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GENETIC VARIATION IN THE mtDNA *12S rRNA* OF EDIBLE NEST SWIFTLET (*Aerodramus fuciphagus*).

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ABSTRACT
Edible Nest Swiftlets (*Aerodramus fuciphagus*) naturally inhabit limestone caves of South East Asia, but of recent, swiftlet ranching in man- made houses has become popular. These swiftlets are of commercial importance due to their unique nests produced wholly of the mucilaginous secretion from the salivary gland. Limited works are available and also lack of knowledge about spatial delineation of the species in Malaysia. The study was carried out to elucidate information on the genetic variability of *A. fuciphagus* analysing the mtDNA *12S rRNA*. For the mtDNA analysis, DNA samples of extracted blood, tissue or nest of 28 EBN swiftlets from six locations were used. The DNA was subjected to PCR amplification. The *12S rRNA* has five haplotypes. The Tajima'sD showed a negative value (P>0.05), while the Fu's and Li's D value was also negative. The median joining network tree for *12S rRNA* showed that EBN swiftlet populations could not be distinguished geographically. The shared haplotypes suggest high gene flow between locations and lack of spatial delineation which makes the marker unsuitable for DNA barcoding. However, the result showed that the overall genetic diversity in the species was relatively high indicating genetically healthy population.
Key words: *Edible Nest Swiftlet, mitochondrial DNA, genetic variability, population.*

INTRODUCTION
Edible Nest Swiftlet (EBN) is mostly confined within the tropical/subtropical region of South East Asia occurring naturally in limestone caves. They are small sized insectivorous swift with distinctive plumage colour of blackish – brown at the back and a paler colour on the abdomen (Camfield, 2004). They are the major producer of edible nests with economic value and their nest are believed to have nutritional and aphrodisiac benefits (Norhayatti et al., 2010, Daud et al., 2021). Studies have shown the occurrences of the birds in different habitats and altitudes in relation to breeding seasonality (Lim et al., 2002). They forage across large ranches and are capable of travelling far from caves used for breeding. In the past few decades, expansion of man – made buildings for the purpose of housing large colonies has been on the increase.
Genetic variation between genomes of individuals or populations represent the genetic diversity within a given animal species (Hiller et al., 2007). This variation can be considered at the level of individual genes or genotypes. The chances of a genetic variant in time and space is influenced by biology and condition through which the population has passed. Mitochondrial DNA (mtDNA) is the genetic material present in the mitochondrial. It has been widely used in phylogenetic studies of animals because it evolves much more than nuclear DNA. MtDNA haplotypes have been the workforce of avian phylogeography, mainstay of intraspecific phylogeography and of systematics and population analysis for years. The rapid pace of sequence changes in mtDNA results in differences between population that have only been separated for brief period of time. This allows a chance of recovering the pattern and tempo of resent historical events with no extensive sequencing effort (Hurst & Jiggin, 2005).
The *12S rRNA* mitochondrial gene is relatively conserved and evolved more slowly than the mitochondrial genome as a whole (Di Finizio et al., 2007, Ameer & Hussein, 2020). Mutation existing at the variable site are reported to make the genes suitable for species discrimination. Adequate knowledge about the delineation among swiftlet species in Malaysia is necessary to evaluate genetic variation and its effect on the rapidly growing industry on local swiftlet population. The study aimed to assess *12S rRNA* mtDNA gene to elucidate the genetic variability of *A. fuciphagus* across locations in Malaysia.

MATERIALS AND METHODS

A total of 28 specimens of Edible Nest Swiftlet (EBN) swiftlet in the form of blood, tissue or nests were used in the study. Blood samples were collected from house farms at Sungkai, Perak (five individual) Tanah Merah, Kelantan (five individuals). Tissues (dead birds) were collected from house farms at Kuala Terengganu, Terengganu (five individuals). Edible nest from house farms at Pitas Sabah (three), Kota Tinggi, Johor (five individual) and nest from cave at Gua Madai, Sabah (five individual). Three Pacific Swallows (*Hirundo tahitica*) were collected to serve as outgroup for comparison. Blood samples (0.1ml) were collected from the right jugular vein using 28-gauge needle and transferred to 1.5ml micro centrifuge tubes. The blood samples were kept in an ice box in the field and later stored in -80^{0}C freezer in the laboratory. Bird nest were collected or purchased and packed in plastic pouches individually.

