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Molecular identification and phylogenetic relationships of seven Satyrinae butterflies in Bangladesh using Cytochrome *c* oxidase subunit I gene

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Abstract

The Satyrinae is a subfamily of Nymphalid butterfly, which is morphologically and ecologically the most diverse group, occurring in all habitats. In the present study, Cytochrome *c* oxidase subunit I (*COI*) gene of seven species of Satyrinae was sequenced, aligned, and used to construct phylogenetic trees. The molecular identification of these Satyrinae species was confirmed by comparing the related sequences in the National Center for Biotechnology Information (NCBI) GenBank. The base compositions of the *COI* sequences were 39.07% T, 16.44% C, 29.83% A, and 14.64% G, revealing a strong AT bias (68.9%). The sequence distance among Satyrinae species ranged from 0.09% to 0.18%. Phylogenetic trees were constructed by the neighbor-joining (NJ) and maximum likelihood (ML) methods, using *Orthetrum sabina* as an outgroup. Both trees had almost identical topologies. The sampled species in Satyrinae exhibited the following relationships: *Melanitis leda* + [(*Mycalesis mineus*+(*Mycalesis gotama*+*Mycalesis anaxias*)) + (*Ypthima baldus* + (*Lethe chandica*+*Elymnias hypermnestra*))], suggesting that *M. leda* might be distantly related with the rest of the Satyrinae species. This clustering result is almost identical to current traditional classification. This study confirms that the *COI* based DNA barcoding is an efficient method for the identification of butterflies including Satyrinae species and, as such, may further contribute effectively to biodiversity and evolutionary research.

Key words: Molecular identification, phylogeny, *COI* gene, butterfly, Bangladesh

INTRODUCTION

The Satyrinae is a subfamily of the Nymphalid butterfly which is highly diverse and found worldwide (Murray, 2001). The adults of the most Satyrinae species fly near the ground, preferably in shaded areas of the garden and forest that feeding on fruits in various stages of decomposition and associated fungi (Kremen, 1994; Viloria, 1998; Murray, 2001). The Satyrinae butterflies exhibit special affinity for certain types of vegetation as open areas, primary or secondary forest (De Vries *et al*., 1997), being considered useful indicators of ecosystem characteristics (Kremen, 1992, 1994; Uehara-Prado *et al*., 2007) and used in population studies (Vila & Bjorklund, 2004; Schmitt *et al*., 2005; Besold *et al*., 2008) and conservation biology (Dennis & Eales, 1997; Bergman, 1999).

There are about 2,500 described species under the subfamily Satyrinae in the world (Pena & Wahlberg, 2008). In contrast, IUCN-Bangladesh recorded and assessed 22 species

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under 6 genera of Satyrinae into threat category (IUCN, 2015). These genera are *Mycalesis, Melanitis, Lethe, Ypthima*, *Orsotriaena* and *Elymnias.* Among the assessed threat category of Satyrinae, 2 species are Data Deficient (DD), 4 are Least Concern (LC), 9 are Vulnerable (VU) and 7 are Endangered (EN) (IUCN, 2015). Although Satyrines are commonly perceived as brown and drab, they are actually diverse in adult coloration. Some are extremely sexually dimorphic and involved in complex mimicry rings (Vane-Wright, 1971). In addition, seasonal polyphenism that is seasonal variation to response to changes in the environment, is common among butterflies including Satyrinae (Goonesekera *et al.*, 2019). The most prominent seasonal forms are the dry season forms (DSFs) and the wet season forms (WSFs). Each form is characterized by a change in the wing pattern and colouration that optimizes survival during the Dry and Wet Seasons, respectively (Braby, 1994; Roskam & Brakefield, 1999). Although the morphological variations, different color forms, and other factors are a strategy to increase fitness of these insects, it creates a considerable problem in correct identification (Goonesekera*et al.*, 2019).

To overcome such problems, a short, standardized 650 bp sequence of the cytochrome *c* oxidase subunit I (*COI*) mitochondrial DNA (mtDNA) has been proposed as a barcoding tool, or at least to confirm species delimitation for taxonomic, ecological and evolutionary studies (Hebert *et al*., 2003; Schindel & Miller, 2005; Ebach & Holdrege, 2005; Costa & Carvalho, 2007; Miller, 2007). Therefore, the present work was carried out to reveal the partial mitochondrial *COI* gene sequences of the available Satyrinae butterflies of Bangladesh for their species identification, genetic divergence and phylogenetic status.

MATERIALS AND METHODS

Sample collection: Butterfly specimens were collected from various locations of Bangladesh (Table 1). The specimens were collected by an insect net in the field and preserved by dehydration in a small envelope. Butterflies were identified morphologically by following the keys described in Bingham (1905, 1907), Wynter-Blyth (1957) and Talbot (1939, 1947). Voucher specimens were prepared as per Brower (1996).

