DNA Barcoding and Analysis of Nine Butterfly Species And Three Moth Species From Entopia Penang Butterfly Farm

A A M Faik¹*, N K M Nagaligam¹, R Roland¹, A M S Abu Bakar², M H Y Meng³, L Y Kuen³ and S Abdul Sani¹

¹ Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
² Veterinary Diagnostics Laboratory, P.O. Box 59, 89457 Tanjung Aru, Kota Kinabalu, Sabah, Malaysia
³ Butterfly House (Pg) Sdn. Bhd., No. 830, Jalan Teluk Bahang, 11050 Penang, Malaysia; www.entopia.com

*Email: ainol@ums.edu.my

Abstract. This study aims to utilize cytochrome c oxidase subunit I (COI) DNA sequences in the classification of 22 butterfly and 8 moth species that were morphologically identified by Entopia Penang Butterfly Farm. COI of the mitochondrial gene has been chosen for evolutionary study of butterflies and moths because COI mitochondrial gene is maternally inherited with little or no recombination. This research revealed that 20 out of 22 butterflies were correctly classified while two butterflies and three moths were misidentified after the sequences were analyzed using Basic Local Alignment Search Tool (BLAST). Morphologically identified Cepora nadina (Sample ID: NCN1) was identified as Cepora iudith while Prioneris thestylis (NPT2) was identified as Prioneris philonome. Three moths; Dysphania malayanus (RDMA1), Nyctemera coleta (RNC1) and Nyctemera coleta (RNC2) were discovered as Bracca matutinata, Nyctemera regularis and Nyctemera baulus respectively. The outcome of this study further classified four butterfly species into subspecies, including Cepora nadina with subspecies andersoni, Prioneris philonome with subspecies themana, Pareronia valeria with subspecies lutescens and Elymnias nesaea classified into subspecies Elymnias nesaea lioneli. Analysis of nine butterfly species and three moth species demonstrates the value in attaching subspecies names to DNA barcode records and further helps to correct misidentified species. Overall, the relationship between butterfly and moth proves that butterfly and moth are distantly related.

1. Introduction
Penang Butterfly Farm was founded in 1986 and later rebranded to Entopia in 2015. The name Entopia was derived from the combination of two words " entomology " meaning " the branch of zoology concerned with the study of insects " [1] and " utopia " meaning " an imaginary place or state of things where everything is perfect " [2]. Entopia created a DNA barcode reference library deposited in the Barcode of Life Data System (BOLD) entitled COILP in 2018 for their cataloged butterflies [3]. This reference library could help to identify the moths and butterflies at the DNA level. In this study, we attempted to create a DNA barcode reference library for the Pieridae, Hesperidae, Satyrinae, and the Riodinae butterfly families along with three moth species in the BOLD by applying DNA barcoding and analyzing their genetic differences.

Butterflies and moths belong to the Kingdom Animalia and Phylum Arthropoda and were categorized as subphylum Hexapoda or six-legged arthropods. Butterflies and moths are further grouped into the Class Insecta followed by the order Lepidoptera or the rough translation of the name is' scale-wing.' The scale wing gives the butterfly and moth a colour and pattern on their wings [4].

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Lepidoptera comprises of butterfly and moth which dominates both in terms of their quantities and
for their ecological and economic significance among the different insect order that lives on the tree
crown at the green parts like leaves, buds and green bark [5]. Lepidoptera has several prominent
characteristics such as four scale-covered wings. Scales are very important for lepidopterans because of
their help in increasing the mass of the wings and thus their heat retaining capacity. Besides that, scales
are also filled the air with serves as insulator and scales also play significant roles in generating thrust
during flight. Furthermore, scales also provide butterflies and moths amazing colour patterns [6]. Other
prominent characteristics are their larvae have chewing mouthparts, while adult mouthparts are
primarily for sucking and some tends to become vestigial. Then, Lepidopterans undergo complete
metamorphosis; their size varies according to the size of wings spread. Their antennae also vary and
normally their variations are clearly visible.

