairwise comparison F_{ST} value of population differentiation revealed the genetic differentiation among all clades was significant (Table 2).

Sequence Variations

Among all sixty-one samples used in the current study, four haplotypes were detected. A total of 492bp fragments from the 16S rRNA of mtDNA genes were successfully aligned for 34 haplotypes from East and Southeast Asia. Among the 492 sites; 47 were sites with gaps, 343 were invariable (monomorphic) sites, and 102 were variable (polymorphic) sites. The variable sites consist of 90 parsimony informative sites and 12 singleton variable sites (Figure 2). However, A/T base contents were significantly higher than the C/G contents (Mean: A=37. 7%, T=21. 2%, C=22. 3% and G=18. 8%).

Genetic Diversity

The genetic diversity and neutrality tests of all haplotypes according to their clade are shown in Table 3. Clade B (Southeast Asia Clade) has the highest haplotype diversity (0.95455) and

nucleotide diversity (0.02403), followed by subclade A1 with (0.92424) and (0.00515), respectively.

Table 2; Pairwise comparison FST value of population differentiation among four clades in East and Southeast Asian swamp eel

	CC	SC A1	SC A2	CB
CC	–			
SC A1	0.97515*	–		
SC A2	0.98044*	0.69556*	–	
CB	0.91095*	0.81519*	0.81994*	–

*P < 0.05; Where CC = Clade C, SCA1= Subclade A1, SCA2= Subclade A2 and CB= Clade B

The high genetic diversity of the 492 bp of 16S rRNA mtDNA marker in this study was similar to the genetic diversity of mtDNA markers used in previous studies on swamp eel population (Collins et al., 2002; Matsumoto et al. 2010). Suggesting that 16S region is a useful genetic marker for this particular species' population genetic studies.

