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Molecular identification of nymphalidae Butterflies in Mukurthi National Park, India

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ABSTRACT

Six species of Nymphalidae butterflies in habiting in Mukurthi National Park (The Nilgiri Biosphere Reserve, Western Ghats, India) were identified through DNA barcoding based on 650bp sequences of the mitochondrial COI gene. The sequences of these native species were compared with data bases available for these six species in India and other countries to improve the understanding of inter and intra species divergence. Neighbour-Joining cluster analysis with one out group, Pachilopta hector, and three different haplotypes revealed pair-wise distance comparison with the nearest neighboring species in BOLD database showed overall nucleotide divergent between 0.01-0.19%. The mean inter and intra specific nucleotide divergence of these six species was calculated to be 0.00827. As per the 10X rule, the threshold value was 0.08%. Therefore, no deep intra species nucleotide divergence was observed and no significant barcoding gap existed between the selected species. In this study, the native species in each haplotype was found to be very close relative to non-native species. The overlapping of the DNA sequences occurs due to less variation.

Keywords: DNA barcoding; Nymphalidae; divergence; overlapping.

INTRODUCTION

Utility of DNA barcoding has a wide dimension of biodiversity and application in various fields including biodiversity, conservation biology, ecology, population studies, phylogeographic, etc. It is more a novel and a rapid method for species description and identification [1] put forward based on the DNA sequences and it undoubtedly serves as a complementary approach for the existing traditional taxonomical practices [2]. Although the conventional morphology-based method of species identification is available, it is on a slow track due to many reasons such as inaccessibility of important type material for comparison, unavailability of old literature, decreasing number of specialists and very importantly, negligible number of students entering in this field.

Along with this, phenotypic variability and convergent evolution cause misidentification and may also lead to overlooking of cryptic species. In addition, most of the times, keys fail to link different life history stages of an organism to its adults; and sexual dimorphism adds more confusion to species identification [3]. The butterfly species have often been considered good surrogates for total biodiversity assessments since they have a substantial role in the stability of food webs. Their subspecies have traditionally been used to recognize ‘moderate’ morphological differentiation correlated with disjunct geographical distributions [4, 5].

However, non-discrete morphological variation and the application to contiguously distributed populations, often make subspecies boundaries ambiguous. India is one of the 12 mega biodiversity countries in the world and one of the richest biodiversity regions with high levels of endemism among the various ranges of taxa. Establishment and development of the DNA barcode reference libraries would be ultimately needed to explore the hidden diversity of the country. Inter-specific variations and intra-specific divergences in the taxa can be well studied and the creation of the databases like BOLD to store the nucleotide information would benefit future researches in delimiting and identifying the cryptic species.

India forms a large part of the Indo-Malayan bio-geographical zone with an extremely diverse terrain, climate and vegetation, which comprises extremes of heat and cold, of desert and jungle, of low lying plains and the highest mountains, of dryness and dampness, islands and continental areas, many of the floral and faunal forms show Malayan affinities with some taxa being unique to the Indian region. In addition, India hosts three of the world's biodiversity hotspots: the Western Ghats, the Eastern Himalayas, and the hilly ranges bordering India and Myanmar, each having numerous endemic species, including many butterfly species (84 species of *Papilionidae* (swallow tail butterflies), 81 species of *Pieridae* (yellow and white butterflies), 439 species of *Nymphalidae* (brush-footed butterflies) and 225 species of Hesperiidae (skipper butterflies).

The Western Ghats region extends along with Tamil Nadu, wherein about 334 species of butterflies of the major five families have been reported. There are 19 species of *Papilionidae*, 34 species of *Pieridae*, 97 species of *Nymphalidae*, 101 species of *Lycaenidae* and 83 species of *Hesperiidae* [6]. Report 145 butterfly species, of which 62 were new species in and around Nagpur, central India. The study area, Mukurthi National Park is a 78.46 km² protected area located in the western corner of the Nilgiri Plateau (11°10' to 11°22' N and 76°26' to 76°34' E), west of Ootacamund hill station in the northwest corner of Tamil Nadu state in the Western Ghats mountain range of South India[7].

