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# GENETIC RELATIONSHIPS OF THE ANABAS (ANABAS SP.) USING MITOCHONDRIAL CYTOCHROME OXIDASE SUBUNIT I MARKER GENE

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## Abstract

Total DNA of the anabas specimens (A1, A2, A3, A4) in Vietnam were extracted using PHUSA-IHHNV kit and according to the process of Phu Sa Biochemical Company. The mitochondrial cytochrome oxidase subunit I (COI) gene were amplified using PCR reaction. The obtained PCR products were checked using 2 % agarose gel electrophoresis and were sequenced with both the forward and reverse primers using an automated sequencer ABI 3730XL of Applied Biosystems by Sangers method. The genetic distances ($D_{ij}$) between the anabas species in Vietnam (A1, A2, A3, A4) are very small from 0.0046 to 0.0156 and they are all in group 4 with *Anabas testudineus* in Malaysia so they are the same *Anabas testudineus.* The researched species of Anabas genus in Vietnam (A1, A2, A3, A4) and some countries in South and Southeast Asia were divided into four groups based on COI marker gene. The groups with far genetic distance include group 2 and group 4 ($D_{24} = 0.1055$), group 1 and group 4 ($D_{14} = 0.1041$), group 1 and group 3 ($D_{13} = 0.0910$). The groups with medium genetic distance include group 2 and group 3 ($D_{23} = 0.0849$), group 1 and group 2 ($D_{12} = 0.0772$). The groups with near genetic distance include group 3 and group 4 ($D_{34} = 0.0285$). The species *Anabas testudineus* in India and *Anabas testudineus* in Myanmar should be re-identified *Anabas cobojius* in India and *Anabas cobojius* in Myanmar belong to group 1.

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**Key words:** Anabas, Cytochrome oxidase I gene, Genetic relationships, Phylogeny tree.

## Background

The *Anabas* genus includes two species of the climbing perch (Anabas testudineus Bloch, 1792) [16], see Figure 1 and the gangetic koi (*Anabas cobojius* Hamilton, 1822), see Figure 2. They are called the anabas. Living areas of the anabas are South and Southeast Asia including India, Sri Lanka, Bangladesh, Myanmar, Malaysia, Thailand, Vietnam and Philippines. They can survive in brackish and freshwater environments [22].

Figure 1. *Anabas testudineus*

Figure 2. *Anabas cobojius*

The anabas is an aquaculture species with many prospects because they are likely to like high suspicion due to the respiratory organs on the gills. The aquaculture of anabas is developing strongly in the Mekong Delta. Therefore it is necessary to classify several species of Anabas genus in Vietnam and identify the names of these species.

The complete mitochondrial genome of *Anabas testudineus* was sequenced with length as 16,603 bp including 13 protein coding genes, 2 ribosomal RNA coding genes, 22 transfer RNA coding genes and a region control [23].

The research of species identification using molecular biological methods in the world has applied in genetic checking and genetic modification [7]. For many species, some genes of mitochondrial genome (mitochondrial DNA), such as COI (cytochrome oxidase subunit I), NADI (nicotinamide dehydrogenase subunit I), COB (cytochrome b) and gene of nuclear genome (nuclear DNA) ITS-2 (internal transcribed spacer 2) which has been considered important molecular markers for species identification and classification [3-5, 7, 8]. However, mitochondrial genes evolve faster nuclear genes. Therefore, mitochondrial genes are often used to analyze the type-generation relationship for low-level taxa such as familia, subfamilia, genus, species, subspecies. In which the COI gene evolved rapidly and was used to analyze the relationship between species of genus or subspecies [19]. In this research, the genetic relationships of the anabas species in Vietnam and neighboring countries were researched using molecular biology method based on COI marker gene to supplement reliable information in the identification and classification of the species of this genus.

# 1. Materials and methods

## 1.1. Sample collection and DNA extraction

The anabas specimens were collected in Vietnam including Tri An reservoir, Dong Nai province (specimens A1); Can Gio district, Ho Chi Minh city (specimens A2); Vam Co Dong river, Long An province (specimens A3) and Dau Tieng lake, Tay Ninh province (specimens A4) by gill net. These specimens were processed and stored at -$20^0$C for total DNA extraction. Total DNA was extracted from fish tissue by PHUSA-IHHNV kit according to the procedure of Phu Sa Biochemical Company. Concentration and purity of total DNA were checked using UV-VIS molecular absorption spectrometer of Bio-Rad measuring two wavelengths 260 nm and 280 nm.

