Towards creating a DNA barcode reference library of geometrid moths from western Himalaya, India

(Lepidoptera, Geometridae)

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The taxonomy of the family Geometridae (Lepidoptera: Geometroidea) is rather well established for most temperate regions of our earth, but tropical areas still need large-scale revisions. The Indian Himalayan region with its unique physical attributes is home to a distinctive faunal diversity but there is a lack of comprehensive study on its Geometridae moth fauna. In the present study a total of 113 species of the family Geometridae collected in the Nanda Devi Biosphere Reserve (a UNESCO World Heritage Site), Uttarakhand, India have been subjected to DNA barcoding (CO1). Distinct Barcode Gaps (difference between intraspecific and interspecific nucleotide divergence) exist between most of the species studied, thus confirming their discrimination. Sequences of species from the Lepidoptera Collection of the Zoologische Staatssammlung München collected from western Himalaya (Himachal Pradesh and Kashmir) and eastern Himalaya (Sikkim) were included into the analysis. Four species from Sikkim show deep genetic (K2P distance) divergence from conspecifics collected in the western Himalaya (Uttarakhand), indicating potential cryptic diversity. Our preliminary assessment was based on DNA barcoding in combination with standard taxonomy, and revealed one new species record for India and distribution extensions of 15 other species within India. The baseline data generated through this study will provide scope to do further extensive research on moths in this region, providing effective geographical distribution data, ecological understanding and foundation for their conservation in the face of habitat degradation and climate change.

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Introduction

The Barcode of Life initiative (Hebert et al. 2003a,b), which started more than a decade ago, has been established as a standardized method for identifying species and for reducing the morphological taxonomic burden, by using a single gene, cytochrome c oxidase subunit 1 (CO1). DNA-based taxonomy – as initially proposed by Tautz et al. (2003) – has, since then, proven to be an immensely useful tool to solve taxonomic problems of lesser known groups (cf. Miller et al. 2016). DNA barcoding adds genetic information to the already established Linnean taxonomy, but sometimes the barcode analysis reveals a mismatch with existing Linnean binomials (Pons et al. 2006). This mismatch or incongruency is an effect of either cryptic species (Hebert et al. 2004a,b); or a lack of expert taxonomic species delineation

(Agapow et al. 2004, Wiens & Penkrot 2002). DNA barcoding, thus far, has been successfully applied across taxa and across countries, including plants (Group et al. 2009), fishes (Ward et al. 2005, Hubert et al. 2008), mammals (Clare et al. 2007), birds (Kerr et al. 2007), insects (Hebert et al. 2004a, Hastings et al. 2008, Hendrich et al. 2014, Morinière et al. 2014, Schmidt et al. 2015, Hawlitschek et al. 2016), crustaceans (Hou et al. 2009) among others. The order Lepidoptera has received particular attention (Janzen et al. 2005, 2009, Hajibabaei et al. 2006, Burns et al. 2008, Silva-Brandão et al. 2009, Hausmann et al. 2011a,b, 2013, 2016, Mutanen et al. 2016 and many more) with currently 1.09 million barcode records on BOLD, the Barcode of Life Data System (Ratnasingham & Hebert 2007). The success of DNA barcoding has encouraged efforts to build DNA barcode libraries across various groups and regions globally (Ekrem et al. 2007, Kerr et al. 2007, Zhou et al. 2009, 2011, deWaard et al. 2010, 2011, Dincă et al. 2011, Kuzmina et al. 2012, Webb et al. 2012, Raupach et al. 2014) also using natural history collections in museums (Strutzenberger 2012, Hebert et al. 2013, Hausmann et al. 2016). DNA barcodes in recent years have shown promising new prospects in answering fundamental ecological questions that govern community assemblage, macroevolutionary processes and conservation of species (Joly et al. 2014, Veldman et al. 2014). The ecological information coupled with the understanding of evolutionary histories from DNA barcodes across taxa can assist in answering complex questions relevant to species assembly and distribution in the era of bioinformatics (Joly et al. 2014). Moreover, it is a valuable tool to understand feeding habits (González-Varo et al. 2014) and target protection of habitats (Kress et al. 2015).