The park is characterized by Montane grasslands and shrub lands interspersed with sholas in a high altitude area of high rainfall, near-freezing temperatures and high winds [8]. The Western Ghats, Nilgiri Sub-Cluster (6,000+ km²), including all of Mukurthi National Park, is under consideration by the UNESCO World Heritage Committee for selection as a World Heritage Site [17]. The genetic diversity is differently patterned within and among region/ biomes, and is related to their histories of climatic changes that have major implications for conservation science. The demographic instability and adaptive challenges produced by different geographical ranges would produce both stochastic and selective effects on the genetic variation and architecture of the living organisms and these consequences can be studied by genetic and phylogeographic approaches. Thus, it can ultimately provide integrated information on how the global biodiversity of these organisms are structured.

The brush-footed butterflies (Nymphaliadae) are one of the groups of lepidopteran insects that have undergone radiation with their closely related species spread across different geographical extents. This group has been the focus of several studied because of their charismatic bright coloration, specific association with host plants (milkweed) during their larval and adult periods and their remarkable mimicry. Therefore, the present study was aimed to explore the variation among the six species of Nymphaliadae butterflies, such as *Danaus chrysippus, Danaus genutia, Acrea violae, Junonia lemonias, Hypolimnas misippus*, and *Tirumala limniace* inhabiting the Mukurthi National Park, India, by using DNA Barcoding based on COI gene and data available for these six species in India and other countries to gain and improve the understanding of inter and intra species variation.

MATERIALS AND METHODS

Taxon sampling and data collection

Based on the available data in the databases, six species of Nymphalidae butterflies were collected. They were *Danaus chrysippus, Danaus genutia, Acrea violae, Junonia lemonias, Hypolimnas misippus*, and *Tirumala limniace* (Fig. 1). The Indian species of these butterflies were collected from Mukurthi National Park (spread over 78.46 km), The Nilgiris, Western Ghats (11.00 N 77.00 E) during the period of one year from January 2011 to December 2011. Samples were collected using sweeping net, just pulled out one or two legs (based on the size of the sample), transferred to the laboratory and stored in 95% ethanol. They were immediately assigned a specimen number and stored at -20^{0}C until extraction of their DNA. The species were identified based on wing shape and colour pattern described in available keys[9].

Genomic DNA extraction and amplification

Genomic DNA was extracted from butterfly tissues of thorax region using manufacturer's protocol of DNeasy kit (Qiagen, INDIA). The presence of DNA was confirmed on 1% agarose gel. PCR amplification was done by using primers of COI gene LEPF1 (5'-ATTCAACCAATCATAAAGATAT-3') and LEPR1 (5'-TAAACTTCTGGATGTCCAAAAA-3'). PCR reaction was carried out in total volume of 50μl containing 4μl DNA template, 20 p mol of each primer and 400 μM of dNTPs, 3.0 mMol/l of $Mgcl_2$, 2.50 units of Taq polymerase and 5 μl of 10X PCR buffer. [10]Thermo-cycling condition was adapted for amplification. (One initial cycle of 1 min at 940C followed by five cycles of 940C for 1 min, 450C for 1 min 30 s, 720C for1 min 15 s, then 30 cycles of 940C for 1 min, 510C for1 min 30 s, 720C for 1 min 15 s, with final step of 720Cfor 5 min). The obtained PCR products were checked on 2% agarose gel.

Sequencing and Data analysis

The amplified DNA product was purified using the PEG-Nacl method [11]. The purified products were sequenced with the forward and reverse primers using an automated sequencer (ABI 3500xL Genetic Analyzer) from Chromos Biotech Pvt. Ltd, Bangalore, India). Sequences were edited to remove reading frame shift, and forward and reverse sequences were assembled using ORF-NCBI. FASTA format of these sequences were used for species identification by adapting BLAST search at NCBI database and the "Species identification tool" at BOLD system (http://www.boldsystems.org/). Sequences were deposited in GenBank with the following accession numbers: JX261941, JX261943, JX261944, JX226065 - JX226068. The Kimura 2-parameter (K2P) model of base substitution was used to calculate pair-wise genetic distance in MEGA 5.2 software and Neighbour-Joining (NJ) clustering analysis was done with one out group, *Pachilopta hector* (Linnaeus). The intra and inter species nucleotide divergence and haplotypes variation was calculated using DnaSP v5 [16]. One-way ANOVA was used to check the statistical significance ($P < 0.05$) by adopting SPSS 11.5 of IBM.

