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*Research Article*

# Genetic Variations of Malaysian And Golden Thai Strains of Climbing Perch and Their Hybrids Based On The Partial Mitochondrial Cytochrome Oxidase Subunit 1 Gene

**Awawu Dasuki[1,2]*, Yuzine Esa[1], Annie Christianus[1] and Mohammad Fadhil Syukri Ismail[1]**

1. Department of Aquaculture, Faculty of Agriculture, University Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia
2. Department of Fisheries and Aquaculture, Faculty of Renewable Natural Resources, Federal University Dutsin-Ma, PMB 5001 Dutsin-Ma, Katsina State, Nigeria
Corresponding author: adasuki@fudutsinma.edu.ng

**ABSTRACT**

Rapid genetic improvements from selective breeding are anticipated in many aquaculture species and mitogenome is used to complement the morphological taxonomy of hybrids to evaluate its genetic structures. A study of the genetic variations within the two strains (Malaysian strain and Golden Thai strain) of *Anabas testudineus* (Bloch, 1792) and their hybrids, phylogenetic trees based on cytochrome oxidase subunit 1 (CO1) of the partial mitochondrial DNA gene were constructed using Maximum Likelihood and Neighbor Joining approaches. The findings support the monophyletic status of the genus with only one haplogroup from which other haplotypes were descended and of a single common ancestor. The individual fishes' phylogenetic relationships revealed two major clades and *Saurida undosquamis* as an outgroup. All the groups had high haplotype diversity, except for the hybrid Malaysian strain × golden Thai strain (0.1540±0.126). This suggests that different fish species in each of these studies had different nucleotide compositions in their mitochondrial genomes. The highest number of haplotypes and the presence of distinct haplotypes in the Golden Thai strain × Malaysian strain hybrid point to the absence of recent or regular gene flow as well as high genetic diversity within the hybrids. This study demonstrated that the mtDNA diversity of *Anabas testudineus* from Malaysia and Thailand had been preserved. Studies on the population genetic diversity and molecular evolution of anabas fish species can benefit from the data provided by this work.

**Key words:** *Anabas testudineus*, haplogroup, mitochondrial genome, phylogenetic relationship

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## INTRODUCTION

There is a definite trend toward achieving food security, as evidenced by the variety of aquaculture species and culture models. (Li, 2015). As a result, it is important to research culturable species that are present in a continent's territorial waters. Having a detailed grasp of the state of resources and their trends as a result of their usage such as the Malaysian indigenous strain *Anabas testudineus*, demonstrating interesting aquaculture potential, is a fundamental first step towards sustainable aquaculture. Farmed fish are included in this category due to genetic interactions between indigenous lines and genetically improved lines for hybrid production.

Rapid genetic improvements from selective breeding are anticipated in many aquaculture species with high fecundity and significant phenetic and genetic variation. The rate of gene flow from the two strains can be determined. However, before genetic improvements through selection can be effectively achieved, there is a need to study genetic variations within the two strains and their hybrids. This will provide a better understanding of the potential strains.

DNA barcoding technique is used to understand the genetic variation and clarification of fish species in addition to morphological techniques (Hebert *et al*., 2016) because it is a cost-effective technique for species identification, thus, its application is fairly widespread. Using mitogenome to complement the morphological taxonomy of hybrids helps to evaluate its genetic structure; the mitochondrial cytochrome oxidase subunit 1 (CO1) gene is employed due to its global usage as a bio identification system for both vertebrates and invertebrates (Clare *et al*., 2007; Whalan *et al*., 2008; Wang *et al.,* 2021). Meanwhile, the CO1 gene was used to define boundaries, providing a benchmark for future management and conservation of *A. testudineus*. The genetic diversity of *A. testudineus* that forms the domestication of its gene pool however,

is poorly understood. The careful selection of suitable natural stocks based on genetic knowledge can greatly enhance natural population management, species restoration, and aquaculture production. (Quattro & Vrijenhoek, 1989). Thus, using the mitochondrial cytochrome oxidase subunit 1 gene, this study aimed to comprehend the genetic variation between two strains of *A. testudineus* and their hybrids.

