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ND5 GENE MARKER REVEALS RECENT POPULATION EXPANSION OF WILD PEARSE'S MUDSKIPPER (*Periophthalmus novemradiatus* HAMILTON) INHABITS SETIU WETLANDS IN EAST PENINSULAR MALAYSIA

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ABSTRACT

Genetic variation and differences in wild Pearse's mudskipper *Periophthalmus novemradiatus* populations from Setiu Wetlands in East Peninsular Malaysia were analysed using the partial mitochondrial DNA ND5 gene sequences. Among the 91 individuals sampled from six different localities, 35 novel putative haplotypes of *P. novemradiatus* were detected. 77% (27) of the haplotypes were unique sequences, with high level of haplotype diversity (H = 0.875) and low nucleotide diversity (π = 0.0037), contributing to the overall highly diversified gene pool of the *P. novemradiatus*. This reflects a large effective population size with current population expansion, which allows the retention of new alleles in populations. However, due to insufficient time, the accumulation of deeper divergent groups among haplotypes was not possible. Hap05 is the most dominant (33%) and widespread haplotype, followed by Hap11 and 31. Low genetic differentiation with high gene flow was detected between sampling sites, and no pattern for isolation by distance was observed despite being territorial creatures. All sites are at the top priority for conservation because they possess unique haplotypes that are only present at the respective location. Further samples collection from other native regions are required to provide full understanding of its genetic distribution and phylogeographical study over larger scale of geographic regions. Heuristic approach to study other species in this area prior to gazetting the Setiu Wetlands as state park is required in order to conserve the biodiversity in-situ.

Key words: Genetic diversity, recent population expansion, mudskipper, mitochondrial DNA, conservation

INTRODUCTION

Mudskippers are categorized in the subfamily Oxudercinae within the family Gobiidae (gobies) and are known to be amphibious where they can stay out of water for some part of the daily life cycle (Murdy, 1989). Recent studies have shown how these fishes adapt to water-to-land transition (You *et al*., 2014; Sakamoto *et al*., 2015). Mudskippers in general are an important indicator species of environmental pollution as they are sensitive to the ambient environment and thus serve as an excellent candidate for detecting pollution level around their habitats (Shirani *et al*., 2012; Ansari *et al*., 2014). Mudskippers are commonly found in mangrove ecosystems and mudflats, relatively small in size and morphologically ambiguous, leading to mis-identification of specimens (Jaafar & Larson, 2008; Jaafar *et al*., 2009), while some suffer from data

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deficiency, and more critically they are very vulnerable to habitat disturbance and degradation.

The wetlands of Setiu in the southern part of South China Sea, situated at the northern corner of Terengganu state, Malaysia is the largest natural wetland in the east coast of Peninsular Malaysia which encompasses a diverse ecosystem that includes freshwater, brackish water, seawater and a 14km lagoon, rich in flora and fauna that support local livelihood. However, the natural vegetation particularly the mangrove forests were reported to be shrunk by 20% since past three years (between 2008 to 2011), due to land clearing for plantation and infrastructure development (Tan, 2016), consequently affecting the whole ecosystem and community surrounding it. Many indigenous fish species inhabiting the area have not been identified nor documented systematically, with the mudskipper being one of them. Despite their significant ecological role, unique life cycle and behaviour, they receive little attention and species mis-identification due to insufficient data affecting species collection and records.

The Pearse's mudskipper *Periophthalmus novemradiatus* Hamilton occurs in tropical countries such as India, Myanmar, Thailand, the Philippines and Malaysia (Murdy, 1989). Its taxonomic keys were described by Murdy (1989) and later being re-diagnosed to differentiate from the valid species of *P. variabilis* Eggert (Jaafar *et al*., 2009), based on morphometric analyses. No prior knowledge on genetic sequence of this particular species is available in the public database except for the DNA barcode sequences from the Java and Bali, Indonesia (Dahruddin *et al*., 2017). Therefore, the present work involves first-time DNA sequencing of mitochondrial DNA ND5 gene, to infer its populations' well being at the Setiu Wetlands based on analyses of genetic diversity and differences. The results provide new DNA sequences into the genetic database and insight of its population health at the Setiu Wetlands, which will be useful as guideline to the authorities in conserving this valuable species within its native ranges.

MATERIALS AND METHODS

Ethics statement

Live specimens from wild populations were obtained from different locations along the two estuaries in Setiu Wetland, Terengganu, Malaysia (Table 1 & Figure 1) using hand net. A small portion of the pectoral fin rays (approximately 0.2 cm x 2 cm) was cut from each individual and preserved in 1.5 ml tubes containing solution of 95% ethanol. Tissue samples were stored in Laboratory of Fisheries Biosystem, School of Fisheries and Aquaculture Sciences at room temperature (~25°C) until further use. No experimentation on the animals was performed and no other ethical issues are applicable to the present research.

