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DNA barcoding of some Indian species of hawk moths based on COI gene (Lepidoptera: Sphingidae)

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Abstract
In the current study, we analysed a partial sequence of 580 bp (approx) of COI gene for seven species belonging to family sphingidae. The study was conducted in the northern India mainly in the districts of Himachal Pradesh, Punjab and Uttarakhand during the period of April, 2015 to December, 2016 the studied sequences have been added to the current database at GenBank NCBI. The database analysis showed mean K2P divergence of 0.59% at intraspecific level, 6.3% at interspecific and 12.2% at intergeneric level. A range of 0.0% to 2.7% was observed during the intraspecific study alongwith the range of 3.6% to 8.2% in the interspecific study, thereby indicating a hierarchal increase in K2P mean divergence across different taxonomic levels.

Keywords: DNA Barcode; mitochondrial gene; phylogeny; Sphingidae.

1. Introduction
Sphingid moths, also known as hawk moths, sphinx moths and hornworms are very important members of order Lepidoptera (Van Nieukerken) [17]. The family Sphingidae is represented by about 1450 species from all over the world with high diversity occurring in tropical regions (Van Nieukerken) [17]. Molecular tools have provided new opportunities to study questions in evolutionary biology (e.g. speciation processes) and in phylogenetic systematics. A mitochondrial fragment, COI, was recently elected as standardised tool for molecular taxonomy and identification (Ratnasingham and Hebert) [14]. Sphingids have become a model group to study taxonomic species boundaries with the onset of DNA barcoding (Janzen *et al.* [10]; Hajibabaei *et al.*) [5]. Hundsdoerfer *et al.* [8] studied the molecular phylogeny of the hawkmoth genus *Hyles.* They used DNA sequences comprising about 2300bp derived from the mitochondrial genes COI, COII and tRNA-leucine to elucidate the phylogeny of *Hyles*. Hundsdoerfer *et al.* [9] gave the whole range of the *Hyles euphorbiae* complex which revealed that it comprised of six distinct mitochondrial lineages in the Mediterranean region based on sequences of the mitochondrial genes encoding COI, tRNA leucine and COII.
In the present study, we analysed a partial 580bp COI sequence from seven Indian species of family sphingidae. COI sequences of these species have been added to the existing database. The dataset has been analysed at different hierarchal level for base composition and sequence divergence.

2. Materials and Methods
Adult specimens belonging to family Sphingidae were collected from different regions of northern India from April, 2015 to November, 2015 and April, 2016 to November, 2016. They were preserved in ethanol. Specimens were identified using relevant literature and expert guidance of Dr. A.P.S. Kaleka of Department of Zoology and Environmental Sciences, Punjabi University, Patiala. DNA was extracted from legs of the specimens following the method of Kambhampati and Rai [11] with minor modifications. A region of COI gene was amplified using primers HCO1490 and LCO2198 (Folmer *et al.*) [4]. Amplification of the target DNA was done by incubating the samples at three steps (denaturation, annealing and extension). Amplification conditions, 1 cycle, 95° C (5 min); 35 cycles, 95°C (1 min), 50° C (1 min), 72° C (90 s); 1cycle, 72° C (7 min). PCR products were visualized on 1% agarose gel with ethidium bromide staining under UV light. The amplified products were sequenced from Yaazh Genomics, Mumbai. Sequences deposited in GenBank by other workers of the congeneric specimens were taken for the alignment purpose.

a. Statstical analysis

All the sequences were aligned in clustal W, and divergence at population, species and genus levels was analysed by K2P model of base substitution. Phylogenetic analysis was carried out using, Maximum Likelihood (ML, Fig. 1) Minimum Evolution (ME, Fig. 2) and Neighbour Joining (NJ, Fig. 3) approaches in MEGA 6 software (Tamura *et al.*) [16].

3. Results

Fourteen COI sequences representing seven species under seven different genera belonging to 3 subfamilies namely Sphinginae, Smerinthinae and Macroglossinae of more than 600 bp along with 200 amino acids were obtained. Sequences were submitted to GenBank database (Table 1).

