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MOLECULAR IDENTIFICATION AND GENETIC DIVERSITY OF TEN PIERID BUTTERFLIES BASED ON MITOCHONDRIAL COI GENE

Muhammad Sohel Abedin, Ananna Ghosh[1], Abdul Jabber Howlader and Md. Monwar Hossain*

DNA Barcoding Laboratory, Department of Zoology, Jahangirnagar University, Dhaka, Bangladesh

ABSTRACT: The IUCN Bangladesh has documented a total of 31 species belonging to the Pieridae family in Bangladesh. There is currently limited knowledge regarding the genetic diversity and molecular relationships among these butterflies. The present investigation involved the generation of mitochondrial COI (mtCOI) gene sequences for ten species of butterflies belonging to the Pierid family. After that, BLAST analysis showed 96% to 100% similarity, and then the sequences were submitted to NCBI's GenBank. MEGA10 and BioEdit were used to identify a 584 bp COI gene fragment with 179 variable sites and 128 parsimony-informative sites. The COI sequences' average base compositions were 38.56% T, 17.12% C, 30.18% A, and 14.12% G. While a significant AT bias (68.74%) existed among the Pierid species. The genetic distance between ten Pierid species was assessed using the Kimura 2-Parameter (K2P) algorithm, and the nucleotide differences ranged from 0.00172% to 0.24261%. Afterwards, the Maximum Likelihood (ML) method was used to construct a phylogenetic tree using ten sequences of Pierid species. These species belongs to two subfamilies, Pierinae and Coliadinae in Bangladesh. On the tree, the Pierinae subfamily of Pieridae formed a paraphyletic arrangement whereas the Coliadinae subfamily was shown to be monophyletic. The data analyses support the following relationships between the two subfamilies as follows: [(*Appias* + *Leptosia*) + (*Pieris* + *Appias*) + (*Delias*) + (*Eurema* + *Catopsilia*) + (*Pareronia*)]. This supported the theory that Pierinae and Coliadinae are sister taxa. Nonetheless, *Pareronia hippia* remains outside the main Pierinae group, requiring further study to resolve this issue. Finally, these studies generated ten mtCOI gene sequences that have the potential to serve as valuable references for the accurate identification of Pierid species. In addition, this result could be used in the future to reveal the subfamily relationships within the Pieridae taxonomic classification.

Key words**:** COI, Genetic diversity, Pieridae, Bangladesh

INTRODUCTION

Butterflies are essential pollinators, and they also cause some harm to crops and forests. Pieridae is one of the most significant and common

**Author for correspondence: <monwar@juniv.edu>, [1]Insect Biotechnology Division, IFRB, Atomic Energy Research Establishment, Dhaka, Bangladesh*

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Lepidoptera family, which serves as pollinators and does a little damage to cultivated crops (Lee 2023). This family is widespread across the globe and comprises approximately 1,200 species (Ackery *et al.* 1999, Vane-Wright 2003, Sobti *et al.* 2007). Pierid adult butterflies are typically medium-sized, white, orange, or yellow in coloration, and their pupae have a highly distinctive morphology (Chapman 1895, Talbot 1939, Mosher 1969). There are numerous studies on the morphological, ecological, and molecular characteristics of Pierid species from the world (Sobti *et al.* 2007). However, the systematic relationship of the Pieridae is inadequately understood, including their molecular data on taxa to a certain extent (Braby *et al.* 2006). Though the family is well-known to be monophyletic (Wahlberg *et al.* 2005, Heikkila *et al.* 2012), but It has only lately been thoroughly researched how the main lineages of the family's phylogeny relate to one another (Braby *et al.* 2006). Prior classifications were based solely on morphological characteristics and non-cladistic methods, and only a small number of researchers have investigated Pieridae's upper-level systematics, which includes figuring out how different subgroups are related to each other, creating a systematic framework (Klots 1933, Ehrlich 1958), and finally, doing the in-depth phylogenetic analyses that are still needed (Braby *et al.* 2006).