Despite their fundamental roles in nature as selective herbivores, pollinators, detritivores, and prey for birds, e. g. migratorial passerines, forest indicator taxa (Summerville et al. 2003a,b, Kitching et al. 2000, Beck et al. 2002, 2017, New 2004) and potential in the definition of conservation priority areas, moths have been strongly neglected in conservation studies. Geometridae, together with the Erebidae, are the most species-rich family of moths worldwide. Geometridae is a cosmopolitan family with the exception of Antarctica and shows diversity peaks in tropical South America, Africa and South-east Asia. This group is a well-established model group for biodiversity studies in temperate and tropical regions (Brehm et al. 2013, 2003a,b, Axmacher et al. 2004, 2009, Barlow, 1982, Barlow & Woiwod 1989 and many more). Geometrids are known to associate with the prevailing environmental conditions while other families like Erebidae include larger portions of species which can colonize human-affected habitats replacing forest species (Summerville 2004, Winfree et al. 2011). Large parts of the diverse Geometridae family may be at the risk of extinction due to destruction and fragmentation of their habitats in the tropics. The taxonomy of geometrid moths is replete with ambiguities. A global review suggests that there is some degree of synonymy at the species level in this family (Scoble et al. 1995) which suggests that taxonomic revisions are impending. There is no comprehensive morphology-based phylogeny available for geometrid moths, but the relationships between subfamilies and many tribes were recently assessed from the analysis of several nuclear genes (Young 2006, Yamamoto & Sota 2007, Regier et al. 2009, Õunap 2011, Sihvonen et al. 2011) largely confirming the traditional classification derived from morphological traits.

