



# COI Gene: A Reliable Tool for Tracing the Phylogeny in Sphingid Moths (Lepidoptera: Sphingidae)

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## ABSTRACT

In the current study, a partial sequence of 536 bp (approx) of COI gene for seven species belonging to family Sphingidae were analysed. The study was conducted on a collection of moths from northern India, mainly in the states of Himachal Pradesh, Uttarakhand and Punjab. The sequences have been added to the database at GenBank NCBI. The analysis showed mean K2P divergence of 0.59% at intraspecific level, 6.3% at interspecific and 12.2% at intergeneric level. The analysis showed a hierarchical increase in K2P mean divergence across different taxonomic levels giving an intraspecific range of 0.0% to 3.6%, interspecific range of 5.8% to 9.3% and intergeneric range of 8.6% to 13.8%.

**Key words:** DNA Barcode, Mitochondrial gene, Phylogeny, Sphingidae.

## INTRODUCTION

Extraordinary numerical, morphological and behavioural diversity within insects has made them potent model systems for examining the connection between ecological phenomena and evolutionary history (Dobler and Farrell, 1999; Farrell *et al.*, 2001; Sequeira *et al.*, 2000; Shaw, 1996a, 1996b). The second largest and more diverse order of class Insecta is Lepidoptera (Benton, 1995). Moths and butterflies (Lepidoptera group) were among the last to arrive on the evolutionary scene around 160 million years ago, following the evolution of flowering plants. There are about 25000 known species of butterflies and over 1, 20,000 moths (Kehimkar, 1997). The superfamily Bombycoidea is represented by six families namely Saturniidae, Brahmaeidae, Endromidae, Bombycidae, Sphingidae and Lasiocampidae. Sphingidae is an important family of moderately sized to very large moths, which are distributed almost over the whole world. The family is represented by 1450 species globally (Van Nieukerken *et al.*, 2011). The involvement of this family as a study group for evolutionary biology has provided new opportunities to explore the phylogenetic systematics. A partial segment of COI gene known as the barcoding region proved to be a standardised tool for tracing the phylogeny of a group. The DNA sequences comprising about 2300bp derived from the mitochondrial genes COI, COII and tRNA-leucine to elucidate the phylogeny of *Hyles* was done by Hundsdoerfer *et al.* (2005). They also 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 536 bp 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 hierarchical levels for base composition and sequence divergence.

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## MATERIALS AND METHODS

The specimens were collected from different regions of northern India from June, 2014 to September, 2014; May, 2015 to November, 2015 and June, 2016 to September, 2016. The samples 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. The extraction of DNA was done following Kambhampati and Rai, (1991) with minor modifications. Legs of the freshly killed specimens were used for the extraction purpose. HCO1490 and LCO2198 primers, (Folmer *et al.*, 1994) were used to amplify a region of COI gene. Three way steps were followed to carry out the amplification of the target DNA i.e. denaturation, annealing and extension. The amplification conditions followed were, 1 cycle, 95°C (5 min); 35 cycles, 95°C (1 min), 50°C (1 min), 72°C (90 s); 1 cycle, 72°C (7 min). Ethidium bromide stained 1% agarose gel was used to visualize PCR products under UV light. The amplified products were sequenced from Yaazh Genomics, Mumbai. For aligning the sample sequences, the sequences of congeneric species were procured from GenBank submitted by other workers. ClustalW method was used to align all the sequences and divergence at population, species and genus levels was done by using K2P model of base

substitution. Phylogenetic analysis was carried out using, Maximum Likelihood (ML, Fig 1) and Neighbour Joining (NJ, Fig 2) approaches in MEGA 7 software (Tamura *et al.*, 2017).

## RESULTS AND DISCUSSION

Fifteen COI sequences representing seven species under six different genera belonging to 3 subfamilies namely Sphinginae, Smerinthinae and Macroglossinae 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 seven genera submitted by other workers were procured directly from GenBank (Table 2). The final aligned data belonged to 29 COI sequences of 536 bp representing 14 species and 7 genera. COI sequence of *Spodoptera litura* (Fabricius, 1775) belonging to family Noctuidae was taken as outgroup. The alignment showed 378 conserved sites, 158 variable sites and 135 parsimony informative sites. The average A+T content was 71% (Tables 3, 5). The nucleotide composition at three positions of codons was calculated. It was observed that third position showed highest AT content (91% of all nucleotides) followed by first position (62%) and the second

(59%). It also showed a strong bias against G with only 0.4% representation at the third position (Table 4).