Despite the similarities shared by Lepidopterans, there are also several differences that present
between butterfly and moth. Firstly, a butterfly has a thickened club while a moth has a simple thread-
like. Secondly, the wing colours of butterfly usually brighter colours while the wing colours of a moth
usually dull colour. Thirdly, a butterfly’s wings are usually held in a vertical form over the body when
they rest while the wings of a moth are held flat, either out at sides or over the back. Next, a butterfly is
known as diurnal fliers (active during the day) while most moth is categorised as nocturnal fliers (active
at night time). In butterflies; the pupa is not enclosed, it presents in naked chrysalis while in a moth, the
pupa is enclosed in a cocoon or concealed under debris, wood or rocks [7]. Cytochrome c oxidase
subunit I gene serves as a standard barcode for almost all animals. COI gene has proved to be highly
effective in identifying birds, butterflies, fishes, flies and many other animal groups [8]. In the inner
mitochondrial membranes, COI is the last enzyme in the electron transport chain, reducing oxygen and
pumping proton. Thus, the changes in the amino acid sequence will changes the protein structure and
may affect energy production in mitochondria [9]. The target gene for DNA barcoding for many
organisms is a 648 base pair region of the mitochondrial c oxidase I (COI) gene. This due to critical
function of mitochondria, the mutation occur at very low rate thus COI gene is conserved, has
“barcoding gap” between individual of different species, maternally inherited with little or no
recombination, small-sized compare to nuclear DNA, lack of introns, proven to be informative in
evolutionary processes studies as it is easy to isolate and consists of conserved sequences that make it
possible to be used as universal primers [10], [11], [12].

Classification or most of the time known as taxonomy is the description and naming of species and
the placement of them within genera and highest taxa. Shelly et., al (2014) [13] stated that morphology
is the classical tool of taxonomy, and hence taxonomic species are traditionally described based on
shared morphological traits. However, it has become difficult and more challenging for humans to
observe, record and analyze morphological characteristics or traits because the communication between
the individuals occurs through chemical, behavioral or other ephemeral signals which last for a very
short time. Therefore, a different tool is required to increase taxonomic accuracy. In this case, DNA
barcoding technique is used to assist in helping to increase accuracy [14].

Canadian zoologist Paul Hebert and his colleagues introduced DNA barcoding as molecular tools to
help identification species [15]. BOLD was launched in 2005 as a workbench and repository with the
support of a growing community of researchers, focusing on building a DNA barcode library for all
eukaryotic life. The system provides an integrated bioinformatics platform that supports all the processes
of the analytical pathway from specimen collection to the identification of species in the barcode library.
2. Materials and Methods

2.1. Species Collection
A total of 9 dried butterfly species and 3 dried moth species provided by Entopia Penang Butterfly Farm (EPBF) were examined. Detailed specimen information of these species is available on BOLD under the project name COILP (Lepidoptera) [3]. Each butterfly species was identified morphologically by Entopia personnel as *Cepora nadina*, *Delias hyparete*, *Hebomoia glaucippe*, *Pareronia valeria*, *Prioneris philonome*, *Prioneris thestylis*, *Cepora iudith*, *Elymnias nesaea* and *Taxila haquinus* while each moth species was identified morphologically as *Dysphania militaris*, *Dysphania malayanus* and *Nyctemera coleta*. There were two to three replicates for each species and they were further stored at -20 ºC.

2.2. DNA Extraction from Butterfly Tissue
The legs of each butterfly and moth sample were removed using forceps. The body was kept in a small zip-lock bag and recorded. Specimens of the donor butterflies and moths were stored in the fridge at -20 ºC. Legs of each butterfly species were crushed using a micropestle in a microfuge containing 50 μl of 1X DNA Lysis Solution (BioLyse DNA Isolation Kit, Biosatria Sdn. Bhd., Malaysia) supplemented with 0.5 μl of 10 mg/ml Proteinase-K. The crushed tissue samples were incubated at 55 ºC for 10 minutes in a shaking thermomixer (Eppendorf) and then cooled to room temperature for 5 minutes. Next, the microfuge tube containing lysate was centrifuged at 13 000 rpm for 10 minutes in a bench top centrifuge. The supernatant containing DNA (20 μl) was added into a new microfuge tube containing 80 μl of ultrapure water.