Coming to the Indian scenario, Gaikwad et al. (2012) and Kumar et al. (2019) barcoded the butterflies from the Western Ghats and the geometrid moths from the eastern Himalaya respectively. DNA barcoding of groups other than Lepidoptera from India has also been attempted in recent years (Kumar et al. 2007, 2012, Lakra et al. 2011, Dubey et al. 2011, Laskar et al. 2013, Gaikwad et al. 2016, Kaur & Sharma 2017). The integration of molecular tools and morphology for taxonomic research, especially for Lepidoptera is still in a nascent state. How do we conserve species with limited knowledge of which species are endangered or even how many species there are? There is lack of a comprehensive inventory of moths from western Himalaya, despite being one of the most biodiverse regions. The Himalayas provide an array of habitats and forest types comprising an excellent scenario to understand the diversity and the related evolutionary processes in this mosaic landscape. Moreover, we find Himalayan-Chinese and Indo-Malayan faunal dominance in the eastern Himalayas as compared to the western Himalayas (Mani 1968). The factors governing the distribution of high altitude insects differ among different mountains systems, depending partly on the present ecological conditions and historical distribution. The wide variations of the geological conditions provide diversified edaphic factors (Mani 1968). Western Himalaya, where the Oriental and Palearctic elements merge (Meinertzhagen 1928), has a unique biodiversity and interesting patterns can be expected. Faunistic inventories are the basis of biodiversity conservation, particularly in threatened and fragmented landscapes like the western Himalaya. An inventory of the moth fauna based on both morphological and molecular characteristics from this area would definitely add valuable information to the existing data and help us look into moth diversity in a comprehensive manner.
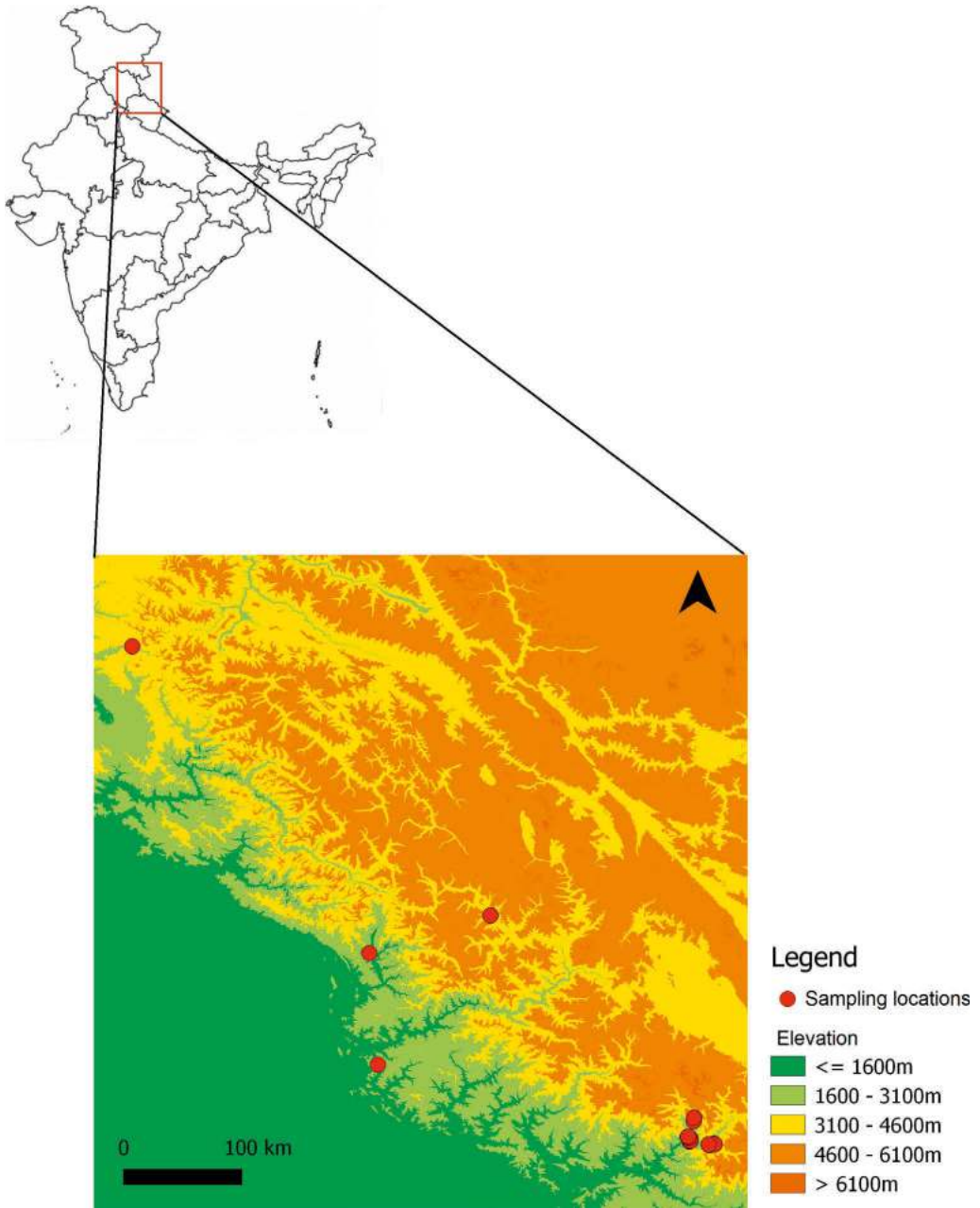
Fig. 1. The elevational gradient map shows the different sampling points from parts of states of Uttarakhand, Himachal Pradesh and Kashmir in the western Himalaya.

The intention behind this study is to combine the exploration of unstudied biodiversity of the study area and using DNA barcoding as an established tool for improving classifications and understand diversity patterns for highly diverse groups like the geometrid moths in India.
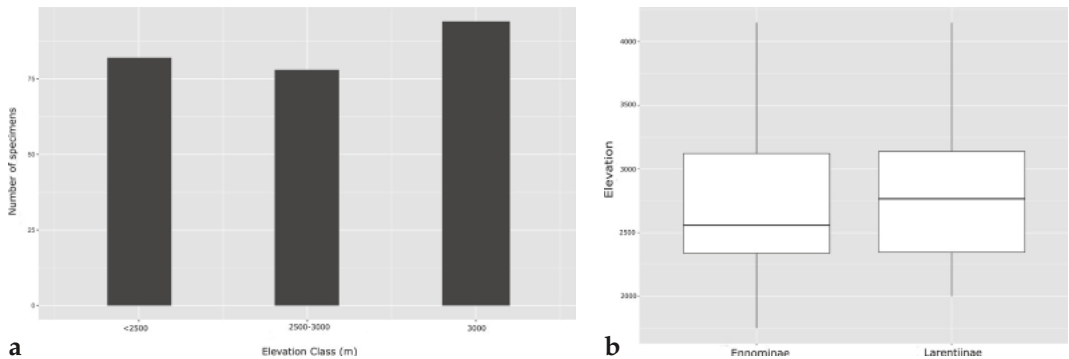
a

b

Fig. 2. a. The graph shows that specimens of Geometridae moths from different altitudes in western Himalayan states of Uttarakhand, Himachal Pradesh and Kashmir were collected with almost equal number of specimens across the elevational classes. **b.** The box-plot shows the elevational distribution of the two most speciose subfamilies (Ennominae and Larentiinae) of Geometridae in the present study. The highest altitude of collected specimens in both of the subfamilies was 4150 m a.s.l.