The data were analysed for sequence divergence at different taxonomic levels. Intraspecific divergence ranged from 0.0% to 3.6% with an average of 0.48% (S.D. 0.98), interspecific divergence ranged from 5.8% to 9.3% with an average of 7.3% (S.D. 1.04) while intergeneric divergence ranged from 8.6% to 13.8% with an average of 11.8% (S.D. 1.57).

The samples studied showed distinct barcodes with no case of barcode sharing. During the BLAST search sequences matched with sequences of congeneric species of family Sphingidae from GenBank proving COI marker to be useful for the diagnosis of the family. An average composition of 73.7% showing the typical A+T bias of insect mitochondrial DNA and cytochrome oxidase genes in particular was given by Clary and Wolstenholme (1985). Brown *et al.* (1994) also showed a bias towards A+T content of 77% nucleotide composition. Similarly, the nucleotide composition bias of 81.79% was given by Cameron and Whiting (2007).

Average K2P intraspecific divergence was found to be  $0.48\% \pm 0.98$  with minimum of 0.0% in *Agrius convolvuli* (Linnaeus, 1758), *Clanis deucalion* (Walker, 1856) and

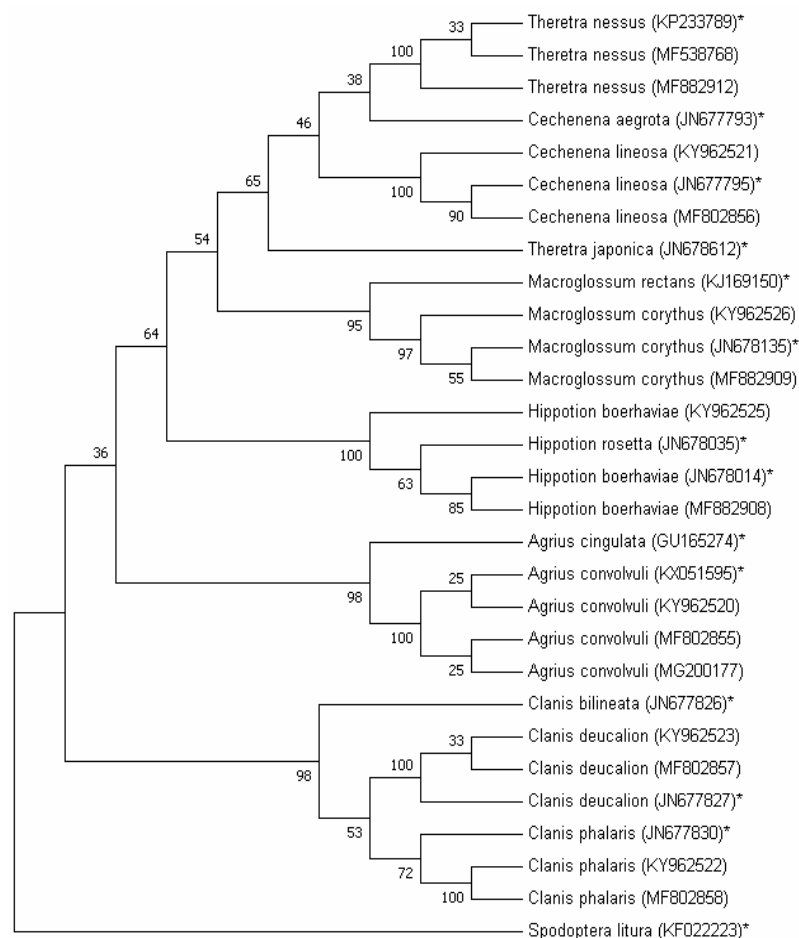
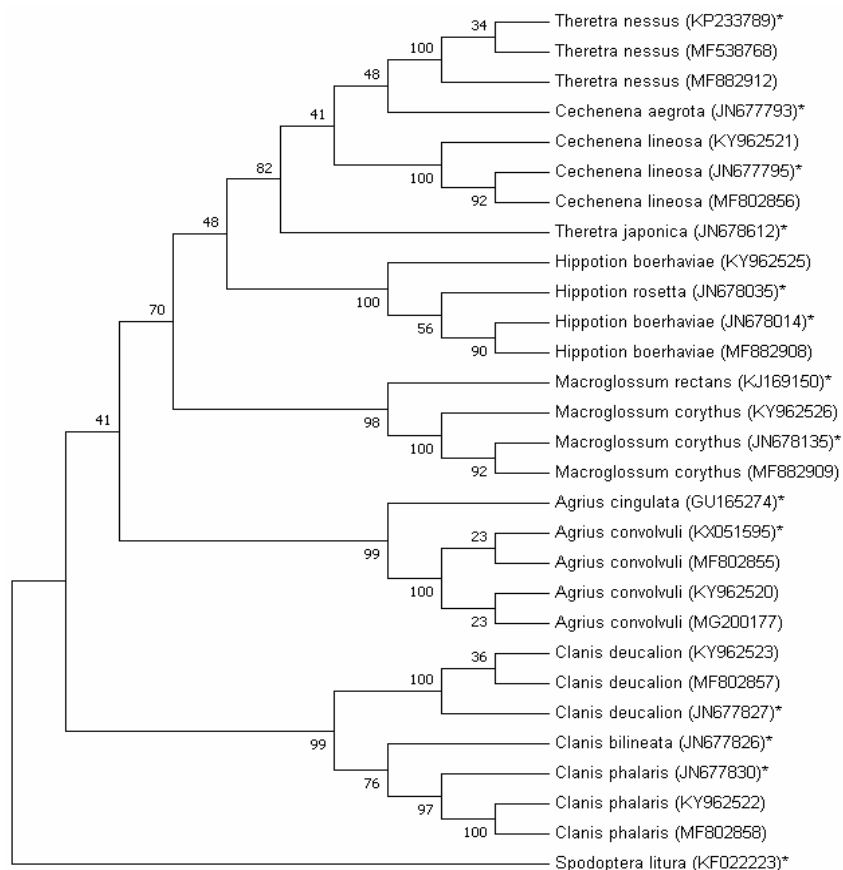