No stop codons or frame shifts were detected, indicating that sequences were not pseudogenes (NUMTs). Fourteen sequences of the same number of species under eight genera submitted by other workers were procured directly from GenBank (Table 2). The final aligned data belonged to 28 COI sequences of 580 bp representing 14 species and 8 genera. Sequence of *Actias maenas* (Doubleday, 1847) belonging to family Saturniidae has been taken as outgroup. The alignment showed 387 conserved sites, 193 variable sites and 158 parsimony informative sites. The average A+T content was 69.2% (Table 3).

The data were analysed for sequence divergence at different taxonomic levels. Intraspecific divergence ranged from 0.0% to 2.7% with an average of 0.59% (S.D. 0.82), interspecific divergence ranged from 3.6% to 8.2% with an average of 6.3% (S.D. 1.62) while intergeneric divergence ranged from 8.1% to 15.6% with an average of 12.2% (S.D. 1.82).

Table 1: Details of species analysed in the present study.

S. No	Taxa	Number of Sample	Collection Place	Collection Month/Year	Accession Number
1.	*Acosmeryx naga* (Moore, 1858)	1	Narkanda	May 2016	KY010197
		1	Dehradun	September 2016	KY073630
2.	*Anambulyx elwesi* (Druce, 1882)	1	Narkanda	July 2015	KU991798
		1	Barog	July 2016	KY0733631
3.	*Cephonodes hylas* (Linnaeus, 1771)	1	Mohali	May 2015	KU355866
		1	Patiala	June 2016	KX500028
4.	*Hippotion celerio* (Linnaeus, 1758)	1	Serighat	June 2016	KX853205
		1	Barog	July2016	KY010199
5.	*Polyptychus trilineatus* Moore, 1888	1	Narkanda	July 2015	KU991799
		1	Chail	July 2016	KY073632
6.	*Psilogramma menephoran* Cramer, 1780	1	Mashobra	July 2016 September 2016	KX906961
		1	Cheog		KY010198
7.	*Theretra alecto* (Linnaeus, 1758)	1	Haridwar	September 2015	KU355867
		1	Subathu	June 2016	KX500027

Table 2: List of taxa whose sequences were downloaded from NCBI for alignment.

S. No	Name	Accession Number	Country
1.	*Acosmeryx castanea*	JN677652	China
2.	*Acosmeryx naga*	JN677655	China
3.	*Actias maenas*	KJ624747	India
4.	*Anambulyx elwesi*	JN677745	Thailand
5.	*Cephonodes hylas*	KC182192	Pakistan
6.	*Cephonodes picus*	JN677808	Philippines
7.	*Hippotion boerhaviae*	KJ168544	France
8.	*Hippotion celerio*	JN678018	Malawi
9.	*Polyptychus trilineatus*	JN678419	Laos
10.	*Polyptychus rougeoti*	JN678416	Cameroon
11.	*Psilogramma mandarina*	GU704616	China
12.	*Psilogramma menephoran*	KJ168330	Malaysia
13.	*Theretra alecto*	JN678599	Philippines
14.	*Theretra japonica*	JN678612	China

Table 3: Percent base composition of the COI segment studied.

Base	A	T	G	C
Average percentage	31.4	37.8	14.7	16.1

Table 4: Pairwise K2P intraspecific divergence.

S.NO	Species (accession no.)	Species (accession no.)	Divergence (%)
1.	*Acosmeryx naga* (KY010197)	*Acosmeryx naga* (KY073630)	0.0
2.	*Acosmeryx naga* (KY010197)	*Acosmeryx naga* (JN677655)*	0.9