Genomic DNA extraction and PCR amplification

Total genomic DNA was isolated from fin tissue using the salt extraction method (Miller *et al*., 1988). The PCR amplification of the mitochondrial DNA NADH dehydrogenase subunit 5 (ND5) gene was performed in 30 µl using 50-100 ng of genomic DNA, 0.05 µM of each primer, 0.17 mM of dNTP. 1.4x PCR buffer, 1 mM $MgCl_2$ and 1.67 U of *Taq* polymerase (all from iNtRON), in an MJ PTC-200 Thermal Cycler (MJ Research, Waltham, MA, USA). Amplification and sequencing of the mtDNA ND5 gene was conducted using the primer pair L12321-Leu (5'- GGTCTTAGGAACCCAAAACTCTTGCT GCAA -3') and H13396-ND5 (5'- CCTATTTTKCG GATGTCYTG-3' (Ruzainah, 2008). The temperature profile for the amplification consisted of initial incubation at 95°C for 1 mins, 30 cycles of 95°C for 15s, 56.8°C for 15s, 72°C for 10s, and final hold

Table 1. Sampling sites, genetic diversity estimates and neutrality tests of *P. novemradiatus* based on mtDNA ND5 gene

Site	Latitude	Longitude	n	Diversity			Neutrality test	
				h (S)	H	π	Tajima's D	Fu's Fs
A	N 5° 41.322'	E 102° 42.079'	10	8 (6)	0.956	0.0035	-0.62	-4.33*
B	N 5° 41.462'	E 102° 41.495'	14	9 (7)	0.879	0.0038	-1.96*	-3.66*
C	N 5° 40.790'	E 102° 42.439'	25	16 (13)	0.900	0.0047	-1.47	-8.96*
D	N 5° 39.743'	E 102° 43.750'	15	7 (5)	0.771	0.0018	-1.77*	-3.38*
E	N 5° 38.133'	E 102° 46.973'	14	6 (3)	0.791	0.0026	-1.00	-1.18
F	N 5° 47.525'	E 102° 36.755'	13	7 (2)	0.910	0.0036	-0.06	-1.58
Total			91	35 (27)	0.875	0.0038	NA	NA
Mean			15.2	8.8 (6)			-1.14	-3.85*

n: sample size, h (S): number of haplotype (number of singleton haplotype), H: haplotype diversity, π: nucleotide diversity.
*significant at $p < 0.05$, NA: not available.