## MATERIALS AND METHODS

### Sample collection

Fin clips (caudal region) from two morphologically distinct forms of *Anabas testudineus* (the Malaysian strains and the Golden Thailand strains) phenotype-groups and their hybrids were analysed. A total of 48 representative specimens were chosen where four groups were established: Golden Thai strain GT (♀) × Golden Thai strain GT(♂); Golden Thai strain GT(♀) × Malaysia strain M(♂); Malaysian strain M(♀) × Malaysia strain M(♂) and Malaysian strain M(♀) × Golden Thai strain GT(♂) were performed using PCR amplification. Fin samples were stored in 1.5 ml micro-tubes using 95 % molecular Biology grade Ethanol. All fish used were subjected to the animal and experimental protocol (R034/2019) as approved by the Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia (UPM).

### DNA extraction, amplification and sequencing

The Genomic DNA was extracted from the fin clippings of each fish individual using Promega, USA, (animal tissues kit) following the manufacturer's instructions. The extractions were used to amplify a partial segment of the mitochondrial DNA (cytochrome oxidase subunit 1) using the published primers given in (Table 1). PCR reactions were performed with a total volume of 25 µL cocktail including 2 µL (100ng) DNA template, 12.5 µL of MyTaq HS Red Mix PCR kit (Bio line, UK), 2 µL of 2 mM $MgCl_2$, 1.5 µL of 0.6 µM of each primer and 5.5 µL double distilled water (ddH2O) with negative control. Thermo cycler (Eppendorf, USA) was used under the following temperature program: initial denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s with a final extension of 5 min at 72 °C as modified from Arisuryanti *et al*. (2019). In other cases, electrophoresis was performed on a 1 % agarose gel to confirm the quality of the PCR product; the product was immersed in gelRed solution (40 ml dH2O + 10 µL gelRed) for 30 min and bands were visualized under a UV transilluminator. The amplicons were sequenced uni-directionally using forward primers at APICAL SCIENTIFIC Laboratories Sdn. Bhd, Selangor, Malaysia.

### Sequence data analysis

The obtained sequences and chromatograms were checked using the BioEdit v7.2.3 software and the sequence were trimmed (Hall *et al*., 1999). The Basic Local Alignment Search Tool Nucleotide (BlastN) utilized by the National Centre for Biotechnology Information (NCBI) was used to identify the edited sequences. By comparing their applicable identities with previously published sequences of the same species that were available in the system, all sequence reads were examined for potential contamination and species identity. Thereafter, multiple sequence alignments were carried out utilizing ClustalX (2.0.10) software (Larkin *et al*., 2007). The aligned sequences' gaps were excluded from the analysis that preceded. DnaSP v6 software was used to assess the polymorphism, nucleotide diversity, haplotype diversity, and standard deviation values for groups of genes in the CO1 region (Rozas *et al*., 2017). A Maximum-likelihood tree was generated using Molecular Evolutionary Genetics Analysis (MEGA) X (Kumar *et al*., 2018) and the GTR model and bootstrap tests (1000 replications).

As an outgroup, four sequences from the mtDNA COI region of the fish *Saurida undosquamis* (MT511732, MT511735-MT511737) were used to demonstrate association with *S. undosquamis*. To identify possible phylogenetic clades, FST distances were generated and used to construct a Neighbor-joining trees of all genetic groups and *S. undosquamis* as outgroup. A medium-joining network was generated by using PopART to visualize the relationships between the haplotypes of all *A. testudineus* (Leigh & Bryant, 2015). This is done to further explore possible relationships between pedigrees. To determine the selective pressure acting on the COI gene in these gene groups, Tajima's D (Tajima, 1989), Fu's Fs (Fu, 1996), and the non-synonymous and synonymous substitutions (dN/dS) were calculated in the hybrids with their parents using DnaSP. Codon-based neutrality tests for each group of genes were performed on MEGA X. Using the BankIt submission tool, a dataset with 37 sequences was submitted to the GenBank database with the accession numbers MT511547-MT511583.