Materials and methods

Abbreviations

BOLD   Barcode of Life Data Systems
CCDB   Canadian Centre for DNA Barcoding
ZSM   SNSB – Zoologische Staatssammlung München, Germany (Bavarian State Collection of Zoology, Munich)

Collecting

Moth specimens were collected in the Nanda Devi Biosphere Reserve, Uttarakhand, India using light-trapping (vertical sheet method). Specimens were collected across the elevational and vegetation gradient of the study area. A part of the collected specimens was pinned, labelled, mounted and identified at the ZSM to provide a reference collection for the DNA barcode library initiative for the western Himalaya. Not all specimens could be used due to legal restrictions in transferring biological material outside the country of collection. Locations of all the specimens used for DNA analysis are shown in Figure 1. Figure 2 shows the elevational distribution.

DNA sequencing

One dry leg was removed from each individual from the specimens collected by the first author and some specimens from the Herbulot collection stored in the ZSM, with sterile forceps and transferred to a 96-well microplate preloaded with one drop of 95 % ethanol in each well. DNA extraction and sequencing were performed at the CCDB, University of Guelph, following standardized high-throughput protocols for DNA barcode amplification and sequencing (Ivanova et al. 2006, deWaard et al. 2008).

Detailed information on the voucher specimens, genetic sequences, images, taxonomic classification, collection data and other relevant information for the specimens collected by the first author is available in the public data set DS-INDIAGEO in the Barcode of Life Datasystems (BOLD; www.boldsystems.org) (Ratnasingham & Hebert 2007, 2013).

DNA sequence analysis

Additional sequences from the specimens collected in Himachal Pradesh, Kashmir and Sikkim regions of India, stored in the Herbulot collection at the ZSM and available (unpublished) in BOLD were included into some analysis steps. Sequence divergences for the barcode region were calculated for only the 224 specimens collected by the first author, using the analytical tools on BOLD Barcode Gap Analysis with BOLD alignment (pairwise deletion) using the Kimura 2 Parameter model (Kimura, 1980). Distances between species are presented as minimum pairwise distances, intraspecific variation as the mean and maximum pairwise distance within that species. BOLD assigns a Barcode Index Number (BIN) (Ratnasingham & Hebert 2013) automatically to sequences >500 bp which belong to the related sequence cluster. BINs very often coincide with the traditional species concepts but can reveal genetic splits within an "established" species or can reveal BIN-sharing ("merge") of two taxa which in previous studies were thought to be two different species. In cases of discrepancy between the sequence based and morphological taxonomy, the specimens were re-examined morphologically to ascertain their identification.