DNA extraction from blood and Tissues was carried out using the QIAmp DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instruction. DNA from the nest was done using Wizard Extraction Protocol. The *12S rRNA* was region was amplified using the primers: forward - 5'AGAACTACGAGCACAAACGC-3' and reverse - 5' GAAGAGGGTGACGGGCGGTATGT-3' (Desjadins and Morais, 1990). The Polymerase Chain Reaction (PCR) was carried out in a thermal cycler (Biometra, Germany). PCR amplification was carried out in 30 µL reactions using 3 µL of 10X Pfu Buffer with MgSO4 (ThermoScientific), 0.6 µL (10 mM) dNTP (Promega), 0.3 µL (2.5U) of Pfu DNA polymerase (ThermoScientific), 0.1 µM of each primer and 50 ng of DNA template. The PCR protocol for the amplification consisted of an initial denaturisation for 5 min at 95°C,8 cycles of 95 °C for 30 s, 64°C for 30 s, 72 °C for 1min, 29 cycles of 95°C for 30 s, 56 °C for 30 s, 72 °C for 1 min followed by final extension at 72 °C for 10min. Confirmation and visualization of the amplified PCR product was done using 1.5% agarose with TAE (Tris – Acetic acid - EDTA) buffer. The gel was stained with ethidium bromide and viewed under a UV transilluminator (BioRad). The amplicon was excised from the agarose gel and purified using GeneAll gel purification kit (Korea). Cloning of the *12S rRNA* mtDNA region was performed using ThermoScientific CloneJET PCR cloning kit, three colonies were picked from each sample. GeneAll HYBRID-Q ^{TM} PLASMID RAPIDPREP (Korea) was used for plasmid extraction. The purified plasmids were sequenced using the service provider (First Base Sdn. Bhd. Malaysia). The sequences were aligned and trimmed using BioEdit V 7.0.9 software to generate consensus sequences. Aligned sequences were examined and phylogenetic analysis were performed using MEGA5 software (Tamura et al., 2011).

RESULTS AND DISCUSSION

The mtDNA 147 bp *12S rRNA* region of *A. fuciphagus* revealed 7 polymorphic sites (Tab1). Five haplotype were detected of which haplotype 1 found in all locations accounted for 82.14% of the population. The remaining haplotypes were unique to one location each. The genetic diversity of *12S rRNA* in *A.fuciphagus* is shown in Tab 2. Neighbour Joining tree constructed for the *12S rRNA* locus shows no clear evidence of geographical clustering. Samples retrieved from GenBank for comparison showed consistency in the branching pattern (Fig1).

They could not be distinguished based on location as the most frequent haplotype was common in all the locations. This is not surprising as slowly evolving regions such as *12S rRNA* exhibit no differentiation among individuals. The Tajuma's D and Fu's and Li's D did not show any genetic structure among locations indicating possible departure from neutral expectation. It could mean that sample size is small or the population had not reached equilibrium with most mutation tending to be unique to a single lineage. Also, negative values were also reported in assessment of mtDNA *cytb* and *ND5* gene loci of the same species (Adebayo et al., 2021). However, pairs of larger colonies in Thailand (Aowphol et.al., 2008), indicated higher genetic variation within those colonies than between colonies and stronger evidence that there is gene flow among sampling locations

Table 1. Haplotypes showing the polymorphic sites, base polymorphism and distribution across location in MtDNA *12S rRNA* region

	Position							Frequency							
Haplotype	45	55	75	86	96	99	105	SP	TM	KT	GM	PT	KG	Total No of samples	% of haplotype
1	T	A	T	C	C	A	C	5	2	5	3	3	5	23	82.14
2	C	.	G	T	G	G	T		1					1	3.57
3	.	C	.	.	.	.	.		2					2	7.14
4	C	.	.	.	.	.	.				1			1	3.57
5	C	G	.	T	.	C	A				1			1	3.57
Total no of haplotype								1	3	1	3	1	1	28	100
H.tahitica	C	.	.	C	C	A	T							3	

KG- Kota Tinggi. A – adenine, C – cytosine, G- guanine, T – thymine. *H. tahitica* – outgroup. Position numbering based on the sequences generated in this study.