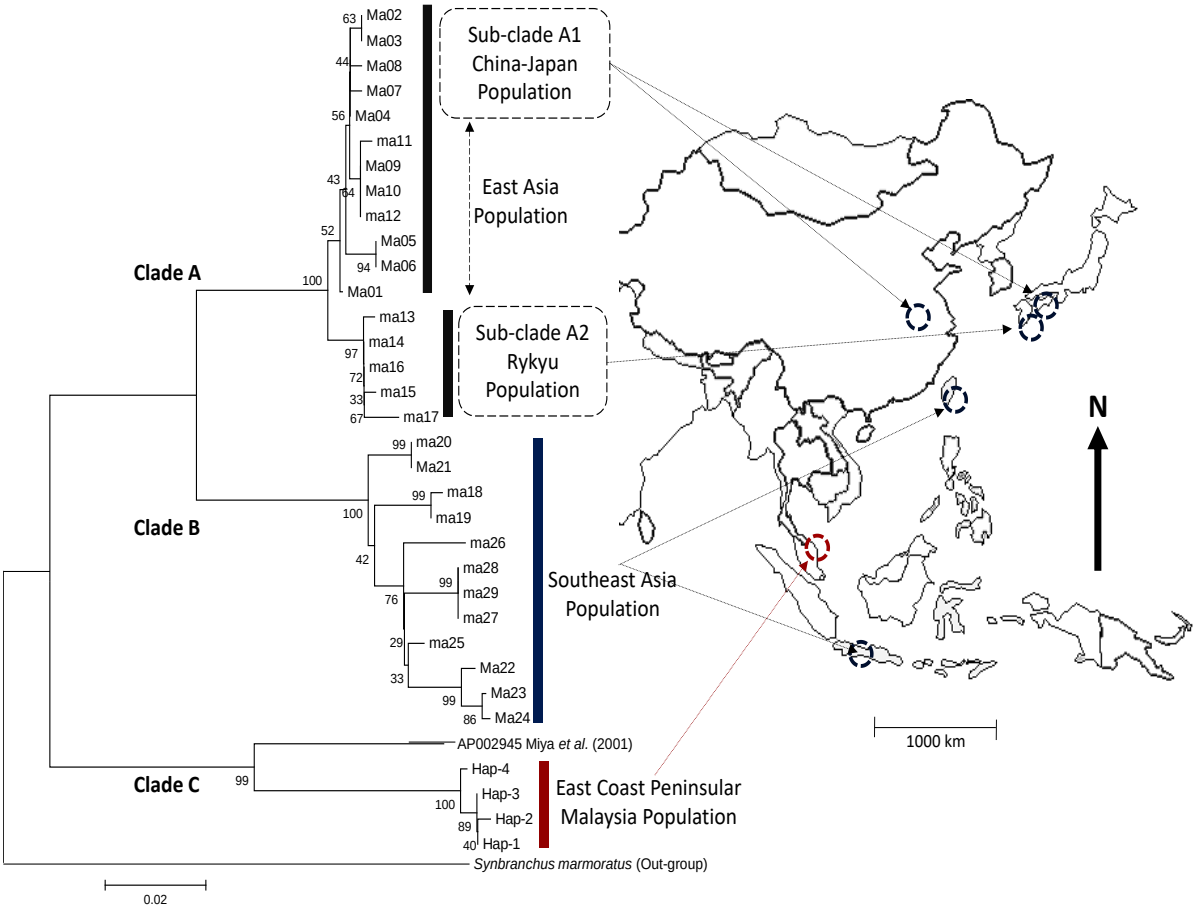

Figure 1: Neighbour-joining tree showing phylogenetic relationships among 4 ECPM, 1 (accession no. AP002945), 29 (accession no. AB494967-494995) haplotypes of swamp eel inferred from sequences of the mtDNA 16S rRNA gene with Synbranchus marmoratus as outgroup. A distance matrix was calculated using the Kimura's 2-parameter model in MEGA v6. The data set bootstrapped 1,000 times, and the appropriate bootstrap values were placed on each branch.

Table 3; Haplotype data from four clades in East and Southeast Asia based on partial fragments of the mtDNA 16S region, haplotype, and nucleotide diversity (mean ± SD), and Neutrality test

Clade	Number of haplotypes	Haplotype diversity	Nucleotide diversity	Fu's *FS*	Tajima's *D*
Clade C (ECPM)	4	0.83333	0.00327	-0.28800	-0.75445
Sub clade A1	12	0.92424	0.00515	-3.38600	-0.83982
Sub clade A2	5	0.90000	0.00436	-2.68000	-1.14554
Clade B	12	0.95455	0.02403	-1.18900	-0.09181