Results

In the present study, we were able to generate 224 CO1 sequences from 250 specimens (90 %). 46 sequences out of 68 specimens (67 %) were added from the collection Herbulot in the ZSM.
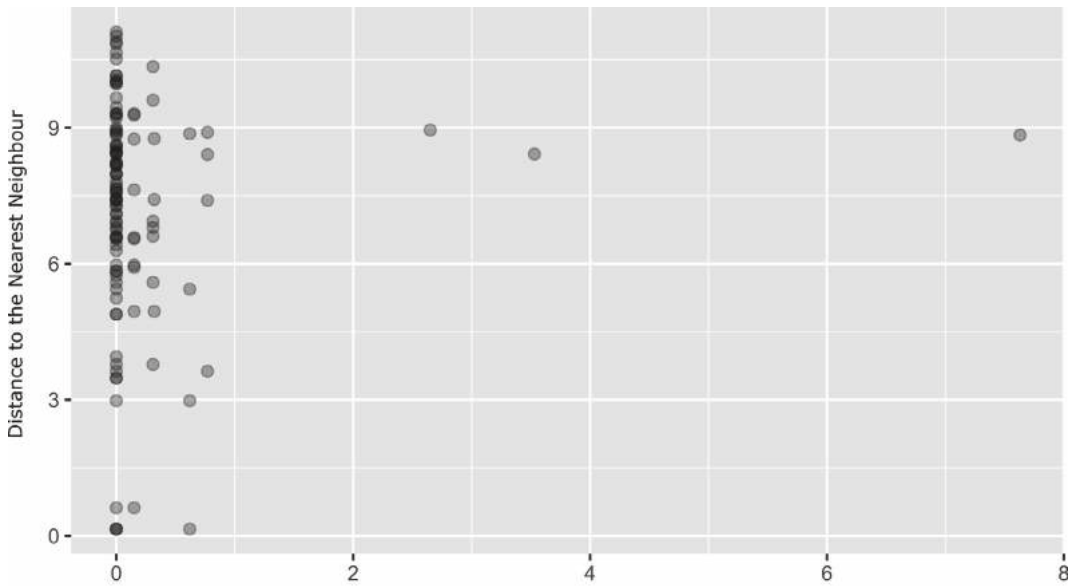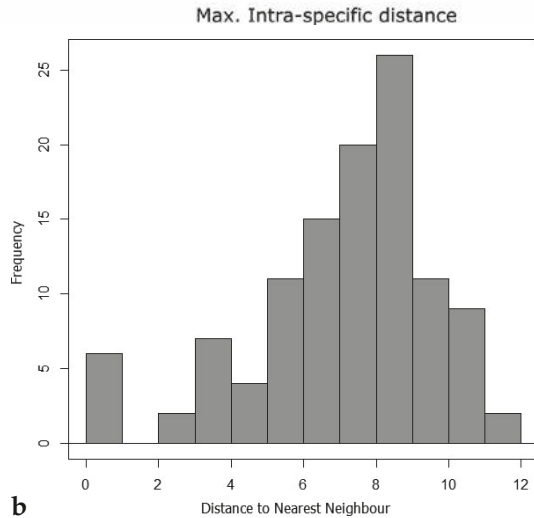
Fig. 3a, b. Barcode Gap Analysis performed with BOLD analytical tool shows the max. intra-specific distances in relation to the distances to the Nearest Neighbour and frequency distribution of the Nearest Neighbour distances in all the specimens collected from Uttrakhand by the first author.

Present study specimens from prov. Uttara-khand, western Himalaya: Out of the 224 DNA barcoded specimens, 35 specimens have only been identified to genus level, the rest (184 = 82.14 %) were assigned morphologically to a Linnean species name, while 5 specimens remain unidentified. Out of the 250 specimens subjected to DNA barcoding, 148 specimens belong to Larentiinae, while 90 belong to the subfamily Ennominae, 8 and 4 specimens to Sterrhinae and Geometrinae respectively. A total of

113 morphological species belonging to 51 genera were assigned to 116 BINs, out of which 63 BINs were singletons. All pre-identified species with multiple records/specimens were placed in a single BIN and successfully discriminated with a distinct Barcode Gap without any overlap in the study area except for *Alcis nudipennis – Alcis leucophaea* (BOLD:AAJ3727), *Opisthograptis mimulina – Opisthograptis* PD01 (BOLD: AAK5702) sharing their BINs. Some species were as-signed to more than one BIN – *Loxaspilates obliquaria*