The family Pieridae is divided into four subfamilies as Pierinae, Coliadinae, Pseudopontiinae, and Dismorphiinae. According to the status of the IUCN in Bangladesh, there are 31 species in the Pieridae family, which is under two subfamilies: Pierinae and Coliadinae. Among them, 23 species belongs to Pierinae and other 8 species to Coliadinae (IUCN 2015). Indeed, one of the least recognized families of butterflies is the Pieridae, and this family is still significant in terms of its biodiversity studies, population survey and significant contributors to evolutionary research (Stavenga *et al.* 2004, Kemp *et al.* 2005), and include some species that are significant in agriculture and visual science, respectively (Arikawa 2017). The morphological changes, various color forms, and other elements are a tactic to improve fitness and the survival rate of these insects, but they can pose a significant challenge in accurate identification (Goonesekeraa *et al.* 2019). In addition, phenotypic variability brought on by aging, sexual dimorphism, seasonal fluctuations, and convergent evolution further confuses the species (Collins and Morris 1985, Kunte *et al.* 2011, Wilson *et al.* 2013). To solve these issues, a brief, standardized 650 bp sequence of the mitochondrial DNA (mtDNA), cytochrome c oxidase subunit I (COI) has been suggested as a barcoding technique, or at the very least to confirm species delimitation and evolutionary investigations (Hebert *et al.* 2003a, Schindel and Miller 2005, Ebach and Holdrege 2005, Costa and Carvalho 2007, Miller 2007). Therefore, the current investigation was conducted to determine the mtCOI gene

sequences of Pieridae butterflies found in Bangladesh. Additionally, the study aimed to reveal genetic divergence and determine the phylogenetic status of this butterfly group.

MATERIAL AND METHODS

Sample collections: Specimens of butterflies were collected from various regions of Bangladesh over the period of 2017 to 2021, as indicated in Table 1. The geographical coordinates of the various places, along with pertinent information such as the collector's name, the date of collection, and additional relevant details, were duly recorded. The specimens were collected from their natural habitat by an insect net and subsequently conserved through desiccation within a small enclosure. Butterflies were morphologically identified using the keys established by Bingham (1905, 1907), Wynter-Blyth (1957), and Talbot (1939, 1947). The voucher samples were generated in accordance with the methodology outlined by Brower (1996).

DNA isolation, amplification and sequencing: The genomic DNA of ten adult Pieridae butterflies was extracted from their legs in accordance with the instructions provided in the Wizard Genomic@ DNA Purification Kit (Promega, USA). The mitochondrial cytochrome c oxidase I (COI) gene region was amplified in PCR using the primers LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). Using the Q2 Green PCR Master Mix, PCR was performed in a thermal cycler from Applied Biosystems (Veriti, USA). The total volume of the PCR reaction mixture was 20 µl, including 10 µl of 2x master mix, 1 µl of forward primer (10 pM), 1 µl of reverse primer (10 pM), 1 µl of template DNA (depending on the quantity of extracted DNA), and 1 µl of nuclease-free water. The cycle's settings were initial denaturation at 95°C for 5 min, 35 cycles of primer annealing at 49°C for 30 sec, primer extension at 72°C for 45 sec, and a final extension at 72°C for 5 min. 1% agarose gel electrophoresis was used to test the effectiveness of the amplification under ultraviolet light (Bio Doc Analyze, Biometra, Anlytic Jena, Germany). An ABI 3500 sequencer was used to sequence the amplified product.

Phylogenetic analysis: The assembled sequences were aligned using the ClustalW programme for multiple alignment in BioEdit version 7.0 (Hall 1999). Using the MEGA10 software, calculations and summaries of nucleotide compositions and estimates of pairwise distances were done. While the Maximum Likelihood (ML) method was used to construct a phylogenetic tree with 1000 bootstrap replicates in MEGA10 (Saitou and Nei 1987, Kumar *et al.* 2018). In the analysis of each species of COI gene sequences, conspecific sequences from two different geographic regions were used (Table 2). The *Aedes aegypti* originating from Bangladesh was employed as an outgroup in the phylogenetic analysis.