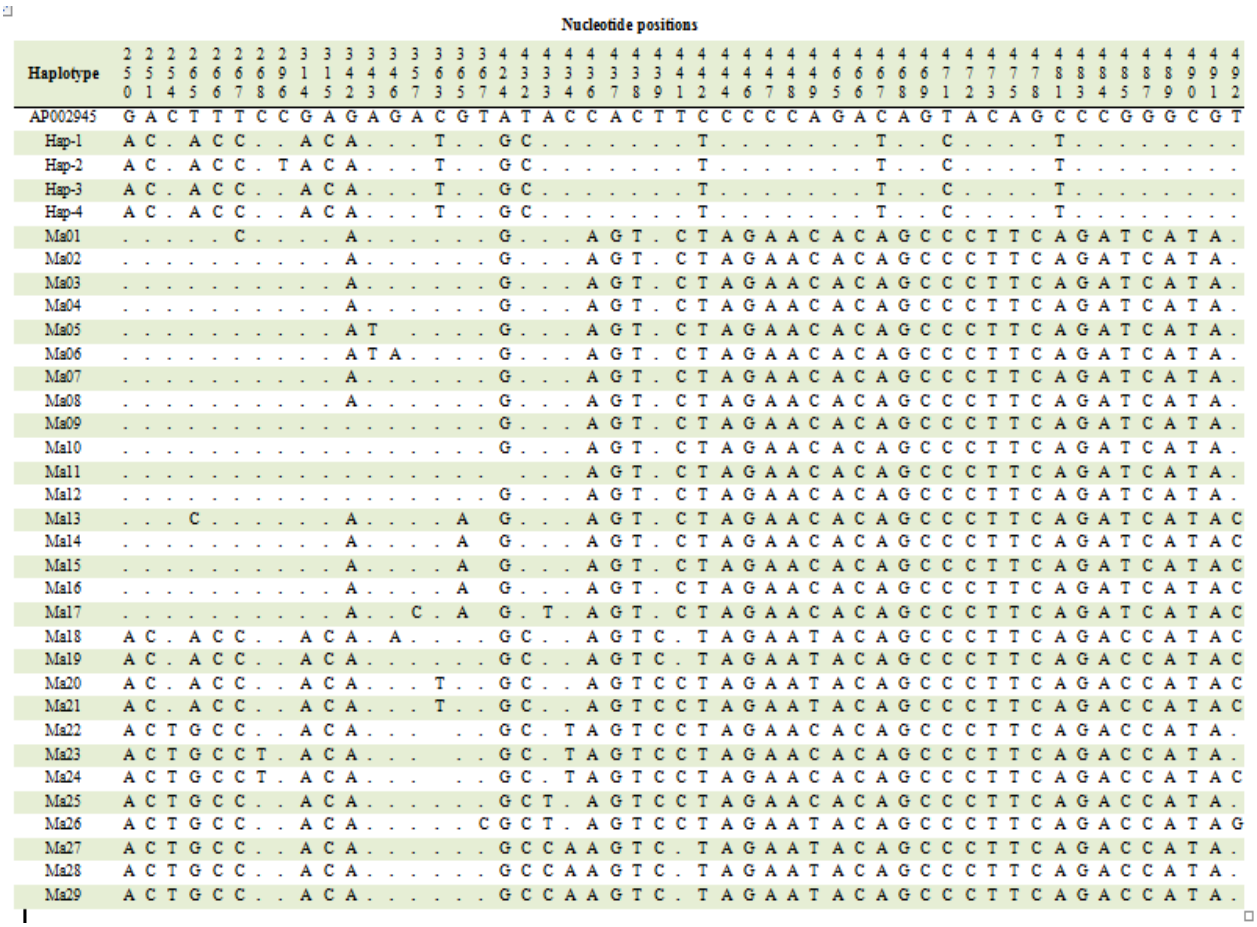

Figure 2: Sequence variations of 34 haplotypes in 16S mtDNA from East and Southeast Asia. Mutations are scored relative to the reference sequence (accession no. AP002945). Dot (.) represents the identical nucleotide with the haplotype AP002945

DISCUSSION

This result shows a high sequence variation between haplotypes and has a subsequent effect on the genetic variation obtained in this study. Miah et al., (2013) described a high level of genetic diversity within the given population as important in handling environmental changes such as epidemic and changes in water temperature. It is further explained that to ensure reestablishment of adaptation, expansion of natural populations, it is essential to maintain the genetic variation in species (Miah et al., 2013).

However, neutrality tests of Tajima's D and Fu's Fs shows a negative non-significant deviation of neutrality in all population (P>0.10). The non-significant negative values of Tajima's, D and Fu's Fs neutrality tests show population might recently experience bottleneck followed by expansion or purifying selection (Tajima, 1983). Ha et al., (2011) explained that demographic contraction or expansion and lack of genetic introgression from exotic populations lead to equilibrium of gene frequencies. The degree of gene flow amongst sympatric clade and their stand as species is still unknown, because very little is known about the ecology of these species.

The pairwise genetic distance between the population shows a clear significant population differentiation of clades in the present study, which suggested that all clades were genetically different. It is strongly supported by the neighbour joining tree result where there is a clear distinction between the clades. The magnitude and geographic pattern of population genetic structure of the species is resulted from historical as well as contemporary events (Dodson et al., 1995). The importance of historical biogeography in shaping intra-species genetic structure is well established, and the capability of mtDNA to maintain a past isolation history is well explained (Avise et al., 1986). As a result of Southeast Asia involvement in the worldwide differences in sea level that happened during the glaciations of Pleistocene, it is quite possible to have more than one clade in that region (Dodson et al., 1995). The Sunda continental shelf connected Peninsular Malaysia and mainland Asia (Clade C of East Coast Peninsular Malaysia) together with greater Sunda Island (Sumatra, Java (Clade C), and Borneo). Clade C is endemic to the Peninsular Malaysia, may be isolated during the high sea level period when the Peninsula was isolated from the mainland. The extensive inter-connections at the periods of glacial maxima might substantially influence and change the distribution of the fauna and flora (Dodson et al., 1995); this could be a reason for wide distribution of swamp eel in these regions.

Ocean current also has a subsequent effect and can help in defining the population differentiation of fish species (White et al., 2010). The ocean current in East and Southeast Asia could also be useful factor for the seed dispersal of swamp eel that can affect the population structures (Figure 3).

Ocean current passing from South to North hindered the seed dispersion between the two adjacent countries of China and Japan (sub-clade A1). Despite prevalence current barrier exist between these countries, yet they retain the same maternal ancestry and remain the same population. Another possibility is that Kyukyu population was in an isolated Island which makes it highly evolved to form sub-clade A2. The Taiwan swamp eel existence in clade B Southeast Asia clade) might possibly be as a result of prevalence current flow from Southeast Asia towards Taiwan. The ECPM does not disturb much with the current flow from either south or north, and this could be a reason that swamp eel in ECPM formed (clade C).

The phylogenetic analysis revealed that swamp eel of East Coast Peninsular Malaysia does not belong to the clade B of Matsumoto et al. (2010) on which previously thought to be Southeast Asia clade. All haplotypes from East Coast Peninsular Malaysia in the current study clustered into clade C.

The differences in reproductive behaviour have supported the findings of the current study that these clades are genetically different populations. It is impossible to align the current study haplotypes sequence with Collins et al., (2002) haplotypes due to a long distance between their nucleotide positions in the 16S rRNA gene. Ten samples collected from Kuala Lumpur provided three distinct haplotypes that were also clustered into one clade together with other haplotype that is also believed to be originated from Southeast Asia (Collins et al., 2002). The current study could not conclude whether samples from Kuala Lumpur shared the same clade with East Coast Peninsular Malaysia due to long distance between their nucleotide positions in the 16S rRNA gene.

