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DNA Barcoding of Lepidopteron Moths from Marathawada Region of Maharashtra

Ravindra Fakirrao Pathre*, Digamber D Bhutekar and Sharad Devidasrao Jadhav

Department of Zoology,

Arts, Science and Commerce College, Ambad, District Jalna - 431 213, Maharashtra, India

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A B S T R A C T

The present study is the first effort to describe diversity and molecular phylogeny of moths from Marathawada region of Maharashtra. A total 55 moth specimens were collected and sequenced from different sampling stations across the region. Our 47 sequences matched with COI sequences already deposited with BOLD. But, 4 sequences did not match with any species but correctly matched with deposited sequences of genus. Our 4 sequences are new record to BOLD but correctly matched with NCBI database. Nearest neighbor distances were greater than 3% for all the species but for two pair of specimens: 1) *Agrotis ipsilon* (EDBLM004/EDBLM005) vs *Agrotis munda* (EDBLM034) it was 2.8, *Agrotis munda* (EDBLM035) vs *Agrotis ipsilon* (EDBLM005) it was 2.8. The largest nearest neighboring distance of 14.47% was observed in *Hydrilodes metisalis vs Agrotis munda.* In present study, we have produced DNA sequences for 21 moth species from Marathwada region of Maharashtra, which may help future conservation efforts and in construction of DNA library of moth species from here.

Key words: DNA barcoding, Marathwada, 55 specimens of moths, *Agrotis munda*

Lepidoptera except Antarctica found in all terrestrial habitats (Gullan and Cranston 2005). Among them, butterflies have been discussed more due to colorful wings (Wang and Fang 2007). Earlier in Maharashtra, there have been very few moth surveys carried out. Hampson *et al*. (1891) recorded about 611 species of moths from Nilgiris, Maharashtra. The moths from Sanjay Gandhi National park, Boriwali, Mumbai were studied by Vaylure *et al*. (2012). Gurule *et al*. (2013) studied 728 species of moths from North Maharashtra. Nimbalkar *et al*. (2015) studied 49 species of moths from Marathwada region of Maharashtra. Kalawate *et al*. (2018) collected 99 species of moths from northern Western Ghats of Maharashtra. In moths, due to the complex morphological characters, identification is difficult (Janzen *et al*. 2005, Hausmann *et al*. 2009, Huemer and Mutanen 2012). Now in species identification, DNA barcoding has been proved to be useful (Hebert *et al*. 2003), Moreover, many workers are studying region-specific lepidopteron diversity (Dinca *et al*. 2011, deWaard *et al*. 2011, Hausmann *et al*. 2011, 2013, Huemer *et al*. 2014, Liu *et al*. 2014, Zahiri *et al*. 2014). Many studies have shown that DNA barcoding could resolve the taxonomic problems in lepidopteran systematic (Hajibabaei *et al*. 2006, Burns *et al*. 2008, Mutanen *et al*. 2012, Jiang *et al*. 2017).

Marathawada region is well known to world by its Ajanta and Ellora caves. It is most diverse region of Maharashtra connected with Sahyadri mountain ranges. Moreover, majority of maize, pulses and cotton production in county is accounted by this region and moth species are important pest here. However so far, this diversity of insects is largely noted by morphological analysis only. Till now, nobody has done DNA barcoding of moths in the region. So, this study aims to produce DNA sequences of moth species. Also, this information on moths would be helpful in quick identification and effective management of some pest species.

MATERIALS AND METHODS

***Corresponding author:** Ravindra Fakirrao Pathre, Assistant Professor and Head, Department of Zoology, Arts, Science and Commerce College, Ambad, District Jalna - 431 213, Maharashtra

e-mail: rfpathre@gmail.com | **Contact:** +91- 7588794162

Marathawada is more diverse region with eight (8) districts. The temperature ranges between 7.8°C (winter) to 42.8°C (summer) as per seasons (Indian meteorological department, regional office Pune, India). The area receives rain from Southeast monsoons. This area has a tropical climate, specially a tropical wet and dry climate with dryness of seven months and rainfall from June to September.