## RESULTS

### Mitochondrial DNA sequence variations

Sequences from 37 individuals of *Anabas testudineus* comprising of Golden Thai strain (5), Malaysian strain (8), Golden Thai × Malaysian hybrid (12) and Malaysian × Golden Thai hybrid (12) respectively were obtained.

The Malaysian line x Golden Thai line hybrids inherited 98.4 % to 98.6 % of all nucleotide positions from the maternal line (Malaysian line). Conversely, 97.5 % to 97.7 % of all nucleotide positions were inherited from the paternal parent (Thai strain). In hybrids of the Golden Thai strain × the Malaysian strain, 7.9 % to 25.1 % of all nucleotide positions were mutated relative to the maternal parent (Thai strain), and 7.0 % to 24. 6 % mutations of all nucleotide positions have mutated relative to the paternal parent (Thai strain).

The final sequence length was 657 base pair after alignment and trimming, with 112 polymorphic sites (17.01 %), 5 sites with little information (0.008 %), and 401 sites (61.04 %) with conserved sites. Average total nucleotide composition C = 28.35 %, T = 31 94 %, A = 21.69 %, G = 18.03 % (Table 2). Using a one-way ANOVA, the results showed that the percentage distribution of nucleotides was not significantly different ($p > 0.05$) between gene groups in the *A. testudineus* population.

The G-C content of the gene is 46.38 %. Meanwhile, in order to exclude the existence of ambiguous gene groups, pairwise analysis was performed within each gene group to show the highest identity within the hybrid of the Malaysian strain and the Golden Thai strain (100-100 %), while, the lowest identity was recorded in the golden Thai strain × the Malaysian strain hybrids (76.6-99.6 %) as shown in (Table 3). Individuals from the

Thai strain had the highest (98.7-99.0 %) nucleotide identity with members of the Malaysian strain, while the Golden Thai strain × Malaysian strain group had the lowest (74.9-92.1 %) nucleotide identity with Thai strain fish.

Table 4 shows the standard diversity index values for *Anabas testudineus* mtDNA analysis. A total of seven haplotypes were observed in the genetic group. The number of haplotypes in the genetic group was highest in Golden Thai strain × Malaysian strain hybrids (6) and lowest in Malaysian strain × Golden Thai strain hybrids (2). Haplotype diversity was high in all groups except for the hybrids of the Malaysian strain × the Golden Thai strain (0.154 ± 0.126). However, low nucleotide diversity values were observed in all groups. All the Fu's Fs values obtained for each gene group were insignificant, whereas Tajima's D values were negative and significant except for the Malaysian strain. The ratio of non-synonymous and synonymous substitutions for each gene group was higher than three, but not significantly different ($p>0.05$). The distribution of haplotypes shows that haplotype 1 (Hap-1) is the most abundant and common of the four genetic groups. Five haplotypes (3, 4, 5, 6 and 7) were found only in Golden Thai strain × Malaysian strain hybrids. Haplotypes 2, 3, 6 and 7 had the lowest number of individuals (Table 5).

The phylogenetic relationships of individual fish revealed two major clades (Figure 1). The four nucleotide sequences from *S. undosquamis* formed the first clade as an outgroup. The second clade consists of 37 *A. testudineus* sequences. Among the genetic groups, two main sub-lineages have been formed.

The first sub-clade showed that hybrid anabas was initially similar to all *A. testudineus* in Thailand, excluding two individuals classified into the second sub-clade (MT511575 and MT511576). The second sub-clade showed that the hybrid anabas were similar to all *A. testudineus* in Malaysia with the exception of one individual (MT511581) grouped with the first sub-clade.

According to the pairwise FST distances (Figure 2), the tree shows that all the genetic groups are derived from a common ancestor. However, the Thai × Malaysia hybrid diverged first. It was followed by a Malaysian × Thai hybrid and finally Thai strain and Malaysian strain. Although the hybrids are separated, they are genetically closer. Figure 3 shows the medium-joining network of the haplotypes observed among the gene groups. The network indicates that the seven haplotypes are linked into one major haplotype group.