Table 2. List of GenBank-retrieved species used in the analysis

Sl No	Species Name	Country	GenBank Accession No.
1	*Pareronia hippia*	India	KX467796
2	*Pareronia hippia*	China	NC071367
3	*Appias olferna*	Malaysia	KF226283
4	*Appias olferna*	Thailand	MW796558
5	*Appias libythea*	India	KJ423038
6	*Appias libythea*	Thailand	ON437315
7	*Appias lyncida*	Malaysia	KF226280
8	*Appias lyncida*	China	EF584853
9	*Pieris canidia*	India	KT175605
10	*Pieris canidia*	Vietnam	OK342235
11	*Delias eucharis*	India	ON358410
12	*Delias eucharis*	Australia	JX978938
13	*Delias pasithoe*	Malaysia	KF226396
14	*Delias pasithoe*	China	KU921271
15	*Eurema hecabe*	India	KP216719
16	*Eurema hecabe*	Korea	GU372559
17	*Catopsilia pomona*	India	EU792482
18	*Catopsilia pomona*	Pakistan	OR140768
19	*Leptosia nina*	India	KJ423032
20	*Leptosia nina*	Malaysia	KF226514
21	*Aedes aegypti*	Bangladesh	OP942188

RESULTS AND DISCUSSION

The current investigation involved the generated of mitochondrial COI gene sequences (mtCOI) spanning around 636 bp from ten species belonging to the Pieridae family. The sequenced gene was compared with the available sequences of the GenBank and the result of BLAST analysis showed 96 to 100% similarity to the same species from different geographical areas. Then, ten COI gene sequences were submitted to the NCBI's GenBank in order to get accession numbers (Table 1).

Nucleotide analysis of COI gene: BioEdit and MEGA10 were used to evaluate the COI gene sequences, and the results revealed a 584 bp COI gene fragment with 128 parsimony-informative sites and 179 variable sites (Table 3). The majority of changes were seen at the second and third positions of the codons. In Table 3, it is seen that out of the informative sites, 36 were ranked in

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

Sl No	Species Name	GPS Coordinates	Date of Collection	Voucher No	GenBank Accession No.
1	*Pareronia hippia*	23°52'32.9"N 90°16'05.9"E	18.07.17	BBV259	MH269418
2	*Appias olferna*	24°19'35.3"N 91°47'00.8"E	09.07.18	BBV308	MK282887
3	*Appias libythea*	23°52'33.6"N 90°16'05.8"E	17.09.18	BBV271	OQ359494
4	*Appias lyncida*	24°19'40.1"N 91°47'01.0"E	31.10.17	BBV0255	MK317932
5	*Pieris canidia*	24°19'40.1"N 91°47'01.0"E	11.05.18	BBV250	MK282888
6	*Delias eucharis*	23°52'26.1"N 90°16'03.3"E	30.12.21	BBV269	OQ254745
7	*Delias pasithoe*	24°19'30.7"N 91°47'14.1"E	31.10.17	BBV0270	MK317930
8	*Eurema hecabe*	23°52'32.9"N 90°16'05.9"E	29.05.17	BBV264	MF784358
9	*Catopsilia pomona*	23°52'32.9"N 90°16'05.9"E	11.05.17	BBV258	MF784359
10	*Leptosia nina*	23°52'33.0"N 90°16'05.7"E	30.12.21	BBV268	OQ254746

the first position, 42 were ranked in the second position, and 46 were ranked in the third position. The average base compositions of the COI sequences were found to be 38.56% thymine (T), 17.12% cytosine (C), 30.18% adenine (A), and 14.12% guanine (G). A significant bias towards the adenine-thymine (AT) base pair was observed, accounting for 68.74% of the total base pairs. The adenine-thymine (A+T) content of the first, second, and third codon positions of the cytochrome c oxidase subunit I (COI) fragment was found to be 59.02%, 57.28%, and 90.04%, respectively (Table 3).

The parsimony informative is employed in molecular phylogenetic and evolutionary studies to elucidate diverse relationships among organisms. Similarly to other protein-coding genes, a significant proportion of changes were detected at the third codon position, as demonstrated by our present findings (Table 3) (Win *et al.* 2015). Additionally, an AT bias was also identified in other Lepidopterans, such as *Pieris* to *Catopsilia*, which aligns with our present results (Table 3) (DeSalle *et al.* 1987, Li *et al.* 2015, Nie *et al.* 2018).