Fig 1: Maximum likelihood tree based on (K2P). Numbers indicate the percentage of 1000 bootstrap replicate.

*Theretra nesus* (Drury, 1773) to a maximum of 3.6% in *Clanis phalaris* (Cramer, 1777) (Table 4). The K2P value was 0.6% for *Macroglossum corythus* Walker, 1856, 0.4% for *Cechenena lineosa* (Walker, 1856) and *Hippotion boerhaviae* (Fabricius, 1775) (Table 6).

A mean of 0.73% (SE=0.033) intraspecific divergence

in family Geometridae was observed by Hausmann *et al.* (2011). Rougerie *et al.* (2014) gave the intraspecific distance in Australian Sphingids ranging from 0.0% to 2.19% (mean=0.3%, SE=0.007) (Table 9). In the family Gelechiinae the intraspecific distances varied from 0.00% to 2.94% (mean = 0.39%, SE=0.01) as reported by Kekkonen *et al.*



**Fig 2:** Neighbour-Joining tree based on (K2P). Numbers indicate the percentage of 1000 bootstrap replicate.

**Table 1:** List of species whose sequences were generated.

Taxa	Number of sample	Collection Place	Collection Month/Year	Accession Number
<i>Agrius convolvuli</i>	1	Ropar	November 2015	KY962520
(Linnaeus, 1758)	1	Palampur	May, 2015	MF802855
<i>Cechenena lineosa</i>	1	Serighat	July, 2014	MG200177
(Walker, 1856)	1	Summer hill	August, 2016	KY962521
<i>Clanis deucalion</i>	1	Cheog	September, 2016	MF802856
(Walker, 1856)	1	Kalsi	September, 2016	KY962523
<i>Clanis phalaris</i>	1	Bilaspur	August, 2015	MF802857
(Cramer, 1777)	1	Kalsi	September, 2016	KY962522
<i>Hippotion boerhaviae</i>	1	Solan	September, 2014	MF802858
(Fabricius, 1775)	1	Subathu	June, 2016	KY962525
<i>Macroglossum corythus</i>	1	Shimla	June, 2014	MF882908
Walker, 1856	1	Patiala	May, 2016	KY962526
<i>Theretra nesus</i>	1	Patiala	May, 2016	MF882909
(Drury, 1773)	1	Ghanhatti	August, 2016	MF538768
	1	Cheog	August, 2016	MF882912