## DISCUSSIONS

**Table 1.** Primer sequences used to amplify mtDNA cytochrome oxidase subunit 1 in two strains of fish

| Sequence | Expected Size | Reference |
|---|---|---|
| FishF2 5'-TCGACTAATCATAAAGATATCGGCAC-3'<br>FishR2 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3' | 650bp | (Ward *et al*., 2005) |

**Table 2.** Percentage mtDNA sequence nucleotide composition of *Anabas testudineus*

| Genetic group/ Nucleotide | A | C | G | T | Total |
|---|---|---|---|---|---|
| Malaysian | 21.61 | 28.14 | 18.52 | 31.73 | 100 |
| Thai | 21.56 | 28.26 | 18.35 | 31.83 | 100 |
| Malaysian strain × golden Thai strain | 21.46 | 28.16 | 18.42 | 31.96 | 100 |
| Golden Thai strain × Malaysian strain | 22.13 | 28.83 | 16.82 | 32.22 | 100 |

**Table 3.** Percentage pairwise similarities among the mtDNA COI sequences of *Anabas testudineus* populations

| | Malaysian | Golden Thai | Malaysian strain × golden Thai strain | Golden Thai strain × Malaysian strain |
|---|---|---|---|---|
| Malaysian | 99.6-100 | | | |
| Thai | 98.7-99.0 | 99.6-100 | | |
| Malaysian strain × golden Thai strain | 98.4-98.6 | 97.5-97.7 | 100-100 | |
| Golden Thai strain × Malaysian strain | 75.4-93.0 | 74.9-92.1 | 76.8-94.0 | 76.6-99.6 |

**Table 4.** Genetic diversity indices of four genetic groups of *Anabas testudineus*

| Genetic group | N | Nh | h±sd | ʎ±sd | dN/dS | Fu's Fs | Tajima's D |
|---|---|---|---|---|---|---|---|
| Thai | 5 | 1 | 0.470±0.218 | 0.0015±0.0005 | $3.49^{NS}$ | -0.146 | -2.759*** |
| Malaysian | 8 | 2 | 0.679±0.121 | 0.0013±0.0004 | $3.47^{NS}$ | -0.146 | $2.420^{NS}$ |
| Golden Thai strain x Malaysian strain | 12 | 6 | 0.800±0.114 | 0.0083±0.0009 | $3.46^{NS}$ | 0.341 | -2.759*** |
| Malaysian strain x golden Thai strain | 12 | 1 | 0.154±0.126 | 0.0390±0.0320 | $3.55^{NS}$ | 5.704 | -2.759*** |
| Total | 37 | 7 | 0.387±0.101 | 0.0134±0.0098 | | | |

N: Number of sampled individuals, Nh: Number of haplotypes, h: Haplotype diversity, sd: Standard deviation, ʎ: Nucleotide diversity, dN/dS: non-synonymous and synonymous substitution rate, NS: Not significant, ***p <0.001