Analysis of genetic distance: With the help of the MEGA10 program, the pairwise distance was determined. Ten sequences of Pierid butterflies had genetic distances ranging from 0.00172 to 0.24261 (Table 4). The lowest genetic distance (0.00172) was found between *Appias libythea* and *Appias olferna*, and their sequence similarity was 99.83%. While the highest genetic distance (0.24261) was found between *Leptosia nina* and *Delias pasithoe* (Table 4). In the present study, the narrow range of interspecific divergence (0.24261%) may be

Table 3. Basic statistics for COI gene sequences in 10 Pierid species.

Position	No. of sites	No. of variable	No. informative	Statistical base frequencies (%)				AT %
				T	C	A	G	
All Positions	584	179	128	38.56	17.12	30.18	14.12	68.74
First Position	195	58	36	26.15	15.89	32.87	25.07	59.02
Second Position	195	59	42	42.66	27.23	14.62	15.48	57.28
Third Position	194	62	46	46.90	8.19	43.14	1.75	90.04

a result of the prevalence of low interspecies hybridization, which is a prevalent occurrence among many butterfly species (Win *et al.*, 2015). While higher levels of interspecific divergence may result from hybridization in areas where it is more common (Comeault and Matute 2018). Nonetheless, sequence divergences between 0% and 1.2% are observed in numerous Papilio species, whereas sequence divergences exceeding 2% are employed for species differentiation in lepidopteran insects (Hebert *et al.* 2003b, Zakharov 2004).

Table. 4. Percentage pairwise distances among ten Pieridae species

Species Name	1	2	3	4	5	6	7	8	9
Pareronia hippia									
Appias olferna	0.15380								
Appias libythea	0.15641	0.00172							
Appias lyncida	0.16452	0.13067	0.13313						
Pieris canidia	0.15561	0.14861	0.15115	0.12044					
Delias eucharis	0.18463	0.19190	0.18911	0.17984	0.18920				
Delias pasithoe	0.20787	0.19245	0.18965	0.15547	0.17391	0.14607			
Eurema hecabe	0.19047	0.17850	0.17575	0.17049	0.16593	0.18511	0.20976		
Catopsilia pomona	0.17411	0.14853	0.14600	0.13990	0.14779	0.21087	0.17152	0.18762	
Leptosia nina	0.22170	0.18630	0.18357	0.17537	0.19268	0.23245	0.24261	0.22148	0.23272

Phylogenetic analysis: Maximum Likelihood (ML) method was used to construct the phylogenetic tree. In the analysis, the phylogenetic tree of the ten species shared a common ancestor. On the tree, the Pierinae subfamily of Pieridae formed a paraphyletic arrangement whereas the Coliadinae was shown to be monophyletic (Fig. 1). In the tree analysis, *Appias libythea* and *Appias olferna* showed very close relationships between them as indicated by their substantial boot strap support (Fig. 1). In nature, this two species can be difficult to differentiate in appearance, and it is probable to their similar genetic affinities as shown in the present results (Fig. 1). Nevertheless, *Appias libythea*, *Appias olferna*, and *Leptosia nina* were shown in a cluster. On the other hand, *Pieris canidia* and *Appias lyncida* demonstrated close connections, as did