3.	*Acosmeryx naga* (KY073630)	*Acosmeryx naga* (JN677655)*	0.9
4.	*Anambulyx elwesi* (KU991798)	*Anambulyx elwesi* (KY0733631)	0.0
5.	*Anambulyx elwesi* (KU991798)	*Anambulyx elwesi* (JN677745)*	1.4
6.	*Anambulyx elwesi* (KY0733631)	*Anambulyx elwesi* (JN677745)*	1.4
7.	*Cephonodes hylas* (KU355866)	*Cephonodes hylas* (KX500028)	0.0
8.	*Cephonodes hylas* (KU355866)	*Cephonodes hylas* (KC182192)*	0.2
9.	*Cephonodes hylas* (KX500028)	*Cephonodes hylas* (KC182192)*	0.2
10.	*Hippotion celerio* (KX853205)	*Hippotion celerio* (KY010199)	0.0
11.	*Hippotion celerio* (KX853205)	*Hippotion celerio* (JN678018)*	0.0
12.	*Hippotion celerio* (KY010199)	*Hippotion celerio* (JN678018)*	0.0
13.	*Polyptychus trilineatus* (KU991799)	*Polyptychus trilineatus* (KY073632)	0.0
14.	*Polyptychus trilineatus* (KU991799)	*Polyptychus trilineatus* (JN678419)*	0.9
15.	*Polyptychus trilineatus* (KY073632)	*Polyptychus trilineatus* (JN678419)*	0.9
16.	*Psilogramma menephoran* (KX906961)	*Psilogramma menephoran* (KY010198)	0.0
17.	*Psilogramma menephoran* (KX906961)	*Psilogramma menephoran* (KJ168330)*	0.2
18.	*Psilogramma menephoran* (KY010198)	*Psilogramma menephoran* (KJ168330)*	0.2
19.	*Theretra alecto* (KU355867)	*Theretra alecto* (KX500027)	0.2
20.	*Theretra alecto* (KU355867)	*Theretra alecto* (JN678599)*	2.5
21.	*Theretra alecto* (KX500027)	*Theretra alecto* (JN678599)*	2.7

*shows the sequences accessed from GenBank

Table 5: Pairwise K2P interspecific divergence.

S.NO	Species (accession no.)	Species (accession no.)	Divergence (%)
1.	*Acosmeryx naga* (KY010197)	*Acosmeryx castanea* (JN677652)*	3.6
2.	*Acosmeryx naga* (KY073630)	*Acosmeryx castanea* (JN677652)*	3.6
3.	*Hippotion celerio* (KX853205)	*Hippotion boerhaviae* (KJ168544)*	5.9
4.	*Hippotion celerio* (KY010199)	*Hippotion boerhaviae* (KJ168544)*	5.9
5.	*Cephonodes hylas* (KU355866)	*Cephonodes picus* (JN677808)*	6.5
6.	*Cephonodes hylas* (KX500028)	*Cephonodes picus* (JN677808)*	6.5
7.	*Polyptychus trilineatus* (KU991799)	*Polyptychus rougeoti* (JN678416)*	8.2
8.	*Polyptychus trilineatus* (KY073632)	*Polyptychus rougeoti* (JN678416)*	8.2
9.	*Psilogramma menephoran* (KX906961)	*Psilogramma mandarina* (GU704616)*	5.6
10.	*Psilogramma menephoran* (KY010198)	*Psilogramma mandarina* (GU704616)*	5.6
11.	*Theretra alecto* (KU355867)	*Theretra japonica* (JN678612)*	7.9
12.	*Theretra alecto* (KX500027)	*Theretra japonica* (JN678612)*	8.1

*shows the sequences accessed from GenBank

Table 6: Pairwise K2P intergeneric divergence.

S.NO	Species (accession no.)	Species (accession no.)	Divergence (%)
1.	*Acosmeryx naga* (KY010197)	*Anambulyx elwesi* (KU991798)	12.9
2.	*Acosmeryx naga* (KY010197)	*Cephonodes hylas* (KU355866)	12.4
3.	*Acosmeryx naga* (KY010197)	*Hippotion celerio* (KX853205)	8.1
4.	*Acosmeryx naga* (KY010197)	*Polyptychus trilineatus* (KU991799)	9.8
5.	*Acosmeryx naga* (KY010197)	*Psilogramma menephoran* (KX906961)	13.3
6.	*Acosmeryx naga* (KY010197)	*Theretra alecto* (KU355867)	10.2
7.	*Anambulyx elwesi* (KU991798)	*Cephonodes hylas* (KU355866)	15
8.	*Anambulyx elwesi* (KU991798)	*Hippotion celerio* (KX853205)	12.3
9.	*Anambulyx elwesi* (KU991798)	*Polyptychus trilineatus* (KU991799)	13.1
10.	*Anambulyx elwesi* (KU991798)	*Psilogramma menephoran* (KX906961)	13.8
11.	*Anambulyx elwesi* (KU991798)	*Theretra alecto* (KU355867)	15.6
12.	*Cephonodes hylas* (KU355866)	*Hippotion celerio* (KX853205)	11.2
13.	*Cephonodes hylas* (KU355866)	*Polyptychus trilineatus* (KU991799)	12
14.	*Cephonodes hylas* (KU355866)	*Psilogramma menephoran* (KX906961)	12.4
15.	*Cephonodes hylas* (KU355866)	*Theretra alecto* (KU355867)	13.3
16.	*Hippotion celerio* (KX853205)	*Polyptychus trilineatus* (KU991799)	11.6
17.	*Hippotion celerio* (KX853205)	*Psilogramma menephoran* (KX906961)	12.4
18.	*Hippotion celerio* (KX853205)	*Theretra alecto* (KU355867)	9.8
19.	*Polyptychus trilineatus* (KU991799)	*Psilogramma menephoran* (KX906961)	11
20.	*Polyptychus trilineatus* (KU991799)	*Theretra alecto* (KU355867)	11.2
21.	*Psilogramma menephoran* (KX906961)	*Theretra alecto* (KU355867)	14.3