All moth samples were collected from various sampling stations during the May 2016 to May 2017. Moths were collected through light traps, using 85-Watt CFL bulb, which is convenient method. White cloth sheet hanging between two vertical poles. All specimens collected in jars and killed using ethyl acetate. Before pinning and spreading on the board a leg clip or a tip of abdomen from specimen was cut and stored in Ethanol (90% alcohol). All ethanol preserved samples were stored in refrigerator for molecular analysis. Subsequent to isolation of DNA, moths were spread and preserved according to standard entomological methods at department of Zoology Arts, Science and Commerce College Ambad. Identification was based on wing shape and color pattern described in available keys/identification guides.

DNA extraction, PCR and sequencing

DNA extraction, polymerase chain reaction (PCR) amplifications and sequencing were performed at the Paul Hebert Centre for DNA Barcoding and Biodiversity, Dr. Babasaheb Ambedkar Marathawada University, Aurangabad, Maharashtra, India. Specimen DNA from leg or tip of abdomen was isolated using automated DNA isolation machine. Then, using polymerase chain reaction (PCR) (ABI thermocycler) obtained DNA was amplified. These primers were used for amplification of COI gene: LEP F1 50 ATT CAACCAATCATAAAGATAT 30 and LEP R1 50 TAAA CTTCTGGATGTCCAAAAA 30. For PCR reaction, total volume of 25µl was taken which contain 2µl DNA template, 10 pmol of each primer and 200 µM of dNTP and 0.2 µl of Taq polymerase (Banglore, Genei). Thermo-cycling was as follows: First cycle of 1 min at 94°C followed by five cycles of 94°C for 1 min, 45°C for 1 min 30 s, 72°C for 1 min 15 s, then 30 cycles of 94°C for 1 min, 51°C for 1 min 30 s, 72°C for 1 min 15s, with last step of 72°C for 5 min. The products were checked by 1% agarose gel and then purified using PEG-NaCl method. Finally, using an automated sequencer (3730 DNA analyzer, ABI, Hitachi), products was sequenced by both, the forward and reverse primers.

Data analysis

ClustalW nucleotide sequence alignment and assembly was carried out using MEGA7. The nucleotide sequences were searched for its similarity using BOLD (www.boldsystems.org) and NCBI blast. Sequence divergence values within and among species, were employed using the Kimura two parameter (K2P) model and using analytical functions on BOLD V3. A phylogeny was inferred using maximum likelihood tree based on K2P model (MEGA7) in which insect *Flatidae* sequence was used as outgroup and nucleotide composition values also obtained. Earlier studies have revealed that the most different species of Lepidoptera show >3% sequence divergence at COI (Hebert *et al*. 2003) and researchers have used a 3% pairwise threshold for species delimitation (Strutzenberger 2011). For the barcode-based identity analysis, we also used a threshold of 3% divergence. A barcode gap analysis was performed using BOLD. Sequences were submitted to BOLD project code [EDBLM]. Sequences from moth species from this region were compared with the sequence of the conspecifics from other geographical areas. It was to check intra and interspecific divergences in such widely distributed area and check if any cryptic or overlooked species showing deep divergence.