## 1.2. Amplify, purify and sequencing

Forward primer (5'-AACCCAAAAGACATTGGCACC-3') and reverse primer (5'-GGCCAAAGAATCAAAACAAGT-3') used to amplify mitochondrial cytochrome oxidase subunit I(COI) gene were designed using Primer-BLAST tool of the National Center for Biotechnology Information (NCBI) and were synthesized at Oligo laboratory of Phu Sa Biochemical Company. The thermocycler conditions were slightly modified as follows: 1 initial cycle of 3 minute at $96^0$C followed by 35 cycles of $96^0$C for 15 seconds and $52^0$C for 15 seconds, $60^0$C for 4 seconds. This is followed by a final step of $4^0$C for 5 minutes [10].

The PCR products were checked using 2 % agarose gel electrophoresis and were sequenced with both the forward and reverse primers using an automated sequencer ABI 3730XL of Applied Biosystems by Sangers method [18]. The bands of PCR product must be clear, the width of the bands is large and size about 650 bp as successful amplification reaction. Then, the sequences of COI gene were edited using GENtle version 1.9.4 software and Nucleotide BLAST tool of NCBI.

## 1.3. Phylogenetic analysis

Phylogenetic analysis was conducted based on the multiple sequence alignment of COI gene of the anabas in Vietnam (A1, A2, A3, A4) and the anabas in India, Bangladesh, Myanmar, Malaysia, Thailand and Philippines collected from GenBank database (Table 2) using ClustalW algorithm [2, 9]. The genetic distances were computed using the Maximum Composite Likelihood method [20]. Phylogenetic tree was built using Neighbor - Joining Tree algorithm [17]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [1]. Evolutionary analyses were conducted in MEGA version 10.0.4 software [6].

## 1.4. Genetic relationships analysis

The groups were classified based on phylogenetic tree and genetic distance between groups ($D_{ij}$). From there, the groups were identified with far, medium and near genetic distances. Single nucleotide polymorphism (SNP) sites were analyzed based on pairwise sequence alignment of COI gene of the anabas in same group or other group using DnaSP version 6.12.01 software. SNP ratio $K_{ij}$ (%) of the anabas i and j is computed by equation (1) [10].

$$K_{ij} = \frac{100S_{ij}}{N_{ij}}$$

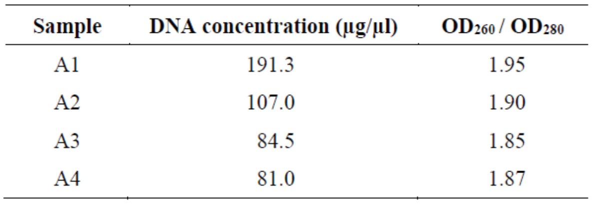
Where $S_{ij}$ is the number of SNP sites; $N_{ij}$ is the total number of sites in pairwise sequence alignment of COI gene of the anabas species $i$ and $j$.

# 2. Results and discussion

## 2.1 Total DNA extraction

The checking results of total DNA samples are high concentrations from 81.0 to 191.3 g/ml. Absorption rates at wavelengths of 260 nm and 280 nm ($OD_{260}$ / $OD_{280}$) are within the limits of 1.8 to 2.0, indicating that good pure DNA samples are not contaminated with proteins and organic substances (Table 1).

Table 1. Results of checking the concentration and purity of total DNA

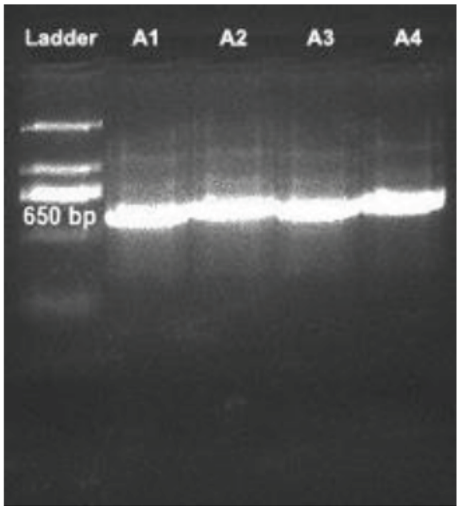


| Sample | DNA concentration (µg/µl) | $OD_{260}$ / $OD_{280}$ |
|---|---|---|
| A1 | 191.3 | 1.95 |
| A2 | 107.0 | 1.90 |
| A3 | 84.5 | 1.85 |
| A4 | 81.0 | 1.87 |