SP- Sungkai, TM- Tanah Merah, GM- Gua Madai and KT- Kuala Terengganu, PT- Pitas,

Table 2. Genetic diversity indices for the MtDNA *12S rRNA* region in six populations across Malaysia.

Population	n	Polymorphic site	Number of haplotype	Haplotype Diversity (H)	Nucleotide diversity (π)	Tajima's D	Fu and Li's D
Sungkai Perak	5	0	1	0.000	0.000	-	-
Kuala Terengganu	5	0	1	0.000	0.000	-	-
Gua Madai	5	2	3	0.700	0.008	-0.973	-0.973
Tanah Merah	5	7	3	0.800	0.037	-0.913	-0.913
Pitas	3	0	1	0.000	0.000	-	-
Kota Tinggi	5	1	1	0.400	0.004	-0.817	-0.817
Total	28	8	5	0.328	0.012	-1.192	-0.540

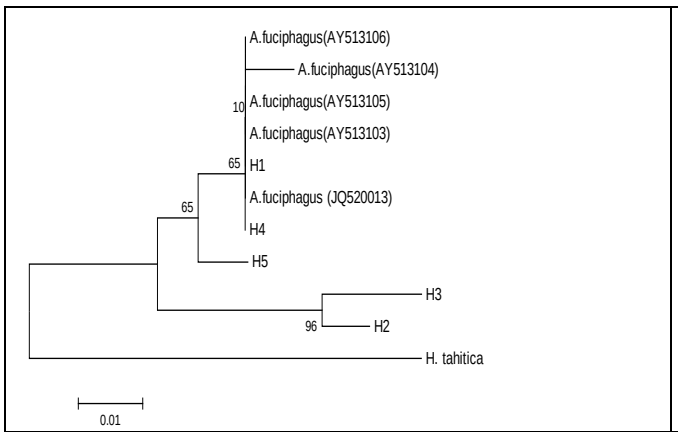

Fig 1. Neighbour Joining phylogenetic trees for the mtDNA *12SrRNA* gene locus of five haplotypes and *A. fuciphagus* retrieved from GenBank. Numbers indicate the Percentage of confidence level for each node estimated at 1000 bootstrap value. *H. tahitica* (outgroup).

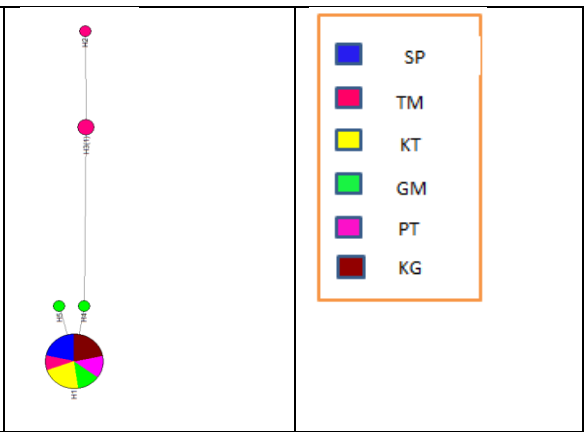

Fig 2. Median Joining Network constructed for the haplotypes obtained from *12S rRNA* gene locus of *A. fuciphagus* (Blue – Sungkai Perak, Pink – Tanah Merah, Yellow – Kuala Terengganu, Green – Gua Madai, Purple – Pitas, Brown – Kota Tinggi).

The median joining network analysis (Fig 2) revealed that other haplotype branched out from the most common haplotype in the locus showing a simple tree network. Similar report was made on the 12SrRNA gene

fragment in *D. Major* (Nakamura et al, 2002). They observed three sequences of D. major had less than 0.6% differences among them. Thus intraspecific variation would present little consequence to the specie- specific amplification of the *12S rRNA* gene fragment observed although such a variation needs to be clarified using a larger number of EBN swiftlet.

CONCLUSION AND RECOMMENDATION

Information on the genetic variability of *12S rRNA* could not be used for differentiation on geographical level. The population structure of EBN swiftlet should also be assessed using nuclear markers, such as microsatellites and SNPs in nuclear DNA region.

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