Table 7: Comparative data of K2P divergence

	Present study		Rougerie *et al.* (2014) [15]		Hausmann *et al.* (2011) [6]	
K2P divergence	**Range (%)**	**Mean distance (%)**	**Range (%)**	**Mean distance (%)**	**Range (%)**	**Mean distance (%)**
Intraspecific	0.0% to2.7%	0.59±0.82	0.0%to2.19%	0.3	N	0.73
Interspecific	3.6% to8.2%	6.3±1.62	1.1%to7.2	N	N	10
Intergeneric	8.1% to15.6%	12.2±1.82	N	7.13	N	13.3

N: Not given by author

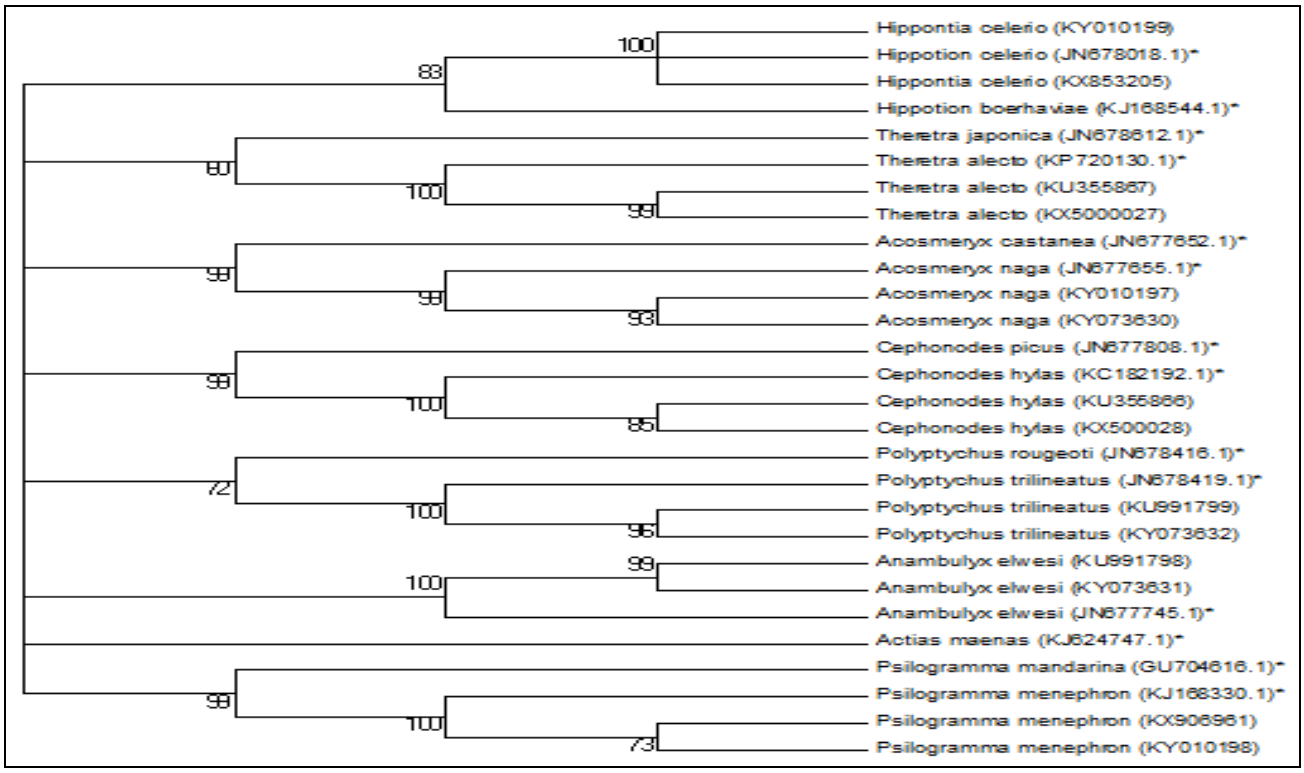

Fig 1: Maximum likelihood tree based on (K2P). Numbers indicate the percentage of 1000 bootstrap replicate.

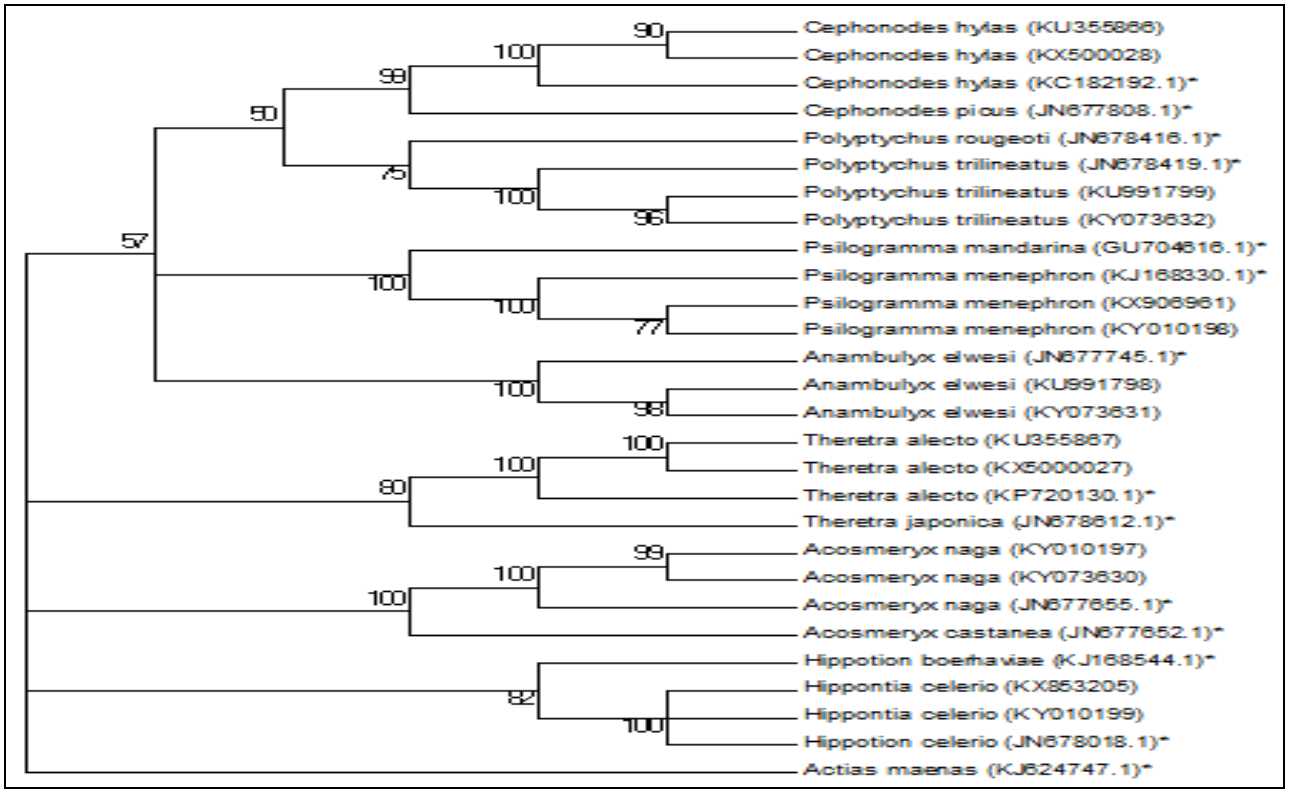

Fig 2: Minimum Evolution tree based on (K2P). Numbers indicate the percentage of 1000 bootstrap replicate.