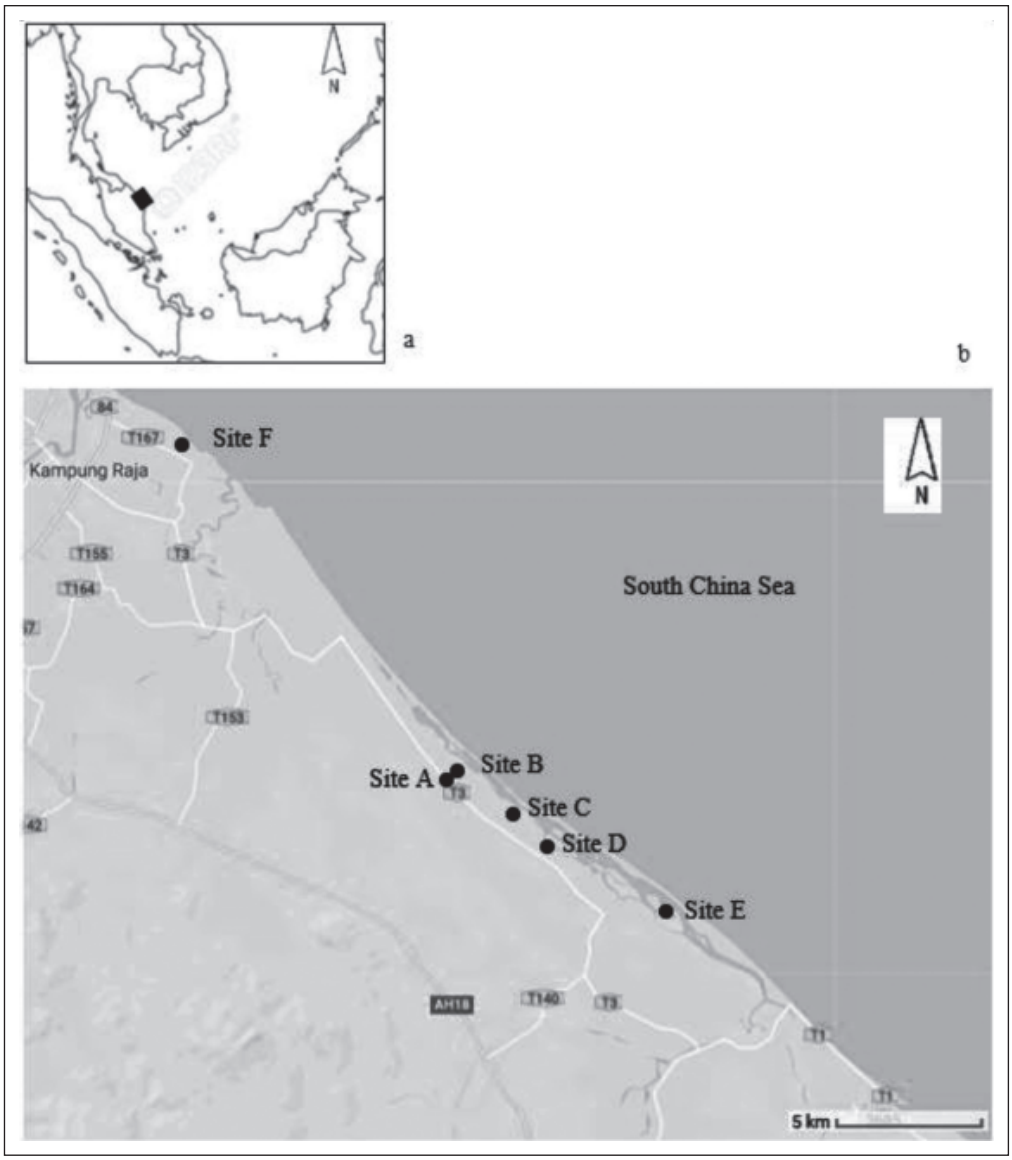

Fig. 1. Sampling sites of *P. novemradiatus* at Setiu Wetlands, as indicated in (a) the South-East Asia map marked with black box and (b) finer scale of the sampling locations at East Coast of Peninsular Malaysia.

at 12°C. The PCR products were visualized on a 1.7% agarose gel, stained with SyBr Safe to confirm successful amplification. All products were sent for DNA sequencing (First BASE Laboratories Sdn Bhd, Selangor, Malaysia) and reading from single DNA strands.

Data analysis

Multiple sequences were aligned and all unambiguous operational taxonomic units (OTUs) were compiled and edited using ClustalW implemented in MEGA 6.0 (Tamura *et al*., 2013). DNA sequences were translated into amino acid sequences to ensure accurate alignment. Haplotype sequences were deposited in GenBank under accession numbers MH536987-537021. The complete aligned dataset was analysed for number of haplotype, haplotype diversity (H) and nucleotide diversity (π) in DnaSP 6.0 (Rozas *et al*., 2017). Selective neutrality tests that evaluate deviation from neutral expectation which may arise from historical population range expansion or mutation-drift disequilibrium was examined through Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) statistics for each locality using Arlequin 3.1 (Excoffier *et al*., 2005).

A hierarchical analysis of molecular variance (AMOVA) was conducted to infer the relative contribution of genetic variation among and within sampling sites. The population pairwise comparison statistic, F_{ST} that calculates relative genetic differentiation between populations was determined in Arlequin 3.5 to evaluate the significance or otherwise of differences among populations and

spatial population structuring. The pairwise F_{ST} was determined based on Tamura-Nei distance method and statistically significant pairwise comparisons were tested with 10,000 permutations. Significant probability values were adjusted by performing the False Discovery Rate Procedure (FDR) at α=0.05 which controls the family wise error rate (FWER), a conservative type I error rate that originates from multiplicity (Benjamini & Hochberg, 1995). Significance levels of all pairwise estimates were adjusted with FDR procedure at = 0.05.

Further to measure the extent of genetic differentiation among populations, haplotypes-based statistics (H_{ST}) and sequence-based statistics (N_{ST} (Lynch and Crease, 1990) and K_{ST}*) were employed, where both statistics' significant level were assessed by permutation test at 1000, respectively (Hudson *et al*., 1992) in DnaSP program (Rozas *et al*., 2003). Using the same program, gene flow estimates (Nm) based on both haplotype-based and sequence-based statistics were derived as in Nei (1973) and Hudson *et al*. (1992), respectively. Pairwise genetic distance between sampling sites was calculated in MEGA 6.0. The relationship between genetic and geographical distance was assessed by use of a Mantel test in IBD v 1.52 (Isolation By Distance) (Mantel, 1967; Bohonak, 2002). Population pairwise F_{ST} values represented genetic distances, and approximate geographical distances between sample locations were measured as linear distance (km) by using Google Earth. Geographic distance was ln transformed, and the strength of the relationship was examined with reduced major axis regression (10,000 randomizations) in IBD v 1.52.