## 2.2. Sequencing of COI genes

The COI genes were successfully amplified by PCR. The PCR products were checked using 2 % agarose gel electrophoresis obtained the clear and wide DNA bands of about 650 bp (Figure 3). The COI genes of A1, A2, A3 and A4 specimens were sequenced and were submitted in GenBank of NCBI having respectively accession number MK351908, MK368519, MK368520 and MK368521 for world-wide publish [11, 12, 13, 14].

Figure 3. Results of PCR products electrophoresis



## 2.3. Genetic relationships analysis

The genetic distance matrix (Figure 4) and the phylogenetic tree (Figure 5) were established using multiple sequence alignment of COI gene of the anabas (Table 2).

Table 2. List of the anabas for multiple sequence alignment of COI gene

In terms of genetic distance between groups $D_{ij} < 0.020$, the researched anabas are classified into four groups:

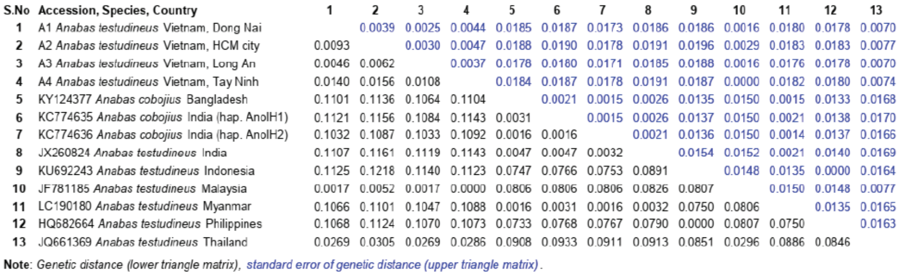
Group 1: *Anabas testudineus* India (JX260824), *Anabas testudineus* Myanmar (LC190180), *Anabas cobojius* Bangladesh (KY124377), *Anabas cobojius* India hap.AnoH1 (KC774635), *Anabas cobojius* India hap.AnoH2 (KC774636);

Group 2: *Anabas testudineus* Indonesia (KU692243), *Anabas testudineus* Philippines (HQ682664);

Group 3: *Anabas testudineus* Thailand (JQ661369);

Group 4: *Anabas testudineus* Vietnam (A1, A2, A3, A4), *Anabas testudineus* Malaysia (JF781185).

*Figure 4. Distance and standard error matrix of the anabas based on COI marker gene*