(2015). A similar pattern was observed in the Elachistinae with intraspecific distances ranging from 0.00% to 2.3% (mean=0.28%, SE=0.01). Singh and Kaur (2017) analysed a partial sequence of 580 bp (approx) of COI gene for seven species belonging to family Sphingidae. They reported an average mean of 0.59% (SD of 0.8) at intraspecific level with a range of 0.0% to 2.7%.

Average interspecific divergence was found to be  $7.3\pm 1.04$  with minimum of 5.8% for *Macroglossum corythus* (MF882909) and *Macroglossum rectans* (KJ169150)\* pair and maximum of 9.3% for *Clanis deucalion* (KY962523) and *Clanis bilineata* (JN677826)\* pair (Tables 7, 9). The minimum interspecific divergence of COI for species diagnosis in insects has been suggested to be 3% by Hebert *et al.* (2003). An average of 16.01% interspecific distance in order Lepidoptera was observed by Hajibabaei *et al.* (2006). Hausmann *et al.* (2011) reported mean of 10% with SE of 0.014 in the family Geometridae. Rougerie *et al.* (2014) observed a range of interspecific distance from 1.1% to 7.2% in family Sphingidae. Singh and Kaur (2017) reported a range of 3.6% to 8.2% of interspecific divergence with a mean of 6.3% (SD of 1.62).

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

Name	Accession Number	Country
<i>Agrius convolvuli</i>	KX051595	France
<i>Agrius cingulata</i>	GU165274	Costa Rica
<i>Cechenena lineosa</i>	JN677795	Malaysia
<i>Cechenena aegrota</i>	JN677793	Laos
<i>Clanis deucalion</i>	JN677827	Nepal
<i>Clanis phalaris</i>	JN677830	Thailand
<i>Clanis bilineata</i>	JN677826	Laos
<i>Hippotion boerhaviae</i>	KJ168544	France
<i>Hippotion rosetta</i>	JN678035	Indonesia
<i>Macroglossum corythus</i>	JN678135	Indonesia
<i>Macroglossum rectans</i>	KJ169150	Australia
<i>Spodoptera litura</i>	KF022223	Philippines
<i>Theretra nessus</i>	KP233789	India
<i>Theretra japonica</i>	JN678612	China

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 *Cechenena lineosa* showed the interspecific distance of 8.4% and 8.6% with *Cechenena aegrota*. The species of genus *Clanis* showed variation during intra as well as interspecific studies. The studied samples of the species *Clanis phalaris* gave an intraspecific variation of 3.6%. The interspecific variation between the studied samples of *Clanis deucalion* (KY962523) and *Clanis phalaris* (KY962522) was found out to be 6.8% while it was 9.3% between *Clanis deucalion* (KY962523) and *Clanis bilineata* (JN677826)\* and 7.4% between *Clanis phalaris* (MF802858) and *Clanis bilineata* (JN677826)\*. This indeed makes them a vital issue to be studied and analysed with different molecular markers including COI as well.

In the present study we did the phylogenetic analysis and generated phylogenetic trees. Both the method i.e. distance based and character based methods of phylogenetic analysis were used to generate trees. In both the trees namely Maximum likelihood and Neighbour joining, different genera clustered separately and congeners clustered together. In both the trees *Spodoptera litura*, a member of family Noctuidae taken as outgroup remained well separated from the studied group.

*Notonagemia analis scribae*, a representative of family Sphingidae 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 as studied by Kim *et al.* (2016). They analyzed nucleotide sequences of 13 protein coding genes performed on 12 species in three families of Superfamily Bombycoidea, including *Notonagemia analis scribae* of family Sphingidae.

