DNA barcoding of Geometridae moths (Insecta: Lepidoptera): a preliminary effort from Namdapha National Park, Eastern Himalaya

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ABSTRACT

The order Lepidoptera is a highly diverse and well-established indicator taxon that helps to monitor anthropogenic threats in the environment and climate change. In India, the taxonomic studies on Lepidoptera have been started long ago, but the molecular studies have been performed rarely, especially in the Himalayan region. The present study generated DNA barcode sequence of 44 Geometridae moths from Namdapha National Park in Eastern Himalaya and identified them through wing pattern and genitalia structure. Among them, the DNA barcode data of 13 Geometridae species are the novel contribution in the global database. All the studied species were revealed sufficient Kimura 2 Parameter (K2P) genetic divergence and distinguished by Bayesian (BA) tree. The genus Proplepsis (subfamily Sterrhinae), Pelagodes and Lophophelma (subfamily Geometrinae) showed distinct clades with their respective species in the BA tree. The BA cladogram successfully separated the studied specimens under three different tribes; Macariini, Eutoeini, and Boarmiini (subfamily Ennominae). The study further revealed the new records of two Geometridae moths, Pelagodes bellula and Hypomecis costaria from India. Nevertheless, more than one clade of Cleora, Hypomecis, and Chiasmia in BA tree; further impelled more rigorous sampling of the studied taxon from different geographical regions for better systematic interpretation.

INTRODUCTION

The order Lepidoptera is one of the megadiverse insect group with approximately 157,424 species under 15,578 genera throughout the globe (van Nieukerken et al. 2011). They are found in all continents, except Antarctica, inhabiting all terrestrial habitats, and associated with higher plants, especially angiosperms (Gullan and Cranston 2005). Due to enormous diversity and functional importance in the eco-system, this insect group is regarded as an indicator taxon and potential focal system for ecological monitoring (Kitcing et al. 2000; New 2004; Summerville et al. 2004; Lomov et al. 2006). Among all lepidopterans, the family Geometridae is the largest group, with 23,000 described and 40,000 estimated species so far (Miller et al. 2016). Geometridae caterpillars, commonly known as loopers or inchworms, feed on single host-plant and govern host-plant abundance and diversity (Ward and Spalding 1993). They can be robustly sampled using standard light trapping and identified by comparing the available literature across the world. Till date, total 1558 Geometridae species have been reported from India (Kirti et al. 2014); among them, 879 species were recorded from pan Indian Himalaya with 70 species from Arunachal Pradesh state in the northeastern region (Sanyal et al. 2018).

Due to the complex morphological characters, hybridization, and mimicry; the accurate species identification of Geometridae moths is difficult (Janzen et al. 2005; Hausmann et al. 2009; Huemer and Mutanen 2012). Although many new species and new records of Geometridae moths has been published every year from India; but an accurate DNA barcode reference library is missing to successfully answer several taxonomic ambiguities and biogeographic questions.

Since the inception of DNA barcoding as a molecular technique for species identification (Hebert et al. 2003), many studies have been targeted to know the region-specific lepidopteran biodiversity (Lukhtanov et al. 2009; Dinca et al. 2011; deWard et al. 2011; Hausmann et al. 2011, 2013; Wilson et al. 2013; Huemer et al. 2014; Liu et al. 2014; Zahiri et al. 2014). Several studies have been shown to resolving the taxonomic quest and the dilemma in lepidopteran systematics (Hajibabaei et al. 2006; Burns et al. 2008; Hausmann et al. 2009; Mutanen et al. 2012; Jiang et al. 2017). The publicly available DNA barcode sequences in the global database proved to be useful for identification of regional faunas (Hajibabaei et al. 2006; Burns et al. 2008; Hausmann et al. 2009; Mutanen et al. 2012; Jiang et al. 2017) and detect the expansion of the range distribution (Mutanen et al. 2012; Hausmann et al. 2013; Huemer et al. 2014; Dinca et al. 2015).
Despite the rich lepidopteran diversity in India, very few attempts were made to generate DNA barcode data of Indian moths. Therefore, the present study preliminarily aimed to generate DNA data of Geometridae moths from the Namdapha National Park in eastern Himalaya to determine the effectiveness of DNA barcoding for species identification. The study contributed DNA barcode data of taxonomically identified Geometridae species in the global database for succeeding research.