DNA extraction, amplification and sequencing: Genomic DNA from these seven Satyrinae butterflies were extracted from the legs of adult butterflies according to the protocol of the Wizard Genomic@ DNA Purification Kit (Promega, Madison,WI, USA). PCR amplification of the mitochondrial cytochrome c oxidase I (*COI*) gene region was performed using the primers LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). PCR was performed in 20 µL of Q2 Green PCR Master Mix (Promega, Madison, WI, USA) in a thermal cycler (Vetteii, USA). The cycle conditions were as follows: initial denaturation (94°C for 5 min), 35 cycles of denaturation (94°C for 1 min), primer annealing (49°C for 1 min), and primer extension (72°C for 1 min), and a final extension (72°C for 4 min). The success of amplification was evaluated by 1% agarose gel electrophoresis under ultraviolet light (Bio Analyzer). The amplification product was sequenced using an ABI 3500 sequencer.

Phylogenetic analysis: The sequences of these seven butterfly species were edited using Chromas version 2.6.2. The assembled sequences were aligned using the ClustalW multiple alignment function in BioEdit version 7.0 (Hall, 1999). Nucleotide compositions were calculated and summarized, and pairwise distances were estimated using the Kimura 2 Parameter (K2P) model with the MEGA10 program (Kimura, 1980; Kumar *et al*., 2018). Phylogenetic trees were constructed using MEGA10 with the Neighbor-Joining and Maximum likelihood (Saitou & Nei, 1987; Kumar *et al*., 2018) methods with 1000 bootstrap replications. *Orthetrum sabina* from Bangladesh was used in phylogenetic analyses as an outgroup (Table 1).

RESULTS AND DISCUSSION

The average fragment length of the *COI* sequences of the seven Satyrinae species was 589 bp. The nucleotide sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank and their accession numbers is given in the Table 1. The sequences were compared with the related sequences of Satyrinae species those were previously deposited in the NCBI's GenBank, and showed 97-100% similarity, which confirms the accuracy of identification of these Satyrinae species from Bangladesh.

Table 1. Information of species from which *COI* genes were sequenced and their GenBank accession numbers

Species Name	GPS coordinates	Date of collection	Voucher No.	GenBank accession No.
Melanitis leda	23°52'33 N 90°16'6 E	06-11-2017	BBV 0300	MK778434
Mycalesis mineus	23°52'33 N 90°16'6 E	18-09-2017	BBV 0294	MK778437
Mycalesis gotama	24°19'44.0"N 91°47'07.6"E	11-05-2018	BBV 0299	MK778435
Mycalesis anaxias	24°19'40" N 91°47'1" E	12-05-2018	BBV 0296	MK757463
Lethe chandica	24°19'40" N 91°47'1" E	11-05-2018	BBV 0309	MK348952
Ypthima baldus	24°19'37.9"N 91°47'05.6"E	10-05-2018	BBV 0286	MK317934
Elymnias hypermnestra	23°52'27.9'N 90 °16'05.3'E	18-09-2017	BBV 0287	MH019977
Orthetrum sabina	23 ° 52'21.8"N 90°16'3.2"E	14-06-2017	DRBV 029	MF784360

The *COI* gene fragment of 589 bp was analyzed; there were 170 variable sites and 88 parsimony informative sites (Table 2). Most variations occurred at the second and third codon position. Among informative sites, 26 were in the first position and 31 were both in the second and third position (Table 2). The mean base compositions of the *COI* sequences were 39.07% T, 16.44% C, 29.83% A, and 14.64% G. There was a strong AT bias (68.9%). The A+T content of the first, second, and third codon positions of the *COI* fragment was 89.91%, 58.23% and 58.45% respectively (Table 2). In general, mitochondrion genome of insects tend to be highly A+T biased (Simon *et al*., 1994). The above result is in accordance with honey bee mitochondrial genomes (84.9%), while in *Drosophila yakuba* this value is 78.6% (Clary & Wolstenholme, 1985; Crozier & Crozier, 1993; Simon *et al*., 1994).

Table 2. Basic statistics of the *COI* gene sequences of the seven Satyrinae species

Position	No. of sites	No. of variable	No. informative	Empirical base frequencies (%)			
				T	C	A	G
All positions	589	170	88	39.07	16.44	29.83	14.64
First position	197	56	26	47.86	8.55	42.05	1.5
Second position	196	57	31	26.53	15.08	31.70	26.67
Third position	196	57	31	42.78	25.72	15.67	15.81

The pairwise distance was calculated by the MEGA10 program. The interspecific nucleotide divergence among the seven species ranged from 0.09% to 0.18% (Table 3). The highest distance of 0.18% was obtained between *Mycalesis gotama* and *Melanitis leda*. The shortest distance of 0.09% was obtained between *Mycalesis gotama* and *Mycalesis minus*. This result is consistent with the finding of Zakharov *et al.* (2004) where the sequence divergences ranged from 0% to 1.2% for many *Papilio* species. This low range of interspecific divergence may be observed due to the presence of interspecies hybridization, which is a familiar phenomenon in many butterflies (Win *et al*., 2015).