2.3. Amplification of COI Region Using Polymerase Chain Reaction (PCR)
In this study, the forward primer, **COIF-650 (5’-GGT CAA CAA ATC ATA AAG ATA TTG G-3’)** and reverse primer **COIR-650 (5’-TAA ACT TCA GGG TGA CCA AAA AAT CA-3’)** were used [16]. The total volume of PCR mixture was 50 μl with a final of 1.5 mM MgCl2, 0.25 μM primer, 0.2 mM dNTP and 1.00 Unit of Taq Polymerase. The amplified product was resolved with 2 % agarose gel electrophoresis. Target band of PCR product of about 700 bp were gel purified (Agarose Gel Extraction Kit, Biotek Corporation, China) and sent for DNA sequencing (First Base Laboratories Sdn. Bhd., Malaysia).

2.4. Data Analysis
Chromatograms of the sequences were verified and trimmed using Seqman (DNASTAR, Inc., Madison, WI, USA). The consensus sequence was constructed and aligned using Molecular Evolutionary Genetics Analysis Version 7 (MEGA 7) [17]. Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) was applied to search regions of local similarity between a query sequence and sequences from the database. Data deposited in BOLD were compared using the Distance Summary and the presence of the barcode gap was determined by maximum intraspecific divergence plotted against nearest neighbour distance using the Kimura 2-parameter (K2P) model (Kimura, 1980). Pairwise distances were calculated using the same K2P model. MEGA 7.0 was also used to construct a phylogenetic tree (including one outgroup) based on the Maximum Likelihood approach using Tamura and Nei model with 1000 bootstrap replicates [18]. The mitochondrial COI sequence of *Lanius cristatus* was used as the outgroup.

3. Results and Discussion

3.1. Result Analysis of DNA sequences
Good quality COI DNA sequences were obtained after DNA sequencing with an average length of 550 bp. All the sequences matched with the COI sequence in the NCBI database ranging from 93%-99% similarity (Table 1). Based on the BLAST result, there were 2 butterflies and 3 moths that were
discovered to be different species compared to morphological identification. Butterfly samples of *Cepora nadina* (NCN 1) and *Prioneris thestylis* (NPT 2) were found to be identified as *Cepora iudith* and *Prioneris philonome* respectively. Morphologically identified moth samples; *Dysphania malayanus* (RDMA 1) was discovered as *Bracca matutinata* with 96 % of percentage identity, *Nyctemera coleta* (RNC 1) and *Nyctemera coleta* (RNC 2) were identified as *Nyctemera leuconoe* and *Nyctemera baulus* with percentage identity of 93 % and 99 % respectively.

Table 1. BLAST result of butterfly samples with NCBI database

| Morphologically Characterized | E-Value | ID (%) | Species Name |
|-------------------------------|---------|--------|--------------|
| **Cepora nadina** (NCN 1)     | 0.0     | 99     | *Cepora iudith* voucher UMKLJJW0057 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Cepora nadina** (NCN 2)     | 0.0     | 99     | *Cepora nadina andersoni* voucher UMKL-JJW0058 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Cepora nadina** (NCN 3)     | 0.0     | 98     | *Cepora nadina* cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Delias hyparete** (NDH 1)   | 0.0     | 99     | *Delias hyparete* voucher YY2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial |
| **Delias hyparete** (NDH 2)   | 0.0     | 99     | *Delias hyparete* cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Hebomoia glaucippe** (NHG 2)| 0.0     | 99     | *Hebomoia glaucippe* voucher UMKL-JJW0092 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Hebomoia glaucippe** (NHG 3)| 0.0     | 99     | *Hebomoia glaucippe* voucher UMKL-JJW0092 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Pareronia valeria** (NPV 2) | 0.0     | 96     | *Pareronia valeria lutescens* voucher UMKL-JJW0104 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Pareronia valeria** (NPV 3) | 0.0     | 92     | *Pareronia valeria lutescens* voucher UMKL-JJW0104 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Prioneris philonome** (NPP1) | 0.0   | 99     | *Prioneris philonome themana* voucher UMKL-JJW0106 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Prioneris philonome** (NPP2) | 0.0   | 98     | *Prioneris philonome* cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Prioneris philonome** (NPP3) | 0.0   | 99     | *Prioneris philonome themana* Voucher UMKL-JJW0106 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Prioneris thestylis** (NPT 2)| 0.0     | 99     | *Prioneris philonome* cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Cepora iudith** (NCI 1)     | 0.0     | 99     | *Cepora iudith* voucher UMKLJJW0067 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Cepora iudith** (NCI 2)     | 0.0     | 99     | *Cepora iudith* voucher UMKLJJW0056 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
| **Cepora iudith** (NCI 3)     | 0.0     | 99     | *Cepora iudith* voucher UMKLJJW0057 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial |
**Table 2. BLAST result of moth samples with NCBI database**