Materials and methods

**Sample collection and morphospecies identification**

Total 44 Geometridae moths were collected from the Namdapha NP (27.48 N 96.45 E) in Changlang district of Arunachal Pradesh (Table 1). The specimens were collected at night time in a single day by installing traditional light trap methods (Fry and Waring 1996). The collected specimens were killed with ethyl acetate vapor and stored in paper envelopes with collection details. The specimens were stretched, pinned, labeled, and dried properly in the laboratory for further morphological studies. The genitalia of studied specimens was also examined for in-depth morphometric analysis. The specimens were identified with the help of available literature (Hampson 1895; Warren 1899; Scoble 1999), published genitalia illustrations (Fletcher and Nye 1979; Holloway 1993, 1996, 1997; Sato 1995), and reference voucher specimens available at the National Zoological Collection (NZC) in the Zoological Survey of India (ZSI), Kolkata. The studied specimens were submitted to the NZC, ZSI, Kolkata, with proper voucher numbers and collateral information.

**Genomic DNA isolation, PCR, and sequencing**

The hind legs of each specimen were collected for DNA analysis and stored in 70% ethanol at −80 °C. The total
Figure 1. Map with red dot showing the collection locality of Geometridae moths in Namdapha National Park, Arunachal Pradesh states in northeast India. Bayesian tree based on partial mtCOI gene shows the species topology of the studied Geometridae moths. Clades with different colours shows the congeners of different genus. Colour clades with dotted lines shows the ambiguous clades of different Geometridae congeners. The generated sequences of this study were indicated by “ZSI LP”. The sequence of *Arctopsyche amurensis* (Trichoptera) was incorporated as the out-group in the BA tree. The habitus, ventral view of genitalia, and aedeagus of the studied species were superimposed beside the tree: (A) *Pelagodes bellula*; (B) *Cleora propulsaria*; (C) *Dalina calamina*; (D) *Darisa lampasaria*; (E) *Hypomecis looptilaria*; (F) *Luxiaria acutaria*; (G) *Racotis inconclusa*; (H) *Lophophelma erionoma*; (I) *Hypomecis costaria*; (J) *Lassaba albibaria*; (K) *Xerodes ypsaria*; (L) *Chorodna testaceata*; (M) *Chiasmia pseudonora*; (N) *Lophophelma vigens*; (O) *Chorodna moorei*. The habitus of the studied species: (P) *Antipercnia belluaria*; (Q) *Fascellina plagiata*; (R) *Petelia medardaria*; (S) *Krananda semihyalina*; (T) *Problepsis albior*. Red arrows are showing the relevant diagnostic characters of the studied species for accurate species level identification.
Genomic DNA was extracted following the Phenol-Chloroform-Isomyl alcohol standard protocol (Sambrook and Russell 2001). The published primer pairs; LCO1490: 5'-GGTCAAAACAATCTAAAAGATTTG-3', HCO2198: 5'-TAAACTTCAGGGTGACAAAAATCA-3' (Folmer et al. 1994), and LepFi: 5'-ATTCAACCAAATCTAAAAGATTTG-3', LepRi: 5'-TAAACTTCAGGGTGACAAAAATCA-3' (Hebert et al. 2004) were used for amplification of a partial fragment of mitochondrial Cytochrome C Oxidase Subunit I (mtCOI) gene in a Verity® Thermal Cycler (Applied Biosystems, Foster City, CA). The PCR reaction was set in a 30-µl total volume containing 20 picomoles of each primer, 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1.8 mM MgCl₂, 0.25 mM of each dNTP, and 1U of Taq polymerase (Takara Bio Inc., Shiga, Japan) with the following cycling parameters: 5 min at 94°C; followed by 40 cycles of 30 s at 94°C, 40 sec at 49°C, 1 min at 72°C, and final extension for 5 min at 72°C. The amplified PCR products were checked in 1.2% agarose gel. The PCR products were purified using a QiAquick R Gel extraction kit (QiAGEN Inc., Germantown, MD), and cycle sequencing products were cleaned by using the standard BigDyeXTerminator Purification Kit (Applied Biosystems, Foster City, CA). Sequencing was done bi-directionally in the 48 capillary array 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) following Sanger sequencing methods in ZSI in-house sequencing facility.