Phylogenetic analyses

In the present study, individual and combined analyses of COI gene sequences were done from 17 samples, including 6 representative species. Species discrimination in DNA barcoding depends on establishment of threshold interspecies nucleotide divergence. The evolutionary analysis was done by MEGA 5.2. For each representative species at least three different haplotypes were used (Figs. 1 and 2). The evolutionary tree was constricted by using the NJ method (1+2; one from the representative species and two from other countries). The optimal tree with the sum of branch length was 0.441. The percentage of replicated trees in which, the associated taxa clustered together was calculated by bootstrap test (1000 replicates). The phylogenetic tree was drawn to scale with branch length in the same units as those of the evolutionary distances. The evolutionary distance was calculated using the Jukes Cantor method The rate of variation among sites was modeled with a gamma distribution (shape parameter = 10). The codon positions read was in the order of $1^{st}+2^{nd}+3^{rd}$+noncoding. All positions containing gaps and missing data were eliminated. In each data set, uniformly 203 positions were read.

RESULTS AND DISCUSSION

The DNA sequences generated from six butterflies species (*D. chrysippus, D. genutia, A. violae, J. lemonias, H. misippus* and *T. limniace*) was approximately 600 bp each, indicated good quality and successful amplification. The identification of sequences by GenBank and BOLD v3 showed 99-100% similarity (*A. violae* - 99%, *T. limniace* - 100%, *H. misippus* - 100%, *J. lemonias* - 99%, *D. genutia* - 99% and *D. chrysippus* - 99%). The E-value was '0' for all the species. Pair-wise distance comparison with the nearest neighbouring species in BOLD database and their overall variance showed between 0.01- 0.19%. Individual species distance with BOLD was in the order of *D. genutia* > *H. misippus* > *A. violae* > *T. limniace* > *D. chrysippus* > *J. lemonias*. The GenBank accession numbers and BOLD- BIN numbers are given in table 1.

The DNA sequences for the selected six butterfly species retrieved from GenBank were actually the representative species of Pakistan, China, Malaysia Finland, Sweden and Australia (Table 2). The intra species nucleotide divergence for each species was calculated from three representative countries, except *A. violae* (for which, only two representatives were available from different geographical locations Malaysia and India (Table 2). Minimum intra species nucleotide divergence of 0.02% was arrived with *D. genutia*, and the maximum intra species nucleotide divergence of 0.15% was found with *J. lemonias.* The mean inter and intra species nucleotide divergence of these six species was calculated as 0.00827. According to [1], we selected an arbitrary threshold of 10X rule and a present

result of the threshold value was 0.08%. Therefore, no deep intra species nucleotide divergence was observed and no significant barcoding gap existed between the selected species. This indicates the fact that the native species in each haplotype was very close relative to non-native species. NJ-clustering analysis to infer the evolutionary history of the species showed several overlapping sequences. For instance, in *T. limniace* and *D. Chrysippus* overlapping was found between the individuals from India and Pakistan with an overall species divergence of 0.03% and 0.14% respectively. Similarly, in the case of *H. misippus* the overall divergence was 0.06% with overlapping found between the species belonging to Sweden and China.

Whereas, in *D. genutia*, the intra species divergence was 0.02% and overlap of the sequence information was observed between the species of India and Australia. Therefore, overlapping of the DNA sequences occur due to less variation within the same species taken from different geographical location. The intra specific nucleotide divergence was maximum in *J. lemonias* (0.15%), and indicates there was no overlapping in the DNA sequence of species taken from three different countries (India, China and Sweden) which in turn indicates there was a high degree of variation. A complementary analysis with the multiple sequence alignment of the selected sequences highlights both similarities and variations (Fig. 3). When the distance in each haplotypes of species calculated by BLASTn was subjected to arrive to a mean value, the similarity between species was on the order of *A. violae* > *T. limniace* > *J. lemonias* > *D. genutia* > *D. chrysipppus* > *H. misippus* (Tables 3 - 6).

Isolation of the genomic DNA, PCR amplification with the appropriate primers, followed by the sequencing yielded good quality of sequences > 650 bp. Generally, the tissue samples taken from the thorax and legs provided better results [12] than the sample taken from the abdomen because of the less proteinaceous substances.