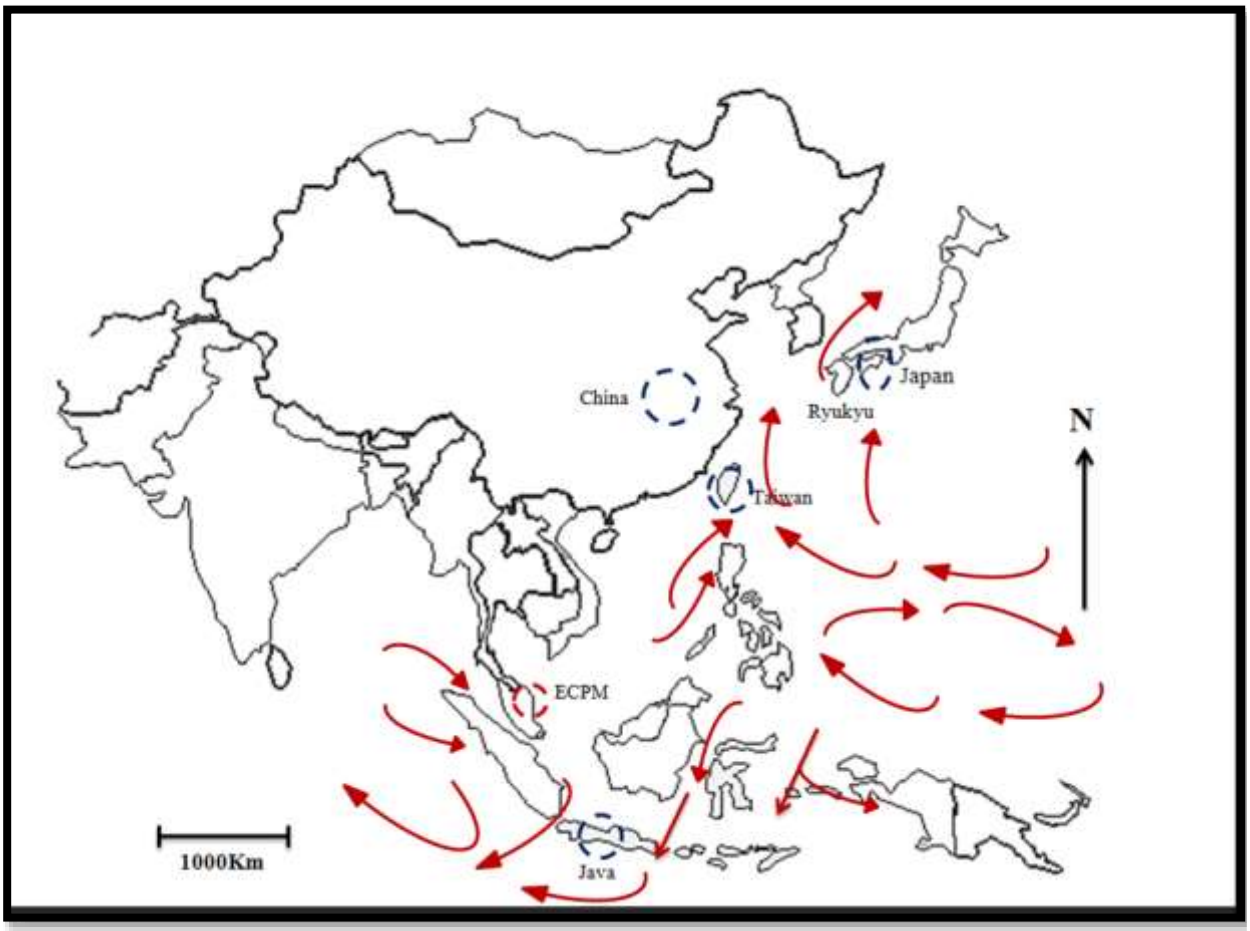

Figure 3: Effects of prevailing ocean current in East and Southeast Asia on population differentiation. The red arrow shows prevailing current direction and red and blue circles show the sampling locations

CONCLUSION

The current research revealed that there is a high genetic diversity of swamp eel population across East and Southeast Asia. Newly clade C discovered in this study is evidence that swamp eel species have a wide geographical distribution across East and Southeast Asia. The findings of this study will contribute and enhance better understanding of population genetic study of swamp eel in Asia that could be useful for swamp eel management and conservation. More sampling areas (e.g. Sabah or Borneo) and countries with no swamp eel genetic information is needed for further studies to reveal more populations structure in Asia Pacific Region.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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