| Morphologically characterized | E-value | ID (%) | Species Name | Details |
|--------------------------------|---------|--------|--------------|---------|
| *Dysphania militaris* (RDM 1) | 0.0     | 99     | *Dysphania militaris* | Sequence ID: KF522381.1. Voucher: AYK-04-0768-01. Cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. |
| *Dysphania militaris* (RDM 2) | 0.0     | 99     | *Dysphania militaris* | Sequence ID: KF522381.1. Voucher: AYK-04-0768-01. Cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. |
| *Dysphania malayanus* (RDMA 1) | 0.0     | 96     | *Dysphania malayanus* | Sequence ID: KF390944.1. Voucher: 11ANIC-04168. Cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. |
| *Dysphania malayanus* (RDMA 2) | 0.0     | 98     | *Dysphania malayanus* | Sequence ID: JF784791.1. Voucher: MM14748. Cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. |
| *Dysphania malayanus* (RDMA 3) | 1 x 10^-15 | 98     | *Dysphania malayanus* | Sequence ID: JF784791.1. Voucher: MM14748. Cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. |
| *Nyctemera coleta* (RNC 1)  | 0.0     | 93     | *Nyctemera regularis* | Voucher: AYK-04-0770-08. Cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. |
| *Nyctemera coleta* (RNC 2)  | 0.0     | 99     | *Nyctemera baulus* | Voucher: RZ387. Cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. |
| *Nyctemera coleta* (RNC 3)  | 0.0     | 100    | *Nyctemera coleta* | Cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. |
3.2. Distance Summary and Barcode Gap Analysis
A significant barcode gap will be exhibited when the sequence divergence within species is lower than the sequence divergence between species [19]. In this study, sequence divergence within species was referred to as maximum intraspecific distance while sequence divergence between species was referred to as interspecific distance or nearest neighbour (NN) distance. All butterfly and 7 moth species exhibited smaller maximum intraspecific distance (%) and did not overlap the nearest neighbour (NN) distance. This indicated the presence of a barcode gap in all butterfly and 7 moth species.

The scatterplot in Figure 1 and Figure 2 were provided to confirm the existence and magnitude of the barcode gap among the butterfly species and moth species respectively. It shows the overlap of the max intraspecific distances versus the interspecific (NN) distances. Points above the line in this scatterplot indicated species with Barcode Gap.

Table 3. Inter- and Intraspecific divergences according to the different taxonomic levels within the COI sequences of the butterfly species.

| Order      | Family     | Species            | Mean Intra-Sp (%) | Max Intra-Sp (%) | Nearest Neighbour | Nearest Species | Distance to NN (%) |
|------------|------------|--------------------|-------------------|------------------|-------------------|-----------------|--------------------|
| Lepidoptera| Nymphalidae| Edyminia nesaea    | 7.58              | 10.97            | COILP082-18       | Cepora ludith   | 13.87              |
| Lepidoptera| Pieridae   | Cepora ludith      | 0.64              | 1.46             | COILP068-18       | Cepora nadina   | 5.01               |
| Lepidoptera| Pieridae   | Cepora nadina      | 2.93              | 2.93             | COILP082-18       | Cepora ludith   | 5.01               |
| Lepidoptera| Pieridae   | Delias hyparete    | 9.20              | 9.20             | COILP082-18       | Cepora ludith   | 13.92              |
| Lepidoptera| Pieridae   | Heberomela glaucopis| 0.58             | 0.58             | COILP087-18       | Taxilia haquinus| 13.04              |
| Lepidoptera| Pieridae   | Pareronia valetia  | 11.24             | 11.24            | COILP080-18       | Cepora nadina   | 17.71              |
| Lepidoptera| Pieridae   | Prileanis philomna | 1.65              | 1.61             | COILP088-18       | Cepora ludith   | 11.29              |
| Lepidoptera| Riodinidae | Taxilia hequinus   | 1.68              | 1.33             | COILP082-18       | Cepora ludith   | 11.92              |

Figure 1. Comparison of maximum intraspecific sequence divergence with minimum interspecific sequence divergence for butterfly species.
Table 4. Inter- and Intra-specific divergences according to the different taxonomic levels within the COI sequences of the moth species.