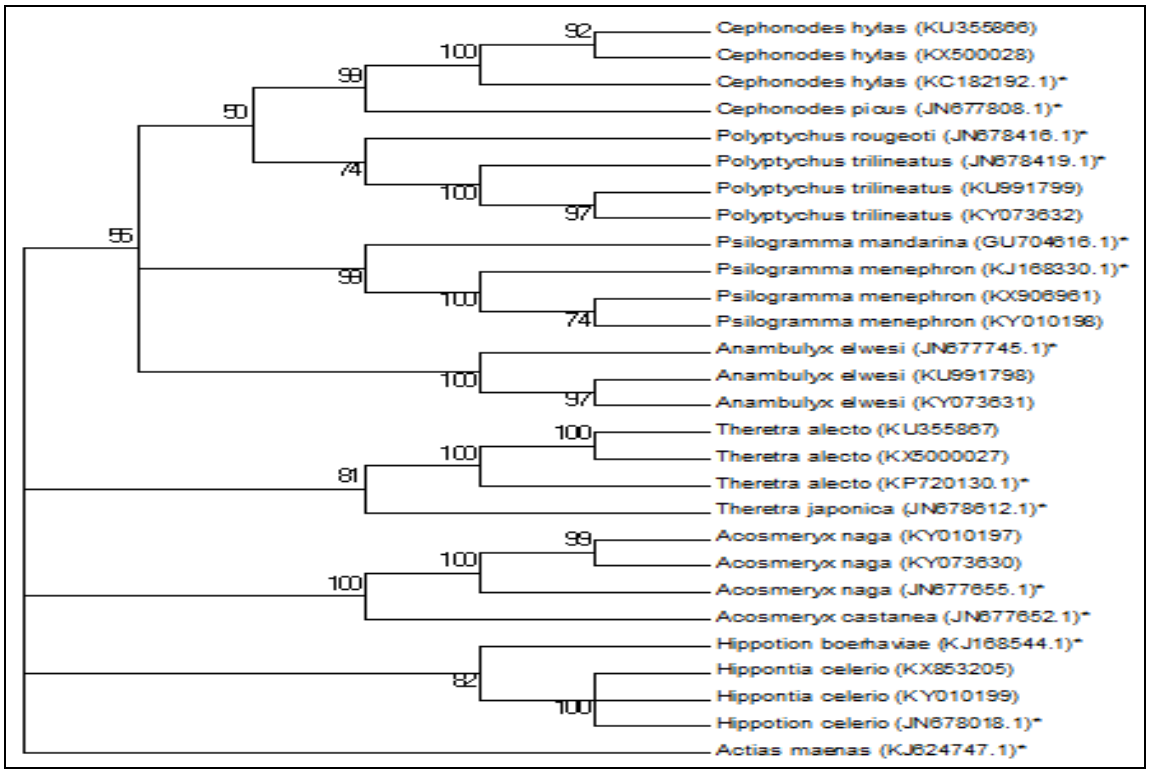

Fig 3: Neighbour-Joining tree based on (K2P). Numbers indicate the percentage of 1000 bootstrap replicate.

4. Discussion

In the present study all the members showed distinct barcodes with no case of barcode sharing. COI marker proved to be useful in the diagnosis of the family Sphingidae as during the BLAST search sequences match with sequences of congeneric species from GenBank. For the ones with no sequence match in the database, the nearest match was always from the family Sphingidae. Clary and Wolstenholme[3] gave an average composition of 73.7% showing the typical A+T bias of insect mitochondrial DNA and cytochrome oxidase genes in particular. Nucleotide composition showed a bias towards A+T content of 77% (Brown *et al.*) [1]. Similarly Cameron and Whiting[2] showed the nucleotide composition bias of 81.79%.