Sequence quality control measure, dataset preparation, and analysis

The generated forward and reverse chromatograms of each specimen were analyzed with SeqScape software version 2.7 (Applied Biosystems) and consensus sequences were obtained after checking deletion, insertion, and stop codons. The similarity search of the generated sequences were performed through BLASTn in GenBank and identification engine in the BOLD database. The generated sequences were submitted to the GenBank and BOLD database through Bankit and BOLD project. Further, to form a combined dataset and construct the phylogeny, 121 published sequences of same or closely related taxa were obtained from the GenBank. One sequence of Arctopsyche amurensis (order Trichoptera) was incorporated in the dataset as out-group. Total 166 sequences were aligned with Clustal X program to form a combined dataset and further analysis (Thompson et al. 1997). The mean genetic divergences were calculated using the Kimura 2 parameter (K2P) in MEGA6.0 (Tamura et al. 2013). The reciprocal monophyly among the studied specimens was tested through Bayesian analysis (BA) with a best-fit model in Mr. Bayes 3 (Ronquist and Huelsenbeck 2003). The bayesian analysis involved Markov Chain Monte Carlo (MCMC) with four chains for 1,000,000 generations, with trees sampled every 100 generations (the first 1000 trees were discarded as burn-in). MCMC analysis was stationary when the maximum standard deviation of split frequencies reached below 0.01 and potential scale reduction factor (PSRF) approached 1.0.

Results and discussion

The unique accession numbers and BOLD Process IDs of each generated DNA barcode sequence were acquired from both GenBank and BOLD database. The similarity search tool preliminarily identified 28% of the studied specimens up to the species level (Table 1). As of now, lots of DNA barcode data are available for lower invertebrate groups, especially for the order Lepidoptera in both GenBank and BOLD system (Scoble and Hausmann 2009). However, due to the lack of exhaustive sampling, wrong morphological identification, and mislabeled DNA barcode data in the global database, the reference DNA sequence library often misled to identify the species through online similarity search tool (Hausmann et al. 2013). Thus, the estimation of species diversity of any specific geographical areas is becoming elusive. In this study, we generated the DNA barcode data of morphologically identified Geometridae moths and screened through similarity search tool, genetic distance, and Bayesian clustering.

Further, due to the phenotypic variations in wing pattern and cryptic diversity (deWaard et al. 2011), the genitalia characters of the studied Geometridae moths were revisited to validate the generated DNA barcode sequences. Based on the 24 singletons and seven analogous barcode index numbers (BINs), the BOLD data system revealed a total of 31 operational taxonomic units (OTUs) in the dataset (Table 1). Out of 44 specimens, 13 were unable to identify up to the species level due to lack of morphological data. Among them, three specimens were identified under subfamily Ennominae, and ten specimens were identified under the genus Cleora, Racotis, Chiasmia, and Petelia. The remaining 31 specimens were identified up to 20 species level by both morpho-taxonomy and molecular approaches. The generated barcode sequences of C. propulsaria, D. lampasarai, H. lioptilaria, H. costaria, R. inconclusa, L. erionoma, L. vigens, P. albiodior, C. pseudonora, C. moorei, D. calamina, L. acutaria, A. belluaria are the new contribution in the global database from this current study. The intra-generic genetic divergence of Pelagodes was 5.4%. The two generated sequences Pelagodes bellula showed 8.1% genetic divergence and close clustering with the Pelagodes aucta in BA tree (Figure 1). Due to the external morphological variation, the genus Pelagodes and the allied group Thalassodes frequently showed cryptic diversity. The genus Pelagodes, Thalassodes, Orotchassodes, and Remiformvalva were previously considered under the genus Thalassodes. Further, based on the structure of the male genitalia and eighth abdominal sternite, many species were shifted from Thalassodes and erected two new genera, Orotchassodes, and Pelagodes (Holloway 1996). In this present study, both morphology and DNA barcode data revealed the new record of P. bellula from northeast India. The presence of P. bellula in the studied region evidenced the occurrence of the species more westward and expand the range distribution in China, Myanmar, and northeast India. The P. bellula is morphologically similar to the Indian species P. aucta, and can be diagnosed with basally broad and pointed costal extension and absence of the sclerotized area in the sacculus of male genitalia (Han and Xue 2011).
The genus Cleora shows 11.1% genetic divergence within the genus in the present dataset which depicts a wide range of intra-generic variation. Most of the Cleora species included in the present dataset, were clustered together in BA tree; however, three sequences of Cleora nesiotes showed separate cluster (Figure 1). The two generated sequences of Cleora propulsaria and three generated sequences of Cleora sp. shows maximum 1% genetic divergence. Thus, based on the genetic distance and BA tree, the generated sequences (ZSI_LP7, ZSI_LP11, and ZSI_LP13) were confirmed as C. propulsaria. The ZSI_LP10 identified as Cleora sp., shows 6.3% to 7.1% genetic divergence with C. propulsaria, and might be a distinct species. Further, the two generated sequences of Cleora sp. (ZSI_LP8 and ZSI_LP9) resulted high genetic divergence (4.4–7.6%) and maintain sister clades with Cleora sabulata and Cleora tenebrata in the BA tree which assumed to be distinct species. The generated sequence of Darisa lampasaria shows 8.7–12.4% genetic divergence with the other Geometridae species in the dataset and depicts a distinct clade in the BA tree (Figure 1). Further, the specimen (ZSI_LP16) was morphologically identified as Petelia medarda. The generated and database sequence of P. medardaria shows maximum genetic divergence (1.2%) and single clades in BA tree. The specimen (ZSI_LP28), which was preliminarily identified as Petelia species shows 2.4–3.4% genetic distance with P. medardaria and clade separately, which might be a distinct species.