Table 1 Showing details of 55 moth specimens studied and species identification by BOLD system

Sample Id	Family	Subfamily	Genus	Species
ACCAB2	Noctuidae	Noctuinae	Agrotis	Agrotis ipsilon
ACCAB-B2			Agrotis	Agrotis ipsilon
GTS6			Agrotis	Agrotis segetum
MRBK5			Agrotis	Agrotis segetum
MCA02			Agrotis	Agrotis munda
MCA2-2			Agrotis	Agrotis munda
MCA2-1			Agrotis	Agrotis munda
CCA11		Amphipyrinae	Sesamia	Sesamia inferens
KTGH2			Sesamia	Sesamia inferens
MRBK3			Sesamia	Sesamia inferens
ACCAB1		Noctuinae	Spodoptera	Spodoptera litura
TWR2			Spodoptera	Spodoptera exigua
TWR2-1			Spodoptera	Spodoptera exigua
ACC3			Athetis	Athetis recluse
CCA10			Athetis	Athetis recluse
CCA6			Athetis	Athetis recluse
ACCB1		Heliothinae	Helicoverpa	Helicoverpa armigera
ACCB2			Helicoverpa	Helicoverpa armigera
ACCB3			Helicoverpa	Helicoverpa armigera

TWR3			Ogdoconta (NCBI Blast)	
CCA02	Geometridae	Ennominae	Chiasmia	-
GRB2		Sterrhinae	Scopula species	-
CCA13		-	Chiasmia species	-
MRBK2		Ennominae	Cleora	Cleora tenebrata
PRA8			Cleora	Cleora tenebrata
TWR1			Chaismia	Chaismia multistrigata
GRB10		Sterrhinae	Traminda	Traminda mundissima
CCA12			Traminda	Traminda mundissima
GRB1		Ennominae	Isturgia	Isturgia disputaria
HBA1			Isturgia	Isturgia disputaria
PRAB2	Erebeidae	Arctiinae	Creatonotos	Creatonotos gangis
CCA04		-	Acantholipes	-
ACC6		Arctiinae	Amata	Amata cysseus
ACCBC1			Amata	Amata passalis
GBS4		Herminiinae	Hydrillodes	Hydrillodes metisalis
CCA01		Arctiinae	Utetheisa pulchella (NCBI Blast)	
MRBK8			Utetheisa	Utetheisa pulchelloides
MRBKB5			Utetheisa	Utetheisa pulchella
JRA2			Utetheisa	Utetheisa pulchella
MRBKB1			Utetheisa	Utetheisa pulchella
MRBKB2			Utetheisa	Utetheisa pulchella
MRBKB3			Utetheisa	Utetheisa pulchella
MRBKB4			Utetheisa	Utetheisa pulchella
PRA2		Calpinae	Culasta	Culasta indecisa
JRA1		Erebinae	Pandesma	Pandesma guenauadi
MRBK4		Erebinae	Mocis	Mocis trifasciata
HBA3		-	Melipotis jucunda (NCBI Blast)	
PRA6		Erebinae	Spirama	Spirama retorta
GTSB3		Lymantriinae	Euproctis	Euproctis lunata
GTSB2			Euproctis	Euproctis lunata
GTS3			Euproctis	Euproctis lunata
JRAB2		Lymantriinae	Lymantria	Lymantria incerta
PRA4	Nolidae	-	Selepa species (NCBI Blast)	
GBS5	Crambidae	Glaphyriinae	Noorda	Noorda blitealis
TWR2-2	Pyrilidae	Galleriinae	Lamoria	Lamoria anella
CCA15	Insect Flatidae as outgroup			

Table 2 Barcode gap analysis for moth species

Name of species	Mean Intra-Sp	Max Intra-Sp	to distance with nearest neighbor	
Noorda blitealis	N/A	N/A	Agrotis munda	11.02
Amata cysseus	N/A	N/A	Mocis trifasciata	10.49
Creatonotos gangis	N/A	N/A	Athetis reclusa	10.14
Hydrillodes metisalis	N/A	N/A	Agrotis munda	9.09
Mocis trifasciata	N/A	N/A	Agrotis munda	8.92
Pandesma guenauadi	N/A	N/A	Mocis trifasciata	9.09
Spirama retorta	N/A	N/A	Pandesma guenauadi	10.59
Utetheisa pulchella	0.32	0.92	Utetheisa pulchelloides	3.94
Utetheisa pulchelloides	N/A	N/A	Utetheisa pulchella	3.94
Cleora tenebrata	0.32	0.32	Mocis trifasciata	12.09
Isturgia disputaria	1.25	1.25	Utetheisa pulchelloides	11.9
Traminda mundissima	0.5	0.5	Spodoptera exigua	13.4
Agrotis ipsilon	1.12	1.12	Agrotis munda	2.8
Agrotis munda	0.43	0.49	Agrotis ipsilon	2.8
Agrotis segetum	0	0	Agrotis ipsilon	4.32
Athetis reclusa	0.57	0.71	Agrotis munda	7.84
Helicoverpa armigera	0.21	0.32	Athetis reclusa	7.89
Sesamia inferens	1.15	1.23	Agrotis munda	6.91
Spodoptera exigua	1.14	1.14	Spodoptera litura	7.31