It was observed that both the types of trees showed similar pattern with little variation in the bootstrap value. All the species showed a similar pattern forming single cluster and giving a divergent distance ranging from 5.8% to 9.3%. Bootstrap values were obtained with 1000 replicates. A higher bootstrap value of more than 70% was showed by majority of branches.

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

Base	A	T	G	C
Average percentage	32	39	14	16

**Table 4:** Percent base composition at the three positions of the codon.

Position	T (%)		C (%)		A (%)		G (%)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
I <sup>st</sup>	28	25-30	14	12-17	34	33-35	25	24-26
II <sup>nd</sup>	44	43-44	27	26-27	15	14-15	15	15-16
III <sup>rd</sup>	45	39-51	8	4-14	46	46-49	0.4	0-2
Average	39	36-42	16	14-19	32	31-33	14	13-15

**Table 5:** Base composition of different taxa.

Taxa	T	C	A	G
<i>Agrius cingulata</i> (GU165274)*	40.6	14.4	31.7	13.3
<i>Agrius convolvuli</i> (KX051595)*	40.6	14.6	31.4	13.5
<i>Agrius convolvuli</i> (KY962520)	40.6	14.6	31.4	13.5
<i>Agrius convolvuli</i> (MF802855)	40.6	14.6	31.4	13.5
<i>Agrius convolvuli</i> (MG200177)	40.6	14.6	31.4	13.5
<i>Cechenena aegrota</i> (JN677793)*	38.0	18.1	30.6	13.3
<i>Cechenena lineosa</i> (JN677795)*	36.9	18.5	31.5	13.1
<i>Cechenena lineosa</i> (KY962521)	37.3	18.1	31.5	13.1
<i>Cechenena lineosa</i> (MF802856)	36.9	18.5	31.5	13.1
<i>Clanis bilineata</i> (JN677826)*	37.6	16.2	32.5	13.7
<i>Clanis deucalion</i> (JN677827)*	37.3	17.0	31.9	13.8
<i>Clanis deucalion</i> (KY962523)	37.3	17.0	31.9	13.8
<i>Clanis deucalion</i> (MF802857)	37.3	17.0	31.9	13.8
<i>Clanis phalaris</i> (JN677830)*	39.3	14.9	32.5	13.3
<i>Clanis phalaris</i> (KY962522)	38.7	15.1	32.7	13.5
<i>Clanis phalaris</i> (MF802858)	38.7	15.1	32.7	13.5
<i>Hippotion boerhaviae</i> (JN678014)*	39.9	14.9	31.5	13.7
<i>Hippotion boerhaviae</i> (KY962525)	40.0	14.8	31.7	13.5
<i>Hippotion boerhaviae</i> (MF882908)	39.9	14.9	31.5	13.7
<i>Hippotion rosetta</i> (JN678035)*	40.0	14.8	31.5	13.7
<i>Macroglossum corythus</i> (JN678135)*	41.0	15.7	29.9	13.5
<i>Macroglossum corythus</i> (KY962526)	40.4	15.7	30.4	13.5
<i>Macroglossum corythus</i> (MF882909)	41.0	15.7	29.9	13.5
<i>Macroglossum rectans</i> (KJ169150)*	41.0	14.8	30.4	13.8
<i>Spodoptera litura</i> (KF022223)*	41.1	15.3	29.9	13.7
<i>Theretra japonica</i> (JN678612)*	38.0	17.5	31.0	13.5
<i>Theretra nessus</i> (KP233789)*	38.9	17.0	30.6	13.5
<i>Theretra nessus</i> (MF538768)	38.9	17.0	30.6	13.5
<i>Theretra nessus</i> (MF882912)	38.9	17.0	30.6	13.5