Table. 3. Percentage pairwise distances among seven Satyrinae species

Species Name	1	2	3	4	5	6
Melanitis leda						
Mycalesis mineus	0.13					
Mycalesis gotama	0.18	0.09				
Mycalesis anaxias	0.17	0.11	0.11			
Lethe chandica	0.16	0.11	0.14	0.14		
Ypthima baldus	0.17	0.13	0.15	0.17	0.15	
Elymnias hypermnestra	0.15	0.11	0.13	0.14	0.11	0.14

The phylogenetic trees for the *COI* gene constructed using the NJ and ML methods showed approximately identical clustering (Fig. 1, Fig. 2). *Orthetrum sabina* was used as an outgroup, and both of the NJ and ML trees revealed two main Clades, A and B. Clade A consisted of 3 species of one genera (*Mycalesis*) viz. *M. mineus*, *M. gotama* and *M. anaxias* and Clade B consisted of three species of three genera viz. *Ypthima baldus*, *Lethe chandica* and *Elymnias hypermnestra*.

In Clade A, the genus *Mycalesis* shows a strongly supported monophyletic group in NJ tree and a well-supported monophyletic group in ML tree. *M. mineus* is a sister group to *M. gotama* and *M. anaxias* with strong support in NJ and a well-supported in ML tree. The relationships of species in *Mycalesis* in Clade A are as follows: [*Mycalesis mineus*+(*Mycalesis gotama*+*Mycalesis anaxias*)] (Fig. 1, Fig. 2).

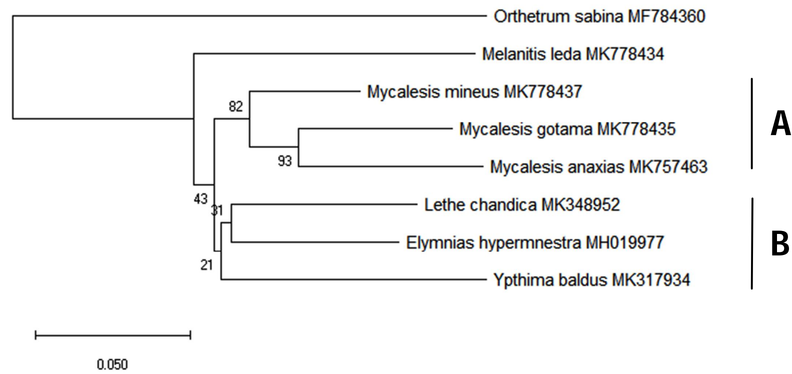

Fig. 1. Neighbor-Joining (NJ) tree of the Satyrinae butterflies based on *COI* gene sequences. Bootstrap values are shown at the branching points. *O. sabina* was used as the outgroup

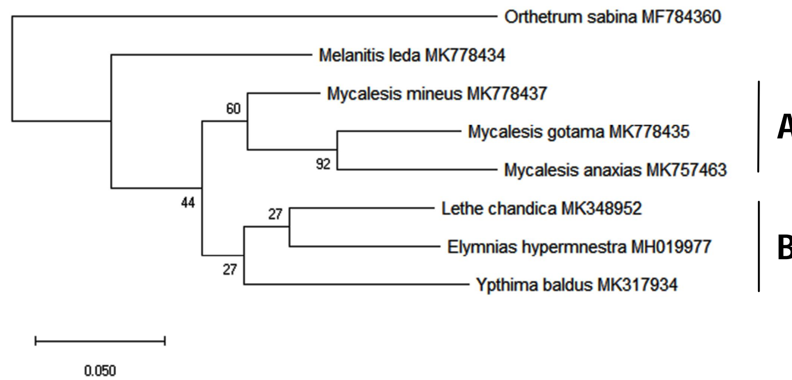

Fig. 2. Maximum Likelihood (ML) tree of the Satyrinae butterflies based on *COI* gene sequences. Bootstrap values are shown at the branching points. *O. sabina* was used as the outgroup

In Clade B, *Ypthima baldus* is resoled as a sister group to *Lethe chandica* and *Elymnias hypermnestra* as well as forms a monophyletic group with very weak support in NJ and ML trees. In Clade B, the relationships among species are as follows: [*Ypthima baldus*+(*Lethe chandica*+*Elymnias hypermnestra*)] (Fig. 1, Fig. 2). *M. leda* is resoled as a sister to the A and B Clade indicating that *M. leda* might have distant relationship with the Satyrinae species of Clade A and B (Fig. 1 and Fig. 2). These findings are in accordance with the finding of Yang *et al.* (2015), and also supported by the traditional classification method. Moreover, the DNA barcode generated in the present study will be available through GenBank to researchers for the species identifications and, as such, may further contribute effectively to biodiversity and evolutionary research.

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