Spodoptera litura	N/A	N/A	Spodoptera exigua	7.31
Lamoria anella	N/A	N/A	Hydrillodes metisalis	14.47

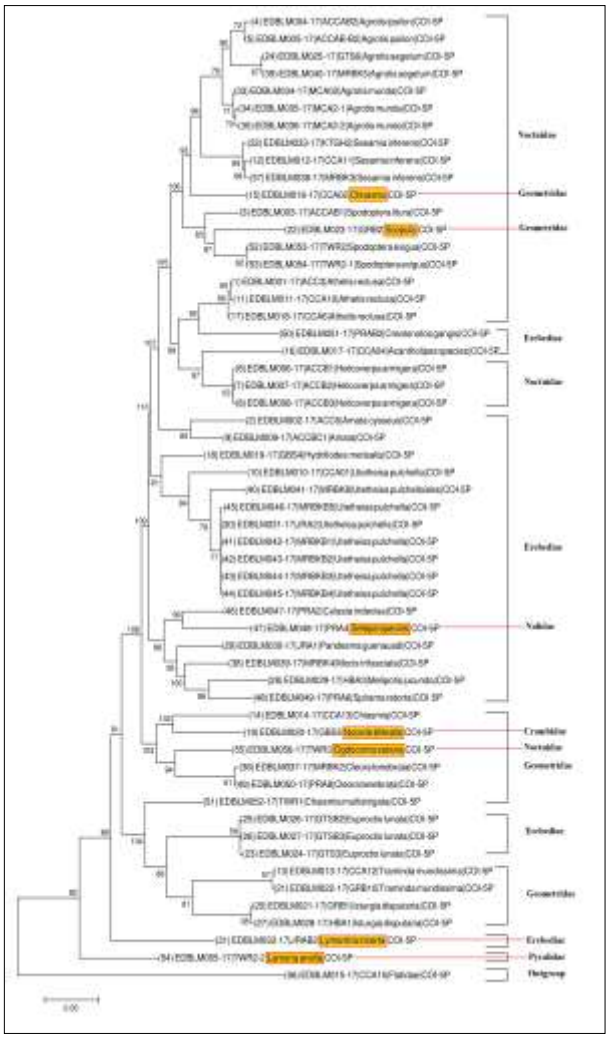

Fig 1 Showing maximum likelihood tree based on Kimura two parameter (K2P) model using MEGA7

RESULTS AND DISCUSSION

We collected 456 moth specimens belongs to 112 morphologically identifiable species, 88 genera, 9 super families and 15 families. A total of 55 COI sequences (Table 1) for 21 species were generated. Our 47 sequences matched with COI sequences already deposited with BOLD. But, 4 sequences not matched with any species but correctly matched with deposited sequences of genus. A Further, our 4 sequences are new record to BOLD but matched with NCBI database. The ClustalW alignment showed 331 conserved sites, 327 variable sites, 230 parsimony informative sites, and 97 singleton sites. All the amplified sequences were 632 bp (mean) with no insertions, deletions, and stop codons. The overall GC content was 29.78 (SE=0.14). GC content at codon positions 1 was 40.02 (SE=0.19), at 2 was 41.92 (SE=0.07), at 3 was 7.48 (SE=0.38) (Table 3). All the sequences were submitted to the BOLD with the project name EDBLM. From the total, 4 sequences represent new record and did not match to the BOLD sequences but correctly matched with NCBI sequences. Genetic divergence increased with taxonomic rank. Intraspecific divergence ranged from 0.0 to 1.25 with a mean of 0.49% (SE=0.01%) (Table 2), while for intragenic distance ranged from 2.8% to 7.67 with a mean of 4.53% (SE=0.05). The distance within families ranged from 6.91% to 17.13 with mean of 10.35% (SE=0.01).