**Table 6:** Pairwise K2P intraspecific divergence.

Species (accession no.)	Species (accession no.)	Divergence (%)
<i>Agrius convolvuli</i> (KY962520)	<i>Agrius convolvuli</i> (MF802855)	0.0
<i>Agrius convolvuli</i> (KY962520)	<i>Agrius convolvuli</i> (MG200177)	0.0
<i>Agrius convolvuli</i> (KY962520)	<i>Agrius convolvuli</i> (KX051595)*	0.0
<i>Agrius convolvuli</i> (MF802855)	<i>Agrius convolvuli</i> (MG200177)	0.0
<i>Agrius convolvuli</i> (MF802855)	<i>Agrius convolvuli</i> (KX051595)*	0.0
<i>Cechenena lineosa</i> (KY962521)	<i>Cechenena lineosa</i> (MF802856)	0.4
<i>Cechenena lineosa</i> (KY962521)	<i>Cechenena lineosa</i> (JN677795)	0.4
<i>Cechenena lineosa</i> (MF802856)	<i>Cechenena lineosa</i> (JN677795)	0.4
<i>Clanis deucalion</i> (KY962523)	<i>Clanis deucalion</i> (MF802857)	0.0
<i>Clanis deucalion</i> (KY962523)	<i>Clanis deucalion</i> (JN677827)*	0.0
<i>Clanis deucalion</i> (MF802857)	<i>Clanis deucalion</i> (JN677827)*	0.0
<i>Clanis phalaris</i> (KY962522)	<i>Clanis phalaris</i> (MF802858)	0.0
<i>Clanis phalaris</i> (KY962522)	<i>Clanis phalaris</i> (JN677830)*	3.6
<i>Clanis phalaris</i> (MF802858)	<i>Clanis phalaris</i> (JN677830)*	3.6
<i>Hippotion boerhaviae</i> (KY962525)	<i>Hippotion boerhaviae</i> (MF882908)	0.0
<i>Hippotion boerhaviae</i> (KY962525)	<i>Hippotion boerhaviae</i> (KJ168544)*	0.4
<i>Hippotion boerhaviae</i> (MF882908)	<i>Hippotion boerhaviae</i> (KJ168544)*	0.4
<i>Macroglossum corythus</i> (KY962526)	<i>Macroglossum corythus</i> (MF882909)	0.6
<i>Macroglossum corythus</i> (KY962526)	<i>Macroglossum corythus</i> (JN678135)*	0.6
<i>Macroglossum corythus</i> (MF882909)	<i>Macroglossum corythus</i> (JN678135)*	0.6
<i>Theretra nessus</i> (MF538768)	<i>Theretra nessus</i> (MF882912)	0.0
<i>Theretra nessus</i> (MF538768)	<i>Theretra nessus</i> (KP233789)*	0.0
<i>Theretra nessus</i> (MF882912)	<i>Theretra nessus</i> (KP233789)*	0.0



**Table 7:** Pairwise K2P interspecific divergence.

Species (accession no.)	Species (accession no.)	Divergence (%)
<i>Agrius convolvuli</i> (KY962520)	<i>Agrius cingulata</i> (GU165274)*	7.0
<i>Agrius convolvuli</i> (MF802855)	<i>Agrius cingulata</i> (GU165274)*	7.0
<i>Agrius convolvuli</i> (MG200177)	<i>Agrius cingulata</i> (GU165274)*	7.0
<i>Cechenena lineosa</i> (KY962521)	<i>Cechenena aegrota</i> (JN677793)*	8.4
<i>Cechenena lineosa</i> (MF802856)	<i>Cechenena aegrota</i> (JN677793)*	8.6
<i>Clanis deucalion</i> (KY962523)	<i>Clanis phalaris</i> (KY962522)	6.8
<i>Clanis deucalion</i> (KY962523)	<i>Clanis bilineata</i> (JN677826)*	9.3
<i>Clanis phalaris</i> (MF802858)	<i>Clanis bilineata</i> (JN677826)*	7.4
<i>Hippotion boerhaviae</i> (KY962525)	<i>Hippotion rosetta</i> (JN678035)*	6.0
<i>Hippotion boerhaviae</i> (MF882908)	<i>Hippotion rosetta</i> (JN678035)*	6.0
<i>Macroglossum corythus</i> (KY962526)	<i>Macroglossum rectans</i> (KJ169150)*	6.2
<i>Macroglossum corythus</i> (MF882909)	<i>Macroglossum rectans</i> (KJ169150)*	5.8
<i>Theretra nessus</i> (MF538768)	<i>Theretra japonica</i> (JN678612)*	8.0
<i>Theretra nessus</i> (MF882912)	<i>Theretra japonica</i> (JN678612)*	8.0