(BOLD:ADF4071, BOLD:ABA2651), *Trichopterigia* PD01 (BOLD:ADF3716, BOLD:ADF2951) and *Euphyia subangulata* (BOLD:AAL5265, BOLD:ADF3104). Figure 3a and b show the distance to nearest neighbour in relation to the max. intraspecific distance and the frequency distribution of nearest neighbour distances obtained from Barcode Gap Analysis on BOLD. 12 BINs (10.3 %) include a different morphological species from outside the study area (requiring further study). There was overlap of BINs (as currently present on BOLD Data Systems) including specimens from mainly the countries of the Indian subcontinent: Pakistan (24 BINs shared), Bhutan (10 BINs), Nepal (6 BINs), while from a biogeographical point of view 50 BINs are shared with Indo-Malayan realm and only 4 BINs are shared with Palearctic countries. Comparison of conspecific CO1 sequences from Himachal Pradesh, Kashmir and Eastern Himalaya (Sikkim, Kanchenjunga) as generated from ZSM (specimens from Herbulot collection) revealed high intraspecific divergence in four species between western (Uttarakhand) and eastern (Sikkim) Himalayas: *Arichanna flavinigra, Heterolocha falconaria, Abraxas superpicaria* and *Ecliptopera substituta* with intraspecific minimum pairwise genetic distance (Kimura-2-Parameter) generated from CO1 sequences in MEGA 7 varying from 3 to 6 % as shown in Figure 4. These four species will be subjected to further detailed taxonomic examination and molecular evidence based on more samples. The highest divergence was in *Heterolocha falconaria* (5.9 %) followed by *Ecliptopera substituta* (5 %), *Abraxas superpicaria* (4.1 %) and *Arichanna flavinigra* (3.1 %). These species show a very characteristic morphology so any chance of misidentification can be ruled out.

Three male and one female specimens of a taxon, initially identified as *Prometopidia conisaria* Hampson, 1902 (new distribution for the state of Uttarakhand) showed 6.6 % genetic divergence and with further investigation of genitalia and type specimen comparison, it was found that the female specimen is possibly a new species in the genus *Prometopidia*. The morphological analysis of these specimens is beyond the scope of this paper and is discussed in detail in another paper (Dey et al. in prep.).

Alcis paghmana Wiltshire, 1967 was identified through sequence matching in BOLD and thereafter morphological confirmation, representing a new distribution record for India. Correlating intra-/ inter-species sequence divergence results and re-examining morphology revealed the following species with new distribution records for Uttarakhand: *Alcis nudipennis, Alcis perspicuata, Arichanna sparsa, Peetula stramineata, Entephria poliotaria, Perizoma variabilis, Costicoma exangulata, Triphosa venimaculata, Perizoma variabilis, Perizoma conjuncta, Perizoma seriata, Electrophaes zaphenges, Entephria punctatissima,* and *Pennithera comis.*

Discussion

This study is a first initiative for assembling a DNA barcode reference library for geometrid moths from western Himalaya, India. Recently, Kumar et al. 2019 has barcoded some geometrid moths from the eastern Himalaya. But our study shows that investigation of DNA barcode variation and BIN-based species delimitation was successful for 113 species of Geometridae and reconfirming the reliability of DNA barcoding as an effective tool for species discrimination in Lepidoptera. Except for two species pairs with <2 % divergence from the nearest neighbour, intraspecific divergence was found to be lower than the distance to the nearest neighbour, thus allowing successful separation. Similarly high success rates for lepidopteran species re-identifications were found for Germany (Hausmann et al. 2011b), Pakistan (Ashfaq et al. 2013, 2017) and Costa Rica (Hajibabaei et al. 2006). Recent studies have explored continent-wide samples also with high (~ 93 %) re-identification success, e. g. for European geometrids (Hausmann et al. 2013) and North American Noctuoidea (Zahiri et al. 2014, 2017). Since our study area holds much higher species richness than other studies cited here, our results strongly support the importance of molecular taxonomy in the geometrid moth taxonomy in the country where the majority of the fauna awaits genetic analysis.