The present dataset resulted intra-generic genetic divergence of Hypomecis is 10.3% and the intra-species genetic divergence of Hypomecis lioptilia is ranging from 0 to 0.5%. Further, most of the Hypomecis species shows clear-cut clustering in the BA tree except Hypomecis taeniota, Hypomecis suasaria, Hypomecis proscora, Hypomecis atactopena, and Hypomecis zalochema. The generated sequences of H. lioptilia and Hypomecis costaria resulted a maximum of 13.6% genetic divergence and clade separately in BA tree (Figure 1). Furthermore, the BA tree shows sister relationship of H. lioptilia and H. costaria with Hypomecis infaustaria, but distant by more than 10% genetic divergence with each other. The study confirmed the range expansion of H. costaria from South East Asia, Sarawak, Borneo up to mainland India. The intra-generic genetic distance of Racotis is 6.8% as compared with the other genus. The generated sequences of Racotis inconclusa (ZSI_LP20) and Racotis sp. (ZSI_LP20A) shows 0.2% genetic divergence and assumed to be the same species. However, the other two specimens of R. inconclusa shows distinct clade in BA phylogeny (Figure 1) with high intra-species genetic divergence (7.3–9.1%), which depicted the presence of the cryptic diversity of R. inconclusa in northeast India. The specimen (ZSI_LP43) is identified as Lophophelma vigens and its distribution pattern is similar to Lophophelma erionoma. The present dataset resulted 9.2% intra-generic genetic distance of Lophophelma and high intra-species genetic divergence (9.1%) in L. erionoma. Further, the generated sequence of L. vigens shows a maximum of 9.7% genetic divergence with L. erionoma in the present dataset. The specimen (ZSI_LP25) was morphologically identified as Lassaba albidaria, which is widely distributed in India (Himachal Pradesh, Sikkim, Uttarakhand, Meghalaya, West Bengal), and other parts of World (China, Pakistan, Nepal). The estimated genetic divergence with L. albidaria and other dataset species were ranging from 8.8 to 13%.