| Order       | Family            | Species                | Mean Intra-Sp (%) | Max Intra-Sp (%) | Nearest Neighbour       | Nearest Species            | Distance to NN (%) |
|-------------|-------------------|------------------------|-------------------|------------------|-------------------------|----------------------------|-------------------|
| Lepidoptera | Erebidae          | Nyctemera baulus       | N/A               | 0                | COILP095-18             | Nyctemera regularis        | 8.63              |
| Lepidoptera | Erebidae          | Nyctemera coleta       | N/A               | 0                | COILP085-18             | Nyctemera regularis        | 10.24             |
| Lepidoptera | Erebidae          | Nyctemera regularis    | N/A               | 0                | COILP096-18             | Nyctemera baulus           | 8.63              |
| Lepidoptera | Geometridae       | Bracca matutinata      | N/A               | 0                | COILP091-18             | Dysphania militaris        | 13.62             |
| Lepidoptera | Geometridae       | Dysphania malayanus    | 25.8              | 25.8             | COILP090-18             | Dysphania militaris        | 9.94              |
| Lepidoptera | Geometridae       | Dysphania militaris    | 2.36              | 2.36             | COILP093-18             | Dysphania malayanus        | 9.94              |

Figure 2. Comparison of maximum intraspecific sequence divergence with minimum interspecific sequence divergence for moth species.

3.3. Phylogenetic Tree of Nucleotide Sequences
The evolutionary history was inferred using the Maximum Likelihood (ML) method. Figure 3 shows the phylogenetic relationship between the butterfly species. Purple dot on the phylogenetic tree indicates the common ancestor of butterfly species in both Clade A and B, while the red dot on the phylogenetic tree shows the most common ancestor for *Cepora iudith*, *Cepora nadina*, *Pareronia valeria lutescens*, *Prioneris philonome themana*, *Hebomoia glaucippe*, *Taxila haquinus* and *Delias hyparete*. *Elymnias nesaea lioneli*, in Clade B is distantly related to all the other butterflies in Clade A. Butterfly with a sample ID NCN1 was morphologically identified as *Cepora nadina*. However, the COI sequence showed that it is identified as *Cepora iudith* with a genetic difference of 1.8% with *Cepora nadina andersoni* (NCN2). The misidentification could result from the similar morphological look of these two butterflies. From this analysis it can be deducted that DNA barcoding is able to differentiate two butterflies with similar morphology.

Maximum Likelihood is also used to infer the evolutionary history of the moth species. Figure 4 shows that the green dot indicates the common ancestor for *Dysphania militaris*, *Dysphania malayanus*, *Nyctemera regularis*, *Nyctemera baulus*, *Nyctemera coleta* and *Bracca matutinata*. Nyctemera genus were morphologically identified as *Nyctemera coleta* but only one was correctly identified. The other two however were identified as *Nyctemera baulus* and *Nyctemera regularis*. The genetic difference
between *Nyctemera baulus* and *Nyctemera regularis* is 2.1%. Meanwhile the pairwise distances between *Nyctemera baulus* and *Nyctemera coleta* is 2.6%. DNA barcoding successfully differentiated these species although morphologically they look similar.

**Figure 3.** The phylogenetic relationship between 8 butterfly species analyzed by Maximum Likelihood method. Clade A indicated the 7-butterfly species which shared the same ancestor that was marked by the red dot while Clade B indicated the one and only butterfly species which was distantly related to all the other butterflies in Clade A.
Figure 4 shows the phylogenetic relationship between the moth species. The green dot indicates the most common ancestor for *Dysphania militaris*, *Dysphania malayanus*, *Nyctemera regularis*, *Nyctemera baulus*, *Nyctemera coleta* and *Bracca matutinata*.

4. Summary

From this study, it was found that morphological identification could be misleading as the species identification through BLAST by using molecular data, pairwise distances and phylogenetic tree had opposed the morphological identification of few species. Therefore, identification of a butterfly should include both phenotype and molecular methods. For this work, all data from the butterfly and three moth species belonging to Entopia Penang Butterfly Farm were deposited into BOLD system. Research involving other butterfly and moth families from Entopia is being carried out and this reference library is expected to help Entopia to easily determine the species of butterflies and moths.

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