The similarity search tool (BLAST) in NCBI database accurately identified the correct species. In the present study, conventional method of identification of species based on the phenotypic characters, such as body shape, coloration, genitalia and wing pattern agreed with the molecular identification using the barcode region. The selected six species of butterflies are morphologically distinctive, but their genetic variation is similar. Therefore, morphological dissimilarity is influenced by environmental factors. Based on the availability of standard CO1 sequences for the selected species in the GenBank and BOLD databases, the geographical locations were chosen. Although, one distinctive species (*A. violae*) was picked from the Indian sub-region, all the other species had their source from very distant geographical locations, such as Australia, Sweden, Pakistan, Malaysia, Finland, and China. Actually, the DNA sequences matched appropriately to the same species but with small degrees of variations, 0-1% with GenBank data. Pair-wise distance variation to the nearest neighbour (NN) in the BIN cluster also indicated low variation, while two species, *H. misippus* and *D. genutia* have variance > 0.1%, and residual species has p-distance < 0.1%.

Comparison of sequence analysis showed apparently higher identity with Pakistan in the case of *T. limniace* with 99.68%, and lower identity match with China in the case of *H. misippus* with 91.21% (Table 3). In the majority of the cases, the sequences from the neighbouring countries like Pakistan and China formed closest clades with our sequences (*T. limniace* (HQ990432) = 99.68%; *D. genutia* (KC755857) = 99.22% and *D. chrysippus* (HQ990426) = 99.03% from Pakistan, and *J. lemonias* (EU368159) = 99.04% from China).

The causes for these variations are perhaps due to dispersal as suggested [13] and these events highly influence their widespread diversification [14]. NJ-clustering analysis served as a strong evidence for exploring and understanding the intra species nucleotide divergence among the species. The analysed 17 individuals formed monophyletic clades representing single species, each of them were similar to the traditional taxonomic identification. All the clades have the intra nucleotide divergence less than 1% and the maximum observed divergence was 0.15% (Fig. 2). Sequence overlapping was observed between *T. limniace* and *D. chrysippus* with the closest geographic region, Pakistan. This indicates that the low intra specific nucleotide divergence may be due to the hybridization of the species lineages within the nearest areas. [15] Suggested that although Pakistan has a diverse butterfly fauna, there was limited overlap of the sequence with that of Central Asia due to the geographical isolation by the long chain of Pamir Mountains, which has ultimately resulted in multiple endemism and segregation of species to a restricted area. On the contrary, the results of our study indicated maximum identity 99.67% and high overlap (0.022% in *D. chrysippus*) of the Indian species with Pakistan species. When the concept of 10X rule was applied to the current study, the threshold value of 3% suggested [1] for species distinction was not achieved. Actually, the obtained threshold value was 0.08%, which is comparatively very low and it cannot be used to delimit the species.

Table 1. Identification review of selected Nymphalidae species through BLASTn identity from GenBank and BOLD identification System (BOLD-IDS).

Species studied	GenBank (BLASTn)			BOLD identification	
	% identity	E-value	Accession no.	BOLD-BIN number	Distance variance of species with BOLD (p-dist)
D. chrysippus	99%	0	JX226068	ABX5122	0.02%
D. genutia	99%	0	JX261941	AAI9595	0.19%
A. violae	99%	0	JX226067	ABY2739	0.09%
J. lemonias	99%	0	JX226065	ABZ7487	0.01%
H. misippus	100%	0	JX226066	AAE5820	0.11%
T. limniace	100%	0	JX261943	AAB6318	0.04%

Table 2. List of voucher and reference sequences downloaded from the GenBank databases with the accession numbers