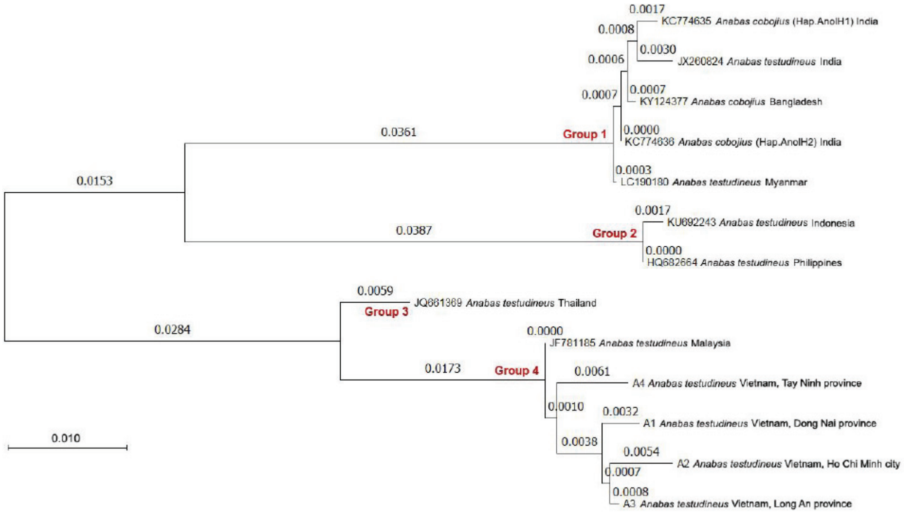


| S.No | Accession, Species, Country | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | A1 *Anabas testudineus* Vietnam, Dong Nai | | 0.0039 | 0.0025 | 0.0044 | 0.0185 | 0.0187 | 0.0173 | 0.0186 | 0.0186 | 0.0016 | 0.0180 | 0.0178 | 0.0070 |
| 2 | A2 *Anabas testudineus* Vietnam, HCM city | 0.0093 | | 0.0030 | 0.0047 | 0.0188 | 0.0190 | 0.0178 | 0.0191 | 0.0196 | 0.0029 | 0.0183 | 0.0183 | 0.0077 |
| 3 | A3 *Anabas testudineus* Vietnam, Long An | 0.0046 | 0.0062 | | 0.0037 | 0.0178 | 0.0180 | 0.0171 | 0.0185 | 0.0188 | 0.0016 | 0.0176 | 0.0178 | 0.0070 |
| 4 | A4 *Anabas testudineus* Vietnam, Tay Ninh | 0.0140 | 0.0156 | 0.0108 | | 0.0184 | 0.0187 | 0.0178 | 0.0191 | 0.0187 | 0.0000 | 0.0182 | 0.0180 | 0.0074 |
| 5 | KY124377 *Anabas cobojius* Bangladesh | 0.1101 | 0.1136 | 0.1064 | 0.1104 | | 0.0021 | 0.0015 | 0.0026 | 0.0135 | 0.0150 | 0.0015 | 0.0133 | 0.0168 |
| 6 | KC774635 *Anabas cobojius* India (hap. AnolH1) | 0.1121 | 0.1156 | 0.1084 | 0.1143 | 0.0031 | | 0.0015 | 0.0026 | 0.0137 | 0.0150 | 0.0021 | 0.0138 | 0.0170 |
| 7 | KC774636 *Anabas cobojius* India (hap. AnolH2) | 0.1032 | 0.1087 | 0.1033 | 0.1092 | 0.0016 | 0.0016 | | 0.0021 | 0.0136 | 0.0150 | 0.0014 | 0.0137 | 0.0166 |
| 8 | JX260824 *Anabas testudineus* India | 0.1107 | 0.1161 | 0.1119 | 0.1143 | 0.0047 | 0.0047 | 0.0032 | | 0.0154 | 0.0152 | 0.0021 | 0.0140 | 0.0169 |
| 9 | KU692243 *Anabas testudineus* Indonesia | 0.1125 | 0.1218 | 0.1140 | 0.1123 | 0.0747 | 0.0766 | 0.0753 | 0.0891 | | 0.0148 | 0.0135 | 0.0000 | 0.0164 |
| 10 | JF781185 *Anabas testudineus* Malaysia | 0.0017 | 0.0052 | 0.0017 | 0.0000 | 0.0806 | 0.0806 | 0.0806 | 0.0826 | 0.0807 | | 0.0150 | 0.0148 | 0.0077 |
| 11 | LC190180 *Anabas testudineus* Myanmar | 0.1066 | 0.1101 | 0.1047 | 0.1088 | 0.0016 | 0.0031 | 0.0016 | 0.0032 | 0.0750 | 0.0806 | | 0.0135 | 0.0165 |
| 12 | HQ682664 *Anabas testudineus* Philippines | 0.1068 | 0.1124 | 0.1070 | 0.1073 | 0.0733 | 0.0768 | 0.0767 | 0.0790 | 0.0000 | 0.0807 | 0.0750 | | 0.0163 |
| 13 | JQ661369 *Anabas testudineus* Thailand | 0.0269 | 0.0305 | 0.0269 | 0.0286 | 0.0908 | 0.0933 | 0.0911 | 0.0913 | 0.0851 | 0.0296 | 0.0886 | 0.0846 | |

**Note**: *Genetic distance (lower triangle matrix), standard error of genetic distance (upper triangle matrix).*

The genetic distances between the anabas in Vietnam (A1, A2, A3, A4) are very small from 0.0046 to 0.0156 (Figure 4) and they are all in group 4 with *Anabas testudineus* Malaysia (Figure 5) so they are the same Anabas testudineus in accordance with the research results of Trinh et al., 2013 and Rainboth, 1996.