**Table 5.** Distribution of mtDNA Haplotypes of *Anabas testudineus* CO1 sequences

| Haplotype Name | Frequency | Members |
|---|---|---|
| Hap_1 | 29 | MT511571_Malaysian_strain, MT511572_Malaysian_strain, MT511581_Malaysian_strain, MT511583_Malaysian_strain, MT511580_Malaysian_strain, MT511579_Thai_strain, MT511578_Thai_strain, MT511577_Thai_strain, MT511576_Thai_strain, MT511575_Thai_strain, MT511574_Malaysian_strain, MT511573_Malaysian_strain, MT511570_MalaysianXThai_hybrid, MT511569_MalaysianXThai_hybrid, MT511568_MalaysianXThai_hybrid, MT511567_MalaysianXThai_hybrid, MT511566_MalaysianXThai_hybrid, MT511565_MalaysianXThai_hybrid, MT511564_MalaysianXThai_hybrid, MT511563_MalaysianXThai_hybrid, MT511562_MalaysianXThai_hybrid, MT511561_MalaysianXThai_hybrid, MT511560_MalaysianXThai_hybrid, MT511559_MalaysianXThai_hybrid, MT511555_ThaiXMalaysian strain_hybrid, MT511556_ThaiXMalaysian _hybrid, MT511557_ ThaiXMalaysian_hybrid, MT511550_ ThaiXMalaysian_hybrid, MT511554_ ThaiXMalaysian_hybrid |
| Hap_2 | 1 | MT511582_Malaysian_strain |
| Hap_3 | 1 | MT511558_ ThaiXMalaysian_hybrid |
| Hap_4 | 2 | MT511551_ThaiXMalaysian_hybrid, MT511553_Golden ThaiXMalaysian_hybrid |
| Hap_5 | 2 | MT511552_ ThaiXMalaysian_hybrid, MT511548_ ThaiXMalaysian_hybrid |
| Hap_6 | 1 | MT511549_ ThaiXMalaysian_hybrid |
| Hap_7 | 1 | MT511547_ ThaiXMalaysian_hybrid, |