Country	Species Name	Gene	GenBank Accession No.	Reference
India	*T. limniace*	COI	JX261943	Paper author
	D. genutia	COI	JX261941	Paper author
	D. chrysippus	COI	JX226068	Paper author
	A. violae	COI	JX226067	Paper author
	J. lemonias	COI	JX226065	Paper author
	H. misippus	COI	JX226066	Paper author
	P. hector (the out group)	COI	JX261944	Paper author
Pakistan	*T. limniace*	COI	HQ990432	Ashfaq*et al.*, 2013
	D. genutia	COI	KC755857	Ashfaq*et al.*, 2013
	D. chrysippus	COI	HQ990426	Ashfaq*et al.*, 2013
China	*J. lemonias*	COI	EU368159	Zhang *et al.*, 2008
	H. misippus	COI	EU368161	Zhang *et al.*, 2008
Malaysia	*A.violae*	COI	KF226261	Wilson *et al.*, 2013
Finland	*T. limniace*	COI	GQ864815	Wahlberg *et al.*, 2009
Sweden	*J. lemonias*	COI	EU053310	Kodandaramaiah and Wahlberg, 2007
	H. misippus	COI	AY788635	Wahlberg *et al.*, 2004
Australia	*D. genutia*	COI	KF405259	Hebert *et al.*,
	D. chrysippus	COI	KF401092	Hebert *et al.*,

Table 3. Similarity comparisons between the haplotypes

Source Organism	Source Location	Comparative locations	Percentage identity (%)	Mean ± standard error	Grade
D. chrysippus	India	India	100.00	98.76 ± 1.394	V
		Pakistan	99.03		
		Australia	97.25		
D. genutia	India	India	100.00	99.06 ± 1.029	IV
		Pakistan	99.22		
		Australia	97.96		
A. violae	India	India	100.00	99.89 ± 0.190	I
		Indian Sub – region	99.67		
		Malaysia	100.00		
J. lemonias	India	India	100.00	99.356 ± 0.557	III
		China	99.04		
		Sweden	99.03		
H. misippus	India	India	100.00	94.356 ± 4.898	VI
		China	91.21		
		Sweden	91.86		
T. limniace	India	India	100.00	98.853 ± 1.716	II
		Pakistan	99.68		
		Finland	96.88		

The results of one way-ANOVA showed some degree of variation ($P < 0.05$) among species. In this study, there was intra species nucleotide divergence between the selected six native species (Fig. 2). However, these variations are not up to the mark of 10X. The results of the present study were comparable with the findings of [10], which emphasised on broader species sampling that possessed threshold divergence value > 3%, but in our study, since,

the species sampling was very small, we could achieved the divergence of < 3% only. Therefore, it is suggested that to attain adequate nucleotide dissimilarity, we need wider sampling with distinct geographical region. However, in this study, all the selected species were undoubtedly identified and discriminated by DNA Barcoding of CO1 gene. Therefore, it is verified that DNA barcoding is a useful tool to sort out even small differences in the DNA sequence of the species.

Table 4. One way ANOVA of species haplotypes

Groups	Sum of Squares	Degrees of freedom	Mean Square	F- value	Significance
Between Groups	60.756	5	12.151	2.407	0.099
Within Groups	60.578	12	5.048		
Total	121.334	17			

Table 5. Multiple comparisons (Bonferroni test) of species haplotypes

(I) FACT	(J) FACT	Mean Difference (I-J)	Std. Error	Significance	95% Confidence Interval	
					Lower Bound	Upper Bound
TL	DC	.0933	1.83451	1.000	-6.6006	6.7873
	DG	-.2067	1.83451	1.000	-6.9006	6.4873
	AV	-1.0367	1.83451	1.000	-7.7306	5.6573
	JL	-.5033	1.83451	1.000	-7.1973	6.1906
	HM	4.4967	1.83451	0.458	-2.1973	11.1906
DG	DC	.3000	1.83451	1.000	-6.3939	6.9939
	AV	-.8300	1.83451	1.000	-7.5239	5.8639
	JL	-.2967	1.83451	1.000	-6.9906	6.3973
	HM	4.7033	1.83451	0.372	-1.9906	11.3973
	TL	.2067	1.83451	1.000	-6.4873	6.9006
DC	DG	-.3000	1.83451	1.000	-6.9939	6.3939
	AV	-1.1300	1.83451	1.000	-7.8239	5.5639
	JL	-.5967	1.83451	1.000	-7.2906	6.0973
	HM	4.4033	1.83451	0.503	-2.2906	11.0973
	TL	-.0933	1.83451	1.000	-6.7873	6.6006
JL	DC	.5967	1.83451	1.000	-6.0973	7.2906
	DG	.2967	1.83451	1.000	-6.3973	6.9906
	AV	-.5333	1.83451	1.000	-7.2273	6.1606
	HM	5.0000	1.83451	0.276	-1.6939	11.6939
	TL	.5033	1.83451	1.000	-6.1906	7.1973
HM	DC	-4.4033	1.83451	0.503	-11.0973	2.2906
	DG	-4.7033	1.83451	0.372	-11.3973	1.9906
	AV	-5.5333	1.83451	0.161	-12.2273	1.1606
	JL	-5.0000	1.83451	0.276	-11.6939	1.6939
	TL	-4.4967	1.83451	0.458	-11.1906	2.1973
AV	DC	1.1300	1.83451	1.000	-5.5639	7.8239
	DG	.8300	1.83451	1.000	-5.8639	7.5239
	HM	5.5333	1.83451	0.161	-1.1606	12.2273
	JL	.5333	1.83451	1.000	-6.1606	7.2273
	TL	1.0367	1.83451	1.000	-5.6573	7.7306