Phylogenetic tree was constructed using Maximum Likelihood (ML) tree building method in MEGA 6.0. The best substitution pattern for the dataset was searched using MEGA. Tamura-Nei with Gamma distribution (TN93+G) was considered as the best DNA substitution model where they yield the lowest BIC score (Bayesian Information Criterion). The confidence levels at each node were assessed by 1000 bootstrap replications (Hall, 2013).

RESULTS AND DISCUSSION

Six populations consisting of 91 *P. novemradiatus* sampled from Setiu Wetlands, southern part of South China Sea were sequenced at partial mtDNA protein coding ND5 gene with final length truncated to 701 bp. Sample size ranged from 10 at site A to 25 at site C, with an average of 15.2 (Table 1). The final alignment of sequences revealed a total of 35 variable sites (37 substitutions and 16 parsimony informative) defining 35 putative haplotypes with 27 (77%) of them being private haplotypes. An average of 8.8 haplotypes with six unique sequences found within each sampling site reflects the overall highly diversified gene pool of the *P. novemradiatus.* High level of genetic diversity was also reported in mudskipper *Boleophthalmus pectinirostris* Linnaeus (Chen *et al*., 2015). In this study, high level of haplotype diversity (H = 0.875) and low nucleotide diversity (π = 0.0037) was estimated, suggesting large effective population sizes with current population expansion from a small population. This allows the retention of new alleles in populations but with insufficient time for the accumulation of deeper divergent groups among haplotypes (Delrieu-Trottin *et al*., 2017). This was in alignment with the significant negative values of neutrality tests, an evidence for an excess rare alleles, suggesting of recent population expansion or from genetic hitchhiking (Tajima, 1989; Ramos-Onsins & Rozas, 2002).

In addition, the population pairwise F_{ST} analysis showed genetic differentiation was averagely low ranging from 0.0015 to 0.2053 (Table 2), where 33.3% of the population pairwise comparisons F_{ST} showed significant structuring after FDR correction at α = 0.05. Pairwise geographic distance between sampling sites was estimated at average 9.4 km (ranging approximately 0.5 to 23.91 km), and no correlation was found between distribution of the gene frequencies over geographic region ($p > 0.05$). This was unexpected knowing that mudskipper is territorial and live in their mud-walled "house" (Clayton & Wright, 1989). The

Table 2. Pairwise Fst estimates between sampling sites *P. novemradiatus*

	Site A	Site B	Site C	Site D	Site E	Site F
Site A						
Site B	0.1480*					
Site C	0.0203	0.0182				
Site D	0.2053*	0.0308	0.0562			
Site E	0.1961*	0.0015	0.0255	0.0258		
Site F	0.2004*	0.0062	0.0279	0.1068*	0.0275	

Significant Fst ($p < 0.05$) based on 10000 permutations of haplotype frequencies among samples, after FDR correction, are indicated with *.

F_{ST} estimates were in concordance to the low and statistically non-significant genetic differentiation estimates of H_{ST}, N_{ST} and K_{ST}*, ranging from 0.007 to 0.074. This in turn reflected a high level of genetic similarity among the sampling sites (Nm=60.82 for haplotype-based statistic and 6.25 for sequence-based statistic). AMOVA analysis shows that 94.2% of genetic variation occurs within sampling site, in support to other genetic differentiation test analyses. The high gene flow between sampling localities may be due to the planktonic larvae that dispersed following the water currents along the coast of East Peninsular Malaysia.

Hap05 is the most dominant (33%) and widespread haplotype where it is present at all six sampling localities, followed by Hap11 and 31. Phylogenetic tree constructed using ML method showed no clear separation of haplotypes according to sampling sites (Figure 2). High gene flow between populations was also observed in the Chinese mudskipper *P. cantonensis* (later was renamed as *P. modestus* Cantor) at Taiwan (Chang & Lee, 1994) but not in Japanese mudskipper *P. modestus* from Japan (Mukai & Sugimoto, 2006).