The success of DNA barcoding depends on the match between the BIN assignments and the taxonomic pre-identification. Neighbour-joining clustering analysis showed high incidence of monophyly in our study results, but the study area is restricted only to western Himalaya, and the sample size was comparatively small. Thus, the relationship between successful species differentiation and geographical range of the samples could not be exploited. We had four species showing intraspecific divergences of 3–6 % collected from two different parts of India, Uttarakhand and Sikkim (at a distance of about 1000 km). Ashfaq et al. (2013, 2017) showed maximum faunal overlap between Pakistan and India, likewise our study reveals the highest number of BINs (24) shared with Pakistan. The case of BIN-sharing of distinct species like that of *Alcis leucophaea* and *Alcis nudipennis* might indicate ancestral polymorphisms with subsequent incomplete lineage sorting (Zahiri et al. 2017) or just a lack of sound taxonomic diagnosis. Discrepancies between DNA barcodes and standard taxonomy
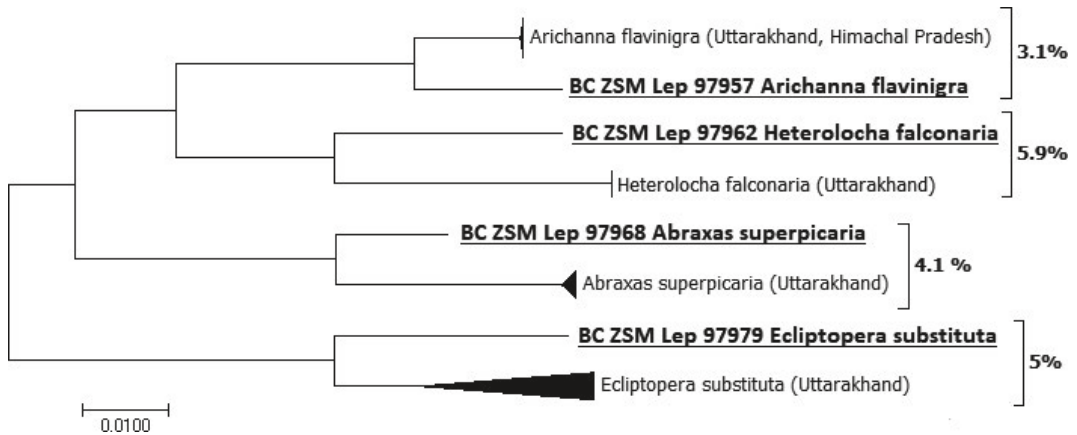
Fig. 4. Neighbour-joining tree (unrooted) showing the genetic divergence between specimens from western and eastern Himalaya found in four species. The names of the branches (bold and underlined) are the specimens from eastern Himalaya (Sikkim) from Collection Herbulot stored in ZSM. Locations of the rest from western Himalaya are given in parentheses.

(once misidentifications are outruled) can be treated as a scope to go in depth both in taxonomy and in the underlying evolutionary processes. Low or no interspecific divergence may suggest potential synonymy (Yang et al. 2016) and species with remarkably high intraspecific divergence demand further in-depth taxonomic analysis as they might include cases of cryptic diversity. However, in our case, low sample sizes and lack of sister species sampling might have affected the intra- and inter-specific distances in some cases giving poorly informative values (Meyer & Paulay 2005). A sample size of 20 samples per species is suggested for a robust DNA barcode analysis (Bergsten et al. 2012, Zhang et al. 2010, Luo et al. 2015).

We have shown that some (4 out of 113) eastern and western Himalayan species show considerable intraspecific divergence which can be explained by the ecological distinctiveness of these two regions. Intraspecific variations in DNA barcodes of species are a key to their unexploited ecological modifications (Joly et al. 2014). The western Himalaya was formed more recently than the eastern Himalaya, and hence there are ecological anomalies in the biogeographical distribution of different species, especially Lepidoptera (Mani 1968) perhaps due to subsequent climatic fluctuations. The environmental factors like the mean elevation, humidity, forest cover, and precipitation are also higher in the eastern than in the western Himalaya (Price et al. 2011). Intraspecific genetic distances might increase with geographic distance but not significantly enough to challenge the efficacy of DNA barcoding (Huemer et al. 2014) and species with discontinuous distribution

not necessarily show effects on genetic divergence correlated with geography (Mutanen et al. 2012). Habitat-induced low dispersal due to discontinuity of physical, geological, ecological and microclimatic conditions might have led to their geographical isolation. Similarly, adaptation to ecological niches is known to cause genetic divergence (Papadopoulou 2008). Study on birds (Price et al. 2003) have shown that species composition varies in different parts of the Himalaya mountains due to local extinctions of populations and that species numbers in the Himalaya decline with the latitudinal gradient. While studies on Himalayan butterflies have shown a significant rarefaction of humid-tropical-forest species westwards of the Sikkim state contrary to the significant increase of the temperate-forest species towards the west (reviewed in Mani 1968). The abundance and number of individuals per species was found to decrease by about five times from the east to the west between western Nepal and Pakistan, where the mountains take a northwards track (Mani 1968, Price et al. 2003, Ghosh-Harihar 2013). Detailed examination of host plant relationships and extending the sampling region to cover more of the distribution range of the investigated species is required to further tease apart the effect of elevation and habitat types from species ecology on cryptic diversity.