Barcode gap analysis revealed intra and interspecific sequences distance in species (Table 2-3) (21, species, 40 sequences analyzed). Here, low intraspecific distance (<3%) suggest low species resolution, thus leading to species overlap. Intraspecific distances could not be determined for the 10 species with just a single representative. Gaikwad *et al.* (2011) studied butterflies from Western Ghats of Maharashtra. Vikas Kumar (2019) also studied Geometridae moths from Namdapha National Park, Eastern Himalaya.

Table 3 Showing distance summary of 55 sequences

	n	Taxa	Comparisons	Min Dist (%)	Mean Dist (%)	Max Dist (%)	SE Dist (%)
Within species	30	11	33	0	0.49	1.25	0.01
Within genus	17	3	24	2.8	4.53	7.67	0.05
Within family	38	3	207	6.91	10.35	17.13	0.01

Table 4 Showing nucleotide frequency distribution

	Min	Mean	Max	SE
G %	13.83	14.45	15.2	0.04
C %	13.7	15.34	19.76	0.15
A %	28.57	30.85	35.56	0.19
T %	30.7	39.36	41.95	0.23
GC %	28.38	29.78	33.74	0.14
GC % Codon Pos 1	37.13	40.02	43.38	0.19
GC % Codon Pos 2	40.61	41.92	43.35	0.07
GC % Codon Pos 3	3.77	7.48	18.18	0.38

Nearest neighbor distances were greater than 3% for all the species but for two species pairs: 1) *Agrotis ipsilon* (EDBLM004/EDBLM005) vs *Agrotis munda* (EDBLM034) it was 2.8, *Agrotis munda* (EDBLM035) vs *Agrotis ipsilon* (EDBLM005) it was 2.8. The max-intraspecific distance was observed with four species, *Agrotis ipsilon* (1.12), *Spodoptera exigua* (1.14), *Isturgia disputaria* (1.25), *Sesamia inferens* (1.23) respectively. The specimens of *Isturgia disputaria* showed maximum intra-species divergence 1.25% collected from different regions while in *Agrotis segetum* it was zero (0%) even when they were collected from distinct geographical area. The largest nearest neighboring distance of 14.47% was observed in *Hydrilodes metisalis vs Agrotis munda*. The average nearest neighbor distance was 8.39% (SE=0.16). The maximum likelihood tree with the highest log likelihood (-6050.81) was obtained (Fig 1). Family Noctuidae formed monophyletic clade with four species, *Chaismia* and *Scopula* (Geometridae) and *Creatonotos gangis* and *Acantholipes* (Erebediae). *Selepa* species (Nolidae) formed monophyletic clade with *Culasta indecisa* (Erebediae). *Noordae blitealis* (Crambidae) found grouped with *Chiasmia* species (Geometridae). The present tree shows that *Lamoria anella* (Pyralidae) is ancestor of all other families.

In present work, genus *Agrotis* formed closed clustering with each other and with species *Sesamia inferens. Athetis reclusa* clustered closely with *Helicoverpa armigera.* All these species are morphological different each other but formed clade with district families show presence of cryptic species in the region. In present study, we have produced first DNA based identification of moths from Marathwada region of Maharashtra, which may help effective management of some pest species of moth from here.

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