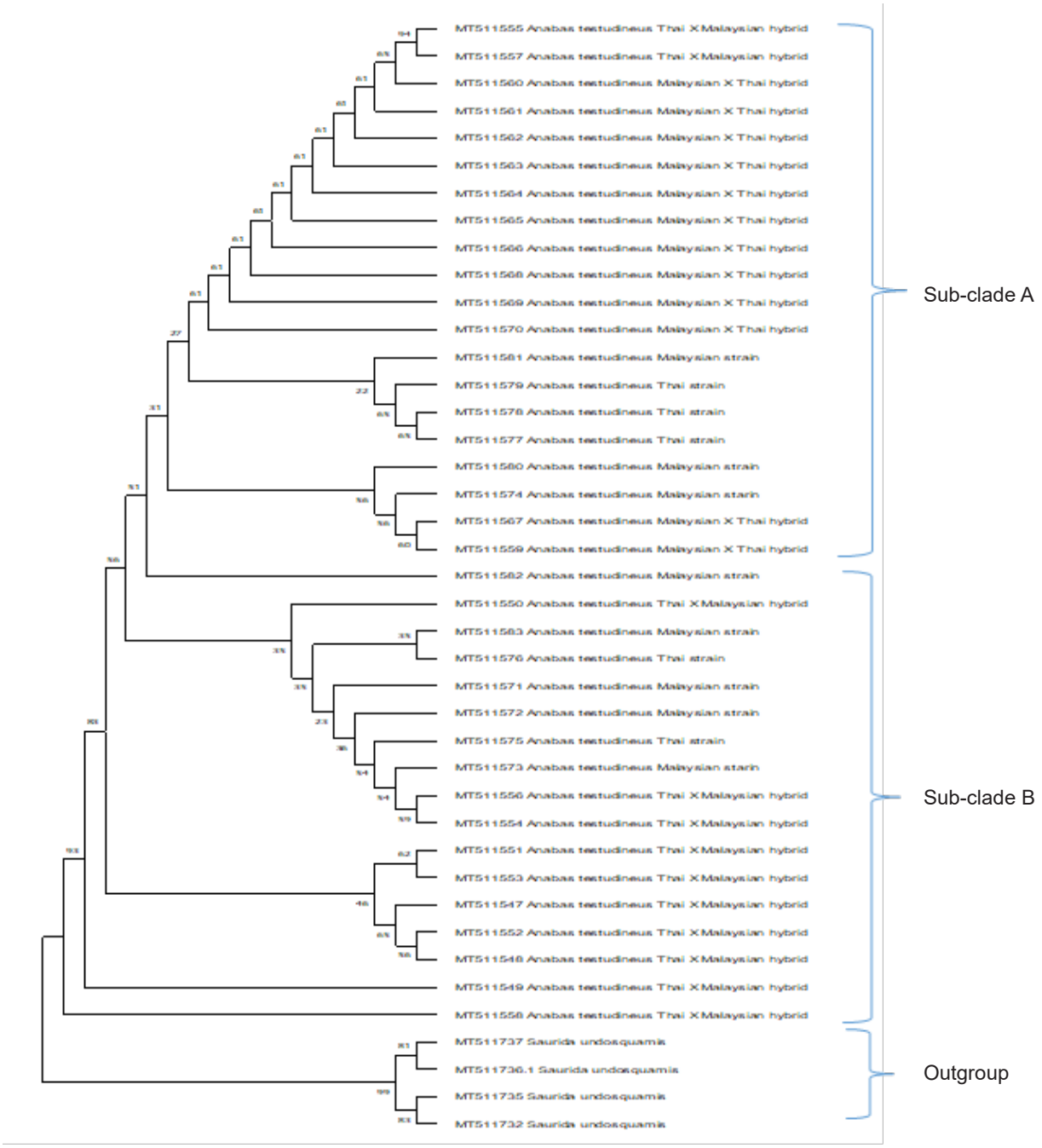


**Fig. 1**. Maximum likelihood tree of 37 individuals constructed under the GTR model and an LRT test.

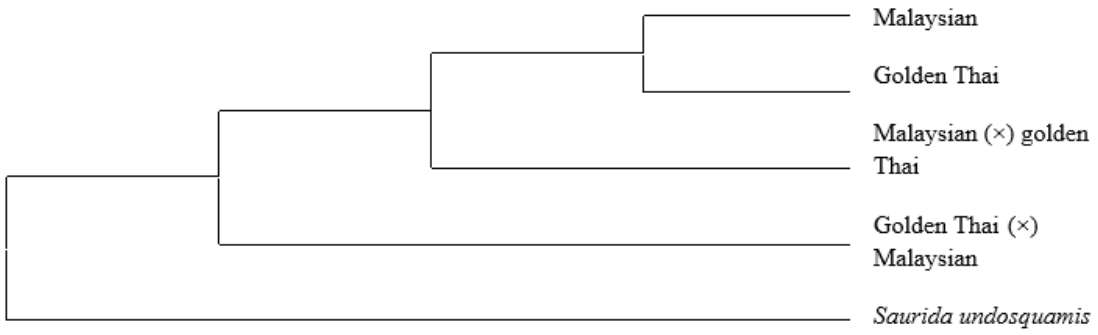


**Fig. 2.** A NJ tree constructed on FST distances of all the genetic groups and *S. undosquamis.*

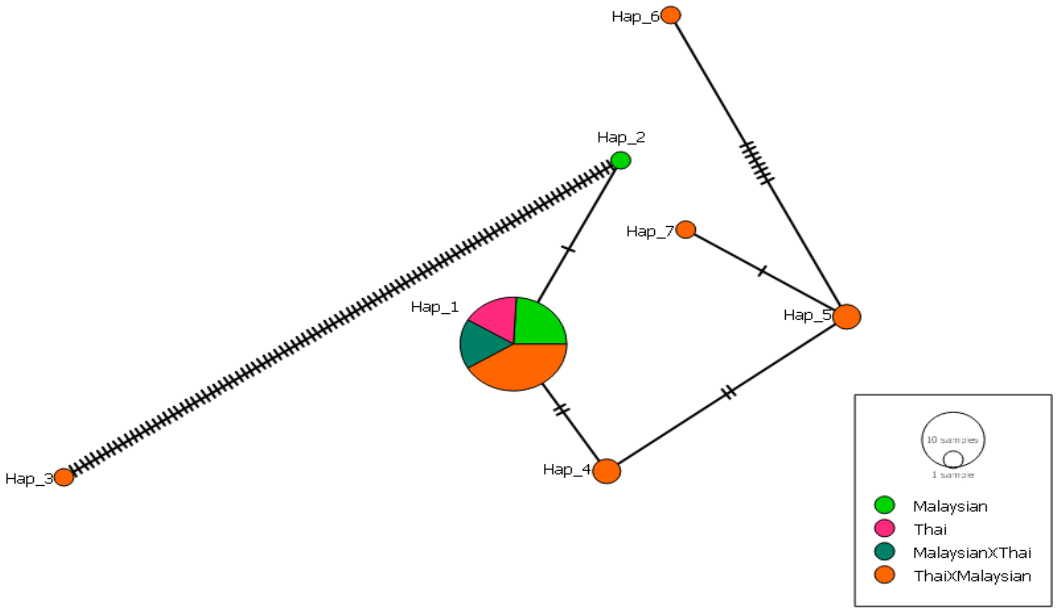


**Fig. 3.** A MJ network analysis of all haplotypes found in the genetic groups.

The mitogenome derivatives in this study was A+T rich. In all gene groups, a consistent pattern of nucleotide composition distribution was observed: T > C > A > G. This reveals that *Anabas testudineus* had a strand bias towards T in all gene groups studied. Chen *et al*. (2016) reported a comparative nucleotide frequencies similar with those obtained in this study while recording the basic composition of comparable patterns (T > C > A > G) for *Brama japonica* Lakra *et al*. (2011) found a similar trend in the nucleotide composition of six Scombrid genera, where T > C > A > G is the overall relative composition of the nucleotides. However, this result is not in line with the default percentage configuration A > G > T > C for *Channa striata* found in Thailand (Boonkusol & Tongbai, 2016). Also, Persis *et al*. (2009) reported the frequency of T > A > C > G nucleotides in CO1 mtDNA from Indian carangid fishes. This means that the nucleotide composition of the mitochondrial genome varies among fish species in all of these studies. The observed variation may be due to the high mutation rate at the mtDNA CO1 locus (Saccone *et al*., 1999). The mitochondrial cytochrome oxidase subunit 1 (CO1) sequences of hybrids and parent strains are very similar to those available on GenBank.

All members of Thai strain lineage and hybrids of Malaysian strain × golden Thai strain had the same haplotype. However, one member of the Malaysian lineage showed distinct haplotypes not seen in other genetic groups. The presence of distinct haplotypes and the greatest number of haplotypes in the Golden Thai × Malaysian hybrids suggests a high genetic diversity of the hybrids as well as a lack of regular or recent gene flow. These results revealed that the mtDNA was more stable in the Malaysian strain × golden Thai strain hybrid compared to the Golden Thai strain × Malaysian strain hybrid. Considering the shared haplotype (Hap-1) between Malaysian strain × golden Thai strain hybrid and Thai strain, as well as a low mutation between Malaysian strain × golden Thai strain hybrid and Thai strain, it implies that CO1 is more of paternal inheritance in Golden Thai strain