JL, *Junonia lemonias*; DG, *Danaus genutia*; TL, *Tirumala limniace*; HM, *Hypolimnas misippus*; DC, *Danaus chrysippus*; AV, *Acraea violae.*

Table 6. Duncan test for species haplotypes

FACT	N	Subset for alpha = 0.05	
		1	2
H. misippus	3	94.3567	
D. chrysippus	3		98.7600
D. genutia	3		99.0600
A. violae	3		99.8900
J. lemonias	3		99.3567
T. limniace	3		98.8533
Significance		1.000	0.583

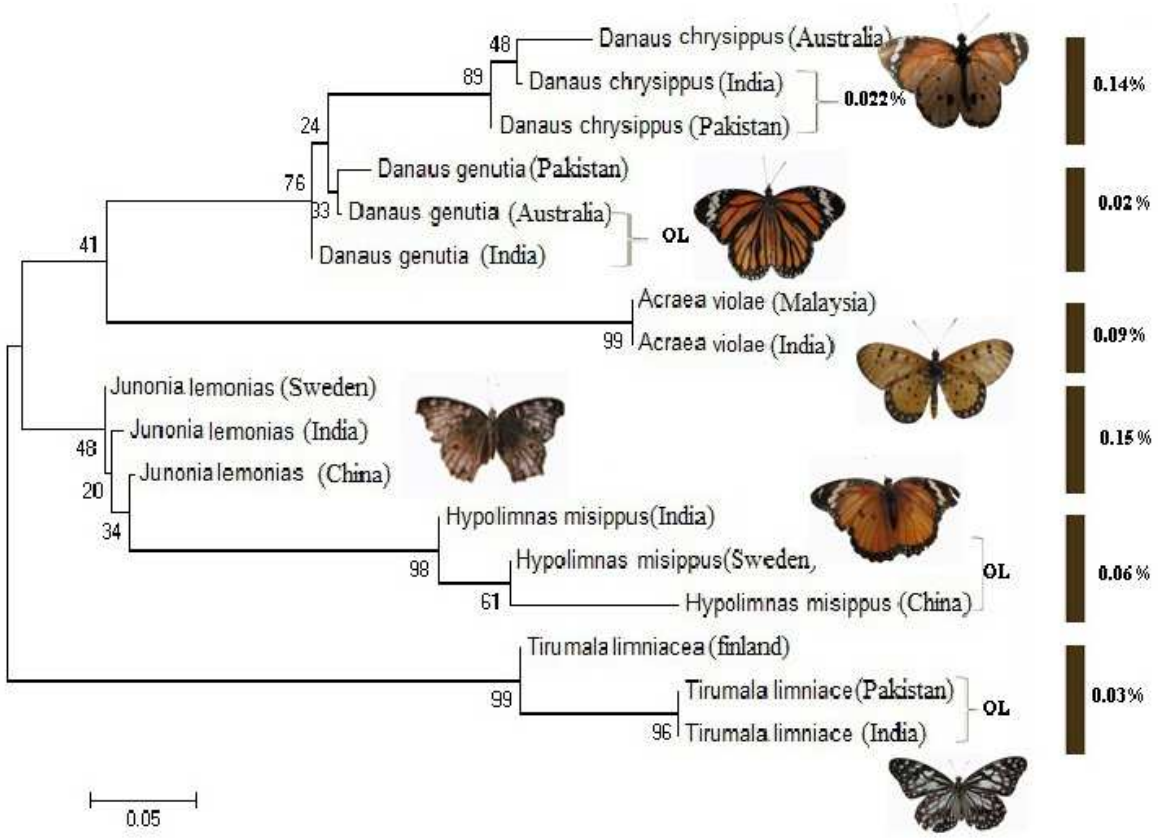
Means for groups in homogeneous subsets are displayed.
Uses Harmonic Mean Sample Size = 3.000.