**Table 8:** Pairwise K2P intergeneric divergence.

Species (accession no.)	Species (accession no.)	Divergence (%)
<i>Agrius convolvuli</i> (KY962520)	<i>Cechenena lineosa</i> (KY962521)	13.3
<i>Agrius convolvuli</i> (KY962520)	<i>Clanis deucalion</i> (KY962523)	12.6
<i>Agrius convolvuli</i> (KY962520)	<i>Clanis phalaris</i> (MF802858)	11.1
<i>Agrius convolvuli</i> (KY962520)	<i>Hippotion boerhaviae</i> (KY962525)	12.2
<i>Agrius convolvuli</i> (KY962520)	<i>Macroglossum corythus</i> (KY962526)	12.2
<i>Agrius convolvuli</i> (KY962520)	<i>Theretra nessus</i> (MF538768)	11.5
<i>Cechenena lineosa</i> (KY962521)	<i>Clanis deucalion</i> (KY962523)	13.8
<i>Cechenena lineosa</i> (KY962521)	<i>Clanis phalaris</i> (MF802858)	13.7
<i>Cechenena lineosa</i> (KY962521)	<i>Hippotion boerhaviae</i> (KY962525)	11.4
<i>Cechenena lineosa</i> (KY962521)	<i>Macroglossum corythus</i> (KY962526)	10.3
<i>Cechenena lineosa</i> (KY962521)	<i>Theretra nessus</i> (MF538768)	8.2
<i>Clanis deucalion</i> (KY962523)	<i>Hippotion boerhaviae</i> (KY962525)	12.0
<i>Clanis deucalion</i> (KY962523)	<i>Macroglossum corythus</i> (KY962526)	12.0
<i>Clanis deucalion</i> (KY962523)	<i>Theretra nessus</i> (MF538768)	13.7
<i>Clanis phalaris</i> (MF802858)	<i>Hippotion boerhaviae</i> (KY962525)	12.0
<i>Clanis phalaris</i> (MF802858)	<i>Macroglossum corythus</i> (KY962526)	12.0
<i>Clanis phalaris</i> (MF802858)	<i>Theretra nessus</i> (MF538768)	13.7
<i>Hippotion boerhaviae</i> (KY962525)	<i>Macroglossum corythus</i> (KY962526)	11.1
<i>Hippotion boerhaviae</i> (KY962525)	<i>Theretra nessus</i> (MF538768)	9.6
<i>Macroglossum corythus</i> (KY962526)	<i>Theretra nessus</i> (MF538768)	8.6

**Table 9:** Comparative data of K2P divergence.

K2P divergence	Singh and Kaur (2017)		Rougerie <i>et al.</i> (2014)		Hausmann <i>et al.</i> (2011)		Present study	
	Range (%)	Mean distance (%)	Range (%)	Mean distance (%)	Range (%)	Mean distance (%)	Range (%)	Mean distance (%)
Intraspecific	0.0 to 2.7	0.59±0.82	0.0 to 2.19	0.3	N	0.73	0.0 to 3.6	0.48±0.98
Interspecific	3.6 to 8.2	6.3±1.62	1.1 to 7.2	N	N	10	5.8 to 9.3	7.3±1.04
Intergeneric	8.1 to 15.6	12.2±1.82	N	7.13	N	13.3	8.6 to 13.8	11.8±1.57

N: Not given by author

The present study added to the database of family Sphingidae but it would be inapt to analyse the species on the basis of the available molecular data. It is recommended that more number of species should be considered to trace the trends of evolution followed in the family.

## CONCLUSION

The current study was based on 15 COI sequences of 7 species belonging to 6 genera of family Sphingidae. It represents the first ever study on the barcoding region of family Sphingidae from north India. We have generated distinct barcodes for each species and thus they can be used for the identification purposes as well. The database analysis shows hierarchical increase in K2P mean divergence across different taxonomic levels.

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