× Malaysian strain hybrid but maternal inheritance in Malaysian strain × golden Thai strain hybrid as inferred from the present study.

The CO1 sequences revealed only one haplogroup from which other haplotypes were derived. The haplogroup infers that all genetic groups originate from a common ancestor. The clustering of all into one major clade suggests the existence of one species with a common maternal mtDNA gene (Scribner *et al*., 2000). This monophyletic trait is also found in other perciformes such as Gobies (Tabassum *et al*., 2016). The low overall FST observed in this study suggests little genetic differentiation between hybrids and their parents. It may be due to conscious human intervention or it may be otherwise dispersed. However, variation within and between species or lineages is greater in fish than in other vertebrates. This study demonstrated that the mtDNA diversities of *Anabas testudineus* from Malaysia and Thailand were preserved based on the findings from Tajima's D and Fu's Fs. However, in order to develop new preservation strategies in accordance with these findings, further analysis should be done using alternative molecular markers and comparisons.

## CONCLUSIONS

The resulting outcome reveals that the CO1 gene displayed a monophyletic status at the genus level with only one haplogroup, however, sharing of haplotypes between the two strains showed a sensitivity to the CO1 marker inferring bio-geographical history and that sustainable management of the indigenous population and hybridization of this native fish may help with domestication-conservation strategy. Additional studies may be conducted with more locations and sampling sites.

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## ETHICAL STATEMENT

All fish used were subjected to the animal and experimental protocol (R034/2019) as approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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