A detailed study of past geological and climatic events coupled with phylogeny can probably explain many of these cases of BIN discordance. The prevailing ecological conditions in the Himalaya mountain system favour a high rate of speciation with higher numbers of endemics than any other mountain chain

(e. g. the Alps, where there are more relict species). Apparently, this can be attributed to the more intensive Pleistocene glaciations in Europe than in Asia (Mani 1968). Past evolutionary patterns were directed towards ecological specializations while at present the high altitude fauna is evolving towards increasing species 'enrichment' (Mani 1968). As for the ecology and habitat requirements of largely unexplored biota, like the Himalayan fauna, it is a difficult task to elucidate even a small fraction of the existing life histories and species interactions.

Need and benefits of a DNA barcode library for India

In tropical regions the taxonomy of hyper-diverse groups of arthropods is a neglected field and, often, morphospecies are surrogates for species (Basset et al. 2012, Ashton et al. 2015), with major constraints due to little availability of taxonomic expertise and resources (Zenker et al. 2016). Thus, an ambitious insect inventory project can quickly overwhelm taxonomists with too many species and specimens. Adding to this, the concentration of expertise to only a few well-known species, leads to 'taxonomic impediment' (de Carvalho et al. 2005). As a result, there is unavailability of fine scale data for conservation for many groups. Along with species diversity, functional diversity and phylogenetic diversity are new dimensions to biodiversity studies but they are not yet particularly well studied in tropical regions among species-rich insect groups (Brehm et al. 2013).

India has a lot of unexplored biodiverse areas since the British left the country. Fragmentary taxonomic records (often very old done by British taxonomists, Hampson 1892–1896, Cotes & Swinhoe 1886, Warren 1889 to name a few) exist for moths from different parts of the country. From Uttarakhand state, some prominent works include Smetacek (2008), Sanyal et al. (2011, 2013, 2017) and Sondhi & Sondhi (2016). Sanyal et al. (2011, 2013) and Dey et al. (2015, 2017) have looked into the diversity and distribution of moth assemblages but none of the studies so far have ventured into the molecular phylogenetics area of research.

The way ahead

Based on the results of the present study, it is clear that the taxonomy of geometrid moth species from the study area is currently obscured in some degree of uncertainty. We need to further investigate correlations between the genetic divergence and morphological characteristics and to try to uncover overlooked species (Janzen et al. 2009) which have not been included in previous taxonomic work.

Species at lower elevations show a wider range of distribution than those at the higher elevations, which also may be reflected and thus measurable by genetic variation. For addressing questions like "how many species are there", geographically large scale reference libraries are required. A logical, great step forward will be to use DNA barcodes to understand large scale ecological patterns in intraspecific variation, and to explore the causes and the outcomes of such variations (Joly et al. 2014). As Janzen et al. (2009) predicted, DNA barcoding will bridge the gap between what is already known and what can be found out.

DNA barcoding has furthermore been shown as a valuable tool for unveiling host-plant associations (Smith et al. 2006, Miller et al. 2007, Matheson et al. 2008, Jurado-Riviera et al. 2009), when collected larvae were fogged or cannot be reared to adult because of food-plant supply constraints or failed rearing (Hausmann & Scalercio 2016). This paper thus opens up possibilities to detect insect-plant relationships using barcodes to connect life histories with species in large scale, which was earlier not possible.

Lastly, very often the conservation efforts are directed towards the Himalayas as a unit, but because of the inherent diverse nature of this mountain system, area-specific conservation should be targeted (Price et al. 2003). A characterisation of such poorly studied groups of insects (e. g. moths) at a molecular level, will allow a large step forward in resolving moth taxonomy using a valuable, integrative approach.

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Appendix

A Neighbour-joining tree based on the Kimura-2-parameter model of all analysed species including the sequences from the materials of the Herbulot collection is provided in the supplementary data (Electronic Supplement S1), details of the specimens from the current study with DNA barcode specimen ID numbers and elevation details are provided as supplementary data (Electronic Supplement S2). Intraspecific variation and distances to nearest neighbours obtained from Barcode Gap Analysis in BOLD of all the 113 species analysed are provided in the supplementary data (Electronic Supplement S3).