Figure 1. Global Geographical position of brush footed butterfly species collected (Indian species) and retrieved (rest of the world) on the basis of available COI gene data in the databases.

Different coloured marking represent the presence of species in different locality.

Figure 2. Phylogeographic analysis of Nymphalidae butterflies using MEGA 5.2.

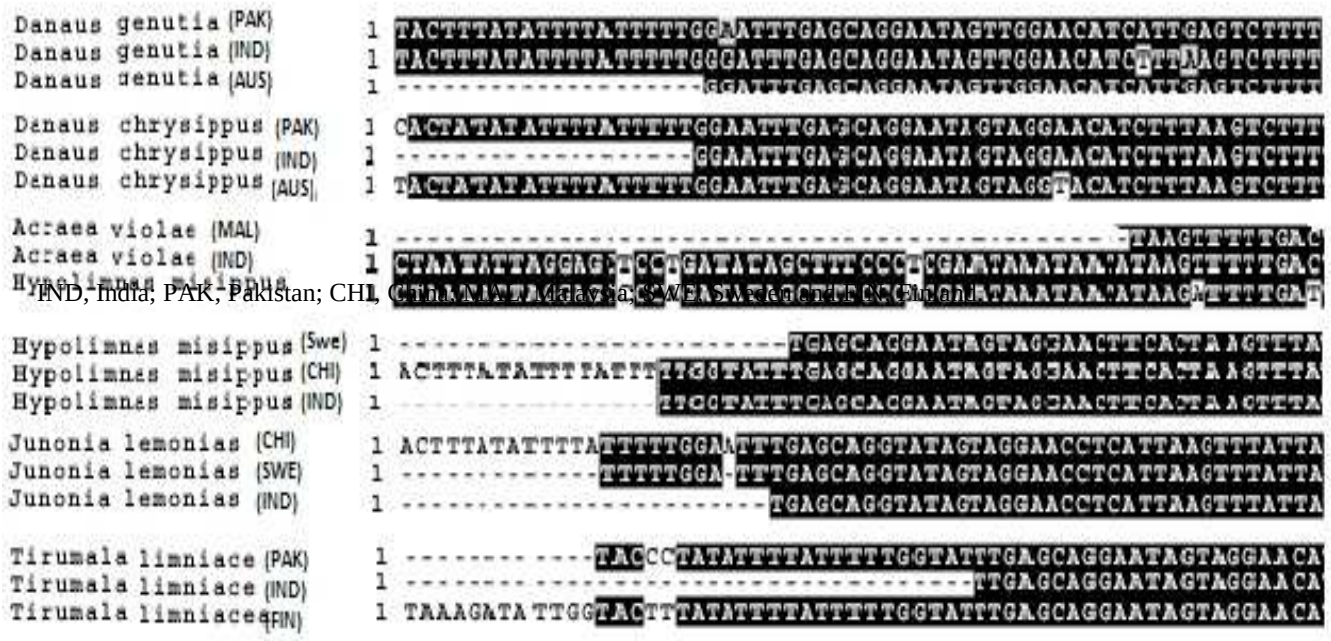
Phylogenetic consensus tree of six Nymphalidae butterflies (with different geographical region) are constructed by NJ method. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. The phylogenetic tree was drawn to scale, with branch length in the same units as those of the evolutionary distances.



OL- Represent overlapping of species.

Figure 3. Multiple sequence comparison of COI gene sequence between three haplotypes taken from different geographical region.

Multiple sequence alignment of consensus sequences for Indian species against the same haplotypes taken from different countries. A multiple sequence alignment was generated by ClustalW and graphically represented by BOXSHADE 3.21. Highly conserved regions which showed 50% identical region were boxed in solid black and light shading indicates conservative substitutions.



IND, India; PAK, Pakistan; CHI, China; MAL, Malaysia; SWE, Sweden; FIN, Finland.

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