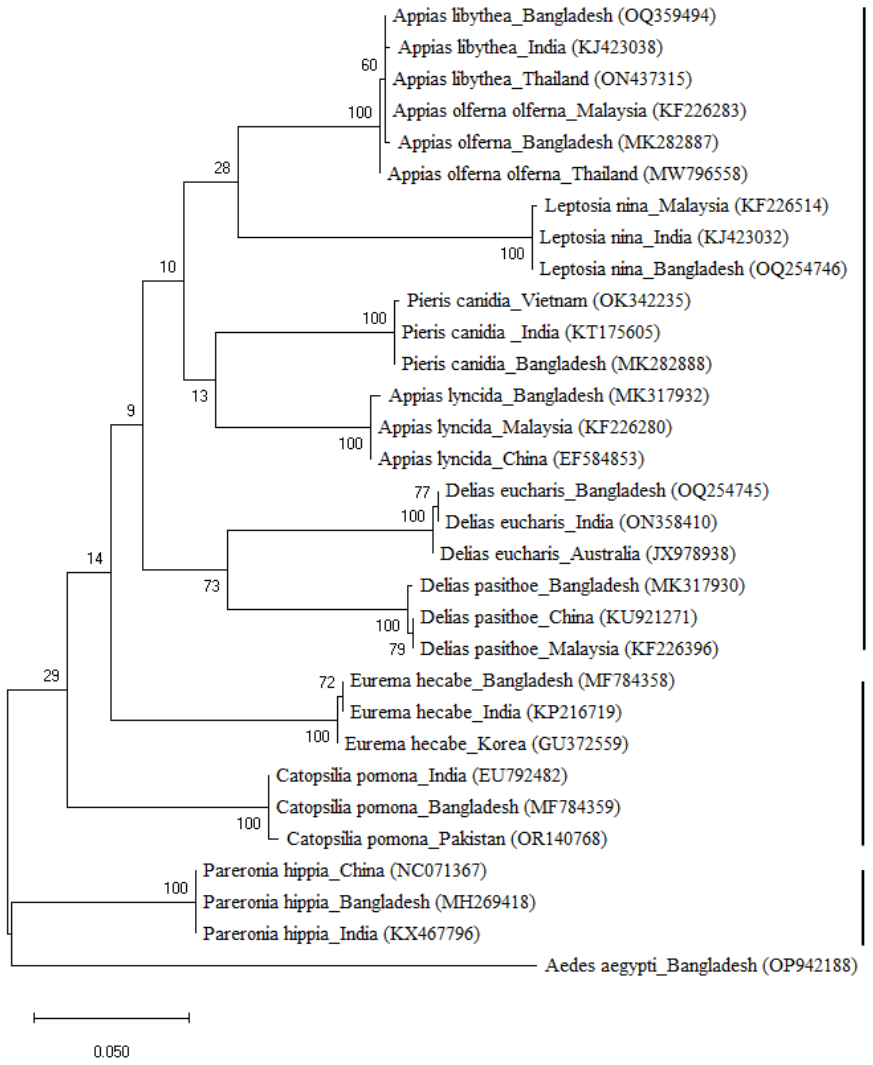

Fig.1: Phylogenetic tree constructed with COI gene sequences using the maximum likelihood (ML) method. Bootstrap values are shown at the branching points. The bar indicates phylogenetic distance.

Delias eucharis and *Delias pasithoe*. While *Eurema hecabe* and *Catopsilia pomona* under the subfamily Coliadinae, were constituted an additional cluster in the analysis. In the tree, only *Pareronia hippia* remarkably showed distance among other Pierinae species. According to the data analysis, the two subfamilies are related as follows: [(*Appias* + *Leptosia*) + (*Pieris* + *Appias*) + (*Delias*) + (*Eurema* + *Catopsilia*) + (*Pareronia*)] (Fig. 1). In the present study, it is shown that Pierinae and Coliadinae are sister taxa, which is also consistent with

the result of Ding and Zhang (2016). Nonetheless, *Pareronia* remain outside the main Pierinae group, and this subfamily played an important role in many evolutionary studies that require further study to resolve this issue in the future (Kemp *et al.* 2005, Braby *et al.* 2006).

CONCLUSION

The current investigation involved the generation and submission of mtCOI gene sequences for ten species of Pierid butterflies to the GenBank database. These generated ten mtCOI genes with the potential to serve as valuable references for the precise identification of Pierid butterflies. In the present study, COI gene sequences of 584bp contained 179 variable sites and 128 parsimony-informative sites. The genetic distance between ten species of Pierid butterfly ranged from 0.00172% to 0.24261%. In Bangladesh, there are two subfamilies of Pieridae, Pierinae and Coliadinae. According to the phylogenetic tree, the Coliadinae subfamily of Pieridae is monophyletic, whereas the Pierinae subfamily is paraphyletic. In the present investigation, the theory supported that Pierinae and Coliadinae are sister taxa. However, *Pareronia hippia* remains outside the primary group of Pierinae, necessitating additional research to resolve this issue.

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