In summary, this study revealed the recent population expansion of the wild *P. novemradiatus*

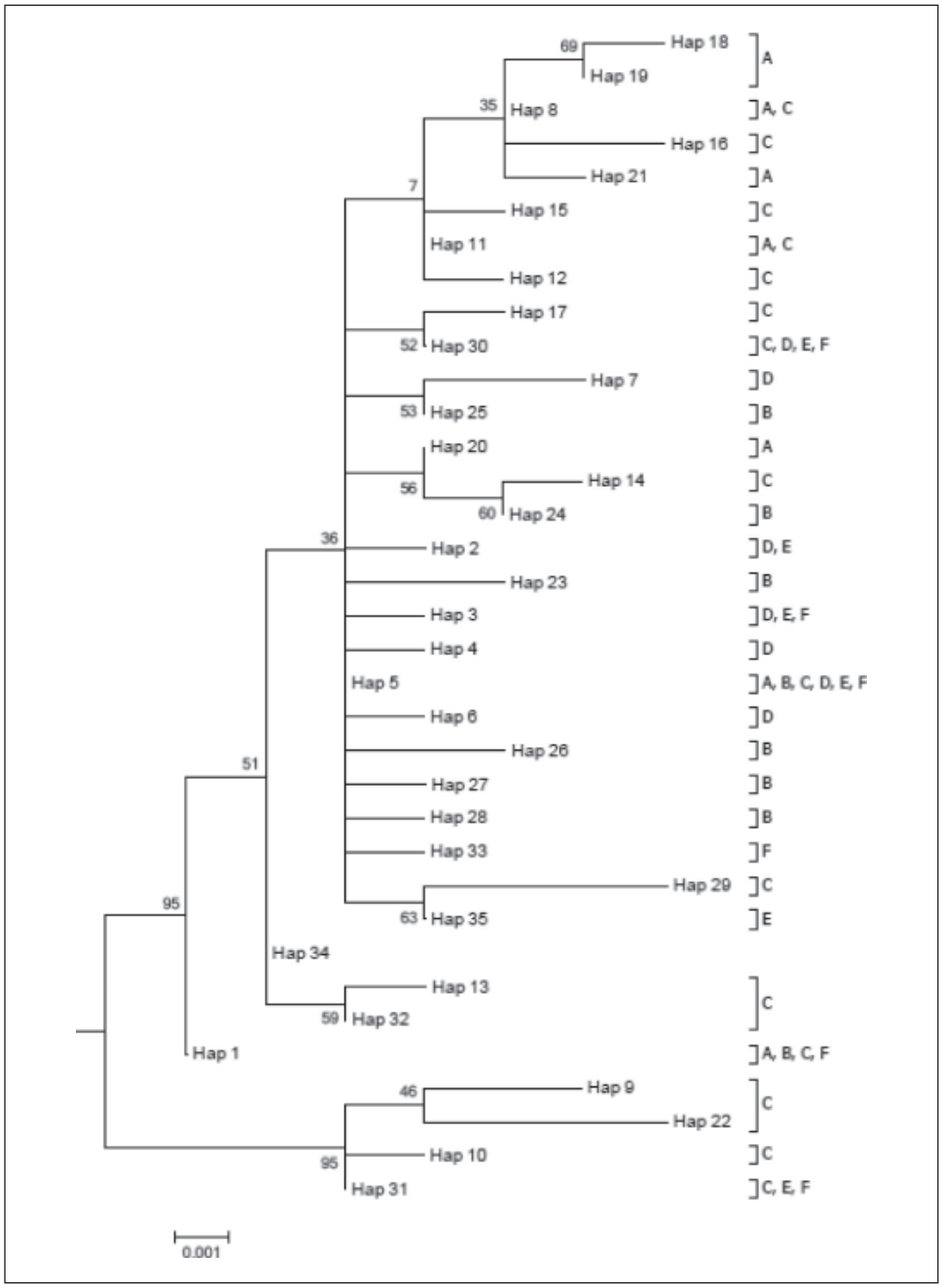

Fig. 2. Gene tree of *P. novemradiatus* haplotypes constructed based on Maximum Likelihood method. The haplotype observed from each sampling localities was marked accordingly.

inhabiting Setiu Wetlands, as indicated by high genetic estimates as supported by multiple test analyses. Only 33.3% of the pairwise populations show significant genetic structuring yet with no pattern of isolation by distance. All sites are at the top priority for conservation because each of them posses unique haplotypes which only are present at the respective location. Further sample collection from other native regions are required to provide full understanding of its genetic distribution and phylogeographical study over larger scale of geographic regions. Heuristic approach to study other species in this area prior to gazetting the Setiu Wetlands as state park is required in order to conserve the biodiversity in-situ.

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