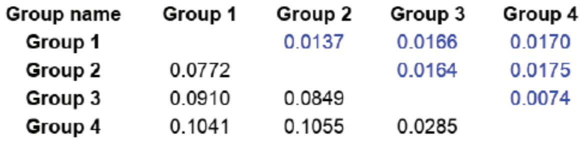
Figure 5. Phylogenetic tree of the anabas based on COI gene



The genetic distance and corresponding standard errors between the groups based on COI gene are shown in figure 6.

Based on the genetic distance matrix of the groups (Figure 6), the groups with far genetic distance include group 2 and group 4 ($D_{24} = 0.1055$), group 1 and group 4 ($D_{14} = 0.1041$), group 1 and group 3 ($D_{13} = 0.0910$). The groups with medium genetic distance include group 2 and group 3 ($D_{23} = 0.0849$),

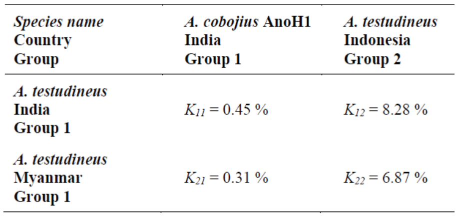
Figure 6. Distance and standard error matrix of the groups based on COI gene



| Group name | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| Group 1 | | 0.0137 | 0.0166 | 0.0170 |
| Group 2 | 0.0772 | | 0.0164 | 0.0175 |
| Group 3 | 0.0910 | 0.0849 | | 0.0074 |
| Group 4 | 0.1041 | 0.1055 | 0.0285 | |

group 1 and group 2 ($D_{12} = 0.0772$). The groups with near genetic distance include group 3 and group 4 ($D_{34} = 0.0285$).

Table 3. SNP ratio $K_{ij}$ (%) using pairwise sequence alignment of COI gene



| *Species name*<br>Country<br>Group | *A. cobojius* AnoH1<br>India<br>Group 1 | *A. testudineus*<br>Indonesia<br>Group 2 |
|---|---|---|
| *A. testudineus*<br>India<br>Group 1 | $K_{11} = 0.45$ % | $K_{12} = 8.28$ % |
| *A. testudineus*<br>Myanmar<br>Group 1 | $K_{21} = 0.31$ % | $K_{22} = 6.87$ % |

Result of genetic diversity analysis using pairwise sequence alignment of COI gene of the anabas was shown in table 3. The SNP ratio of *Anabas testudineus* India (group 1) and Anabas cobojius India hap. AnoH1 (group 1) is $K_{11} = 3/664 = 0.45$ %; The SNP ratio of Anabas testudineus Myanmar (group 1) and Anabas cobojius India hap. AnoH1 (group 1) is $K_{21} = 2/651 = 0.31$ %; The SNP ratio of Anabas testudineus India (group 1) and Anabas testudineus Indonesia (group 2) is $K_{12} = 54/652 = 8.28$ %; The SNP ratio of Anabas testudineus Myanmar (group 1) and *Anabas testudineus* Indonesia (group 2) $K_{22} = 45/655 = 6.87$ %. The SNP ratio $K_{12} = 8.28$ % and $K_{22} = 6.87$ % is much larger than $K_{11} = 0.45$ % and $K_{21} = 0.31$ % so *Anabas testudineus* India, *Anabas testudineus* Myanmar should be re-identified *Anabas cobojius* India, *Anabas cobojius* Myanmar respectively belong to group 1 (Gangetic koi group).

# Conclusions

The genetic distances between the anabas species in Vietnam (A1, A2, A3, A4) are very small from 0.0046 to 0.0156 and they are all in group 4 with *Anabas testudineus* in Malaysia so they are the same *Anabas testudineus.* The researched anabas in Vietnam and some countries in South and Southeast Asia were divided into four groups using COI marker gene. The groups with far genetic distance include group 2 and group 4 ($D_{24} = 0.1055$), group 1 and group 4 ($D_{14} = 0.1041$), group 1 and group 3 ($D_{13} = 0.0910$). The groups with medium genetic distance include group 2 and group 3 ($D_{23} = 0.0849$), group 1 and group 2 ($D_{12} = 0.0772$). The groups with near genetic distance include group 3 and group 4 ($D_{34} = 0.0285$). The species of *Anabas testudineus* in India and *Anabas testudineus* in Myanmar should be re-identified *Anabas cobojius* in India and *Anabas cobojius* in Myanmar respectively belong to group 1.

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