Average K2P intraspecific divergence was found to be 0.59%±0.82 with minimum of 0.0% in *Hippotion celerio* (Linnaeus, 1758) to a maximum of 2.7% in *Theretra alecto* (Linnaeus, 1758) (Table 4). The K2P value was 0.9% *Acosmeryx naga* (Moore, 1858), 1.4% for *Anambulyx elwesi* (Druce, 1882), 0.2% for *Cephonodes hylas* (Linnaeus, 1771), 0.9% for *Polyptychus trilineatus* Moore, 1888 and 0.2% for *Psilogramma menephoran* Cramer, 1780. (Table 4).

Hausmann *et al.* [6] found out it with the mean of 0.73% (SE=0.033) in family Geometridae while Rougerie *et al.* [15] gave the intraspecific distance in Australian Sphingids ranging from 0.0% to 2.19% (mean=0.3%, SE=0.007) (Table 7). Kekkonen *et al.* [13] reported that the intraspecific distances in the Gelechiinae varied from 0.00% to 2.94% (mean = 0.39%, SE=0.01). A similar pattern was observed in the Elachistinae with intraspecific distances ranging from 0.00% to 2.3% (mean=0.28%, SE=0.01).

Two specimens of *Theretra alecto* from the present study showed 0.2% of sequence divergence, while these specimens showed higher divergence with the sequences reported from other countries with K2P value of 2.5% and 2.7%.

Average interspecific divergence was found to be 6.3%±1.62 with minimum of 3.6% for *Acosmeryx naga* and *Acosmeryx castanea* pair and maximum of 8.2% for *Polyptychus trilineatus* and *Polyptychus rougeoti* pair (Table 5). The minimum interspecific divergence of COI for species diagnosis in insects has been suggested to be 3% by Hebert *et al.* [7] Hajibabaei *et al.* [5] gave the average interspecific distance of order lepidoptera (16.01). A mean of 10% with SE of 0.014 was given by Hausmann *et al.* [6] However a range of interspecific distance from 1.1% to 7.2% was observed by Rougerie *et al.* [15].

All of the studied species showed the interspecific divergence that is quite higher than the suggested threshold value signifying them to be well separated species.

In the present study, two specimens of *Theretra alecto* showed the interspecific distance of 7.9% and 8.1% with *Theretra japonica*. The intraspecific distance also showed a variation of 0.2%, 2.5% and 2.7% between the two specimens studied alongwith the one taken from the other part of the world respectively. This indeed makes them a vital issue to be studied and analysed with different molecular markers including COI as well.

We have endeavoured to generate phylogenetic trees for the present work. In all the trees namely Maximum likelihood, Minimum evolution and Neighbour joining, different genera clustered separately and congeners clustered together. In maximum likelihood tree *Actias maenas*, a member of family Saturniidae showed a close relation to all the members of the group studied than to the *Psilogramma* group showing a close relation to its sister family.

Kim *et al.* [12] analyzed nucleotide sequences of 13 protein coding gene performed on 12 species in three families of Superfamily Bombycoidea, including *Notonagemia analis scribae* of family Sphingidae. *Notonagemia analis scribae* grouped together with two within familial species, *Sphinx morio* and *Manduca sexta*, with the highest nodal support (BI, 1.0; ML, 100%), forming the Sphingidae monophyletic group. It was observed that both the trees (minimum evolution and neighbor joining) that are distance based methods of phylogenetic analysis showed similar pattern. *Hippotion celerio* showed a similar pattern in both the trees forming a

single clade even with the one reported from different region showing 0.0% divergence. All the other species showed a similar pattern forming single cluster and giving a divergent distance ranging from 3.6% to 8.2% the interspecies studied. Bootstrap values were obtained with 1000 replicates. Majority of branches showed bootstrap values higher than 70%.

However, it would be inapt to analyse the species on the basis of the available molecular data. In order to discuss the taxonomic status of the species a further study on more number of species should be considered.

5. Conclusion

The present study bequeaths 14 COI sequences of 7 species belonging to 7 genera of family Sphingidae. This is the first ever molecular study for seven species which has generated distinct barcodes for each and thus can be used for their identification. The database analysis shows hierarchical increase in K2P mean divergence across different taxonomic levels.

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