However, due to the lack of published sequences in the database, L. albidaria shows distinct clade in BA tree (Figure 1). The intra-generic genetic distance of Prolepsis was 8.5% with seven species in the present dataset. The Prolepsis albidor shows distinct clade in BA tree and seems to be a sister species of Prolepsis ocellata with 6.2% genetic divergence. The intra-generic genetic distance of Krananda was 8.3% with the database sequence of Krananda extranotata. Further, the two generated sequences of Krananda semihyalina shows high genetic divergence (4.9%) within the species. The K. semihyalina and K. extranotata shows 9.1 to 10.8% genetic divergence and clustered separately in BA tree (Figure 1). The genetic distance of Xerodes with other studied genera in the dataset was ranging from 9.4 to 12.5%. The two specimens (ZSI_LP29 and ZSI_LP33) morphologically identified as Ennominae species clustered closely in the BA tree and maintain 9.7% genetic divergence with each other. The three specimens (ZSI_LP34, ZSI_LP36, and ZSI_LP37) were identified as Chiasmia species, among them one specimen (ZSI_LP36) was identified up to the species level as Chiasmia pseudonora by male genitalia and the remaining two specimens were identified up to genus level (Gayal 2010). The intra-generic genetic distance of Chiasmia was 9.9% as compared with other studied genera. The morphologically identified C. pseudonora (ZSI_LP36) and Chiasmia sp. (ZSI_LP34) are resulted low genetic divergence (0.5%) and clustered closely in the BA tree, thus the ZSI_LP34 was considered as C. pseudonora. Further, two closely related species, C. pseudonora and Chiasmia hypomochla resulted 8.9 to 9.1% genetic distance in the dataset. Further, the generated sequences of Chiasmia sp. (ZSI_LP37) clustered with two published database sequences of Chiasmia goldiei and maintain 6.8% genetic divergence with each other. The BA tree shows two distant clades of Chiasmia species with 9.7 to 12.2% genetic divergence.

The genetic distance of Fascellina was ranging from 9.1 to 11% as compared with other studied genera in the dataset and showed distinct clade in BA tree. The genus Chorodna shows 5.1% genetic divergence as compared with other genera in the dataset. The two identified species Chorodna moorei and Chorodna testaceata shows 4.9% genetic divergence and clustered separately in BA tree (Figure 1). Further, the two database sequences of Chorodna strixaria shows a maximum of 6.7 and 6.2% genetic divergence with C. moorei and C. testaceata, respectively. The genus Dalima shows 8.1 to 11.8% inter-generic genetic distance in the dataset. The BA tree shows the close relationship of Dalima species with Chorodna species with a maximum of 9.7% genetic divergence. The genus Luxiaria resulted 9.1% intra-generic and maximum of 13.6% inter-generic genetic divergence in the dataset. The generated sequence of Luxiaria acutarai shows 10.2% and 7.5% genetic divergence with the two published database sequence of Luxiaria ochrophora and Luxiaria phyllosaria, respectively. The BA tree shows a distinct clade of Luxiaria species with the generated sequences of L. acutarai. The four generated sequence (ZSI_LP79, ZSI_LP80, ZSI_81, ZSI_82) were morphologically identified as Antipercnia belluaria. The inter-generic divergence of the genus Antipercnia was depicted 11.1 to 14.7% with other studied genera. The four generated sequences of A. belluaria were clustered.
distinctly in BA tree with a maximum of 0.2% genetic divergence within the species.

This study is the first and preliminary DNA barcode-based assessment of Geometridae moths from the eastern region of the Indian Himalaya. Due to limited species coverage, the study did not discuss the in-depth phylogenetic relationship of any hierarchical level of Geometridae moths. In the present dataset, the genus *Prolepsis* of subfamily Sterrhinae and two genera, *Pelagodes* and *Lophophela* of the subfamily Geometrinae formed a distinct clade from rest of the Ennominae species in the BA tree. Further, the studied specimens under three different tribes of subfamily Ennominae clustered closely in the resulted cladogram. The generated and database sequences of *Chiasmia* taxa under Macarini tribe, *Lassa* taxa under Eutoeini tribe, and the remaining studied genera under Boarmini tribe shows close clustering with each other. The members of Macarini and Eutoeini tribes were frequently considered as a monophyletic within Boarmini lineage. Further, the members of Boarmini were often resulted ambiguous clades as compared with the known taxonomical classification and considered as paraphyletic (Jiang et al. 2017). In the present study, the members of *Cleora*, *Hypomecis*, and *Chiasmia* shows similar uncertain clades (Figure 1) with high morphological variations. Hence, more exhaustive sampling from broad geographical areas is required to get a clear insight into the evolutionary relationship of the Indian Boarmini members and other related taxa in Ennominae clade.

**Disclosure statement**

The authors declare that they have no competing interests.

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**Author contributions**

Conceived and designed the experiments: VK, SK, KT, KC; Performed the experiments: RC, OS, RR, AP; Morphological examination: AS, AR; Analysed the data: SK, VK, KT, AP; Contributed chemicals and analysis tools: VK, KC; Wrote the paper: SK, VK, AS, KC; All authors reviewed the manuscript.

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