BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, March 2023. Vol. **20**(1), p. 109-119

Phylogenetic Analysis of the Economically Important Hymenopterans using cytochrome oxidase 1 Enzyme Sequences

Charul[1], Sunali Bandral[1], Shivalika[1], Vikas Dogra[2], Mohd. Feroz[3], Umer Bin Farook[4] and Rakesh K. Panjaliya[1]*

[1]Department of Zoology, University of Jammu, J and K, India.
[2]Govt. College for Women, Parade, Jammu, J and K, India.
[3]Department of Zoology, AAAM Degree College Bamina, Srinagar, J and K, India.
[4]Faculty of Agriculture, SKAUST, Kashmir, India.

http://dx.doi.org/10.13005/bbra/3073

(Received: 11 July 2022; accepted: 17 December 2022)

Hymenoptera is the fourth diverse and the most economically important insect order comprising of bees, wasps, ants, sawflies etc. Being an important part of ecosystem, their conservation is of utmost importance. The first step towards conservation strategies is the identification of the species. The traditional morphological approach can sometimes lead to misidentification due to a lack of expertise. DNA barcoding using the small genomic fragments has been identified as an efficient tool in the identification as well as the phylogenetic analysis of the species. In the present study, we used the COI gene sequences as a tool for the characterization of Hymenoptera from different parts of the Jammu region. The collected samples were proceeded for the isolation of DNA, PCR for amplification of the COI gene, and then sequenced by Sanger dideoxy method. A total of 22 COI sequences belonging to 18 different species were successfully generated. Among which eight species sequences (Tachytes sp., Bombus trifasciatus, Rhynchium carnaticum, Ropalidia brevita, Lasioglossum marginatum, Camponotus pennsylvanicus, Tapinoma melanocephalum, Formica rufibarbis) are the novel contribution in the global database. NJ tree using the K2P model with 1000 bootstrap supporting values has been used to study the phylogeny of the species. Sequence analysis shows high AT content (67-77%) in the COI region of Hymenopterans. The generated COI sequence analysis also revealed less than 1% intra-specific divergence in the examined taxa, while the interspecific distances ranged between 8% to 38%. This study added significantly to the databases of DNA barcodes of Hymenopterans species from Jammu region.

Keywords: COI gene; DNA barcoding; Hymenoptera; Phylogenetic study.

Hymenoptera is one of the four mega-diverse insect orders after Coleoptera, Lepidoptera, and Diptera, comprising up to a million undescribed extant species and 153,000 described ones[1, 2]. The order includes ants, bees, wasps, etc. which are of great worth to all terrestrial ecosystems and of economic importance for humanity[3, 4]. Bees and some species of wasps are the most effective pollinators of agricultural and wild plants and help sustain our food supplies[4, 5, 6]. Various wasp species are the predators or parasitoids of crop pests and can be used as a substitute for chemical pesticides have benefits for the environment and are cost-effective[4, 7, 8, 9]. The importance of ants

*Corresponding author E-mail: rakeshpanjaliya@jammuuniversity.ac.in

in the ecosystem is also widespread due to their interactions with different plants, feeding on other insects and small invertebrates, seed dispersal, and seed-predator habit[10, 11].

A sharp decline in the population of these insects has been observed globally due to anthropogenic activities like deforestation, urbanisation, and application of pesticides in agricultural fields, leading to various adverse effects like habitat fragmentation, climate change and degradation of the insect's habitat[10, 11]. For the maintenance of biodiversity and ecological integrity and considering the spanning human needs and interests of these creatures, their conservation is the need of the hour. And the first step in conservation is the accurate identification of the species. The traditional taxonomic methods used for the identification require the expertise of the field with a thorough knowledge of taxonomic terms to avoid misidentifications. Not only this, the technique has limitations in the identification of morphologically cryptic species.

Moreover, the available keys also work for a particular life stage or sex[12]. To overcome the limitations of the morphological taxonomic approach, a molecular technique involving the generation of species-specific barcodes was introduced. Hebert et al., 2003 first established the use of the Mitochondrial COI gene to generate bio-identification barcodes in eukaryotic animals. The mitochondrial COI gene is considered an effective 'barcode' in species identification and delimitation because of its faster rate of evolution, which helps in the study of closely related taxa that have recently diverged[13, 14, 15]. The present study aims to generate the DNA barcodes for the Hymenoptera species from the Jammu region to study phylogenetic relations among them. The study helps in estimating hymenopteran diversity in the region and helps formulate conservation strategies for this species diversity.

MATERIALS AND METHODS

Taxon sampling and vouchering

About 53 samples were collected from different areas of the Jammu region by using sweeping nets and handpicking. The insects were anesthetised using ethyl acetate and preserved in 70% molecular grade alcohol for both morphological and molecular examination. The samples were examined under the Olympus SZ2-ILST for morphological identification, and voucher IDs were assigned to them.

Molecular interrogations, sequence annotation and dataset preparation

For DNA isolation, 1-2 legs depending upon the size and finally chopped, are used in the case of bees and wasps, while an intersegmental abdominal cut was made in the case of ants. The tissue was lysed overnight in buffer ATL and proteinase-K at 56ºC with 150rpm (revolutions per minute) in Stvart SBS40 shaking water bath (Cole- Paramer Ltd. Stone, ST15 OSA, UK). After tissue lysis, the total genomic DNA was extracted by using DNeasy Blood & Tissue Kit (Hilden, Germany). Polymerase Chain Reaction (PCR) was performed in applied biosystems ProFlex PCR system by life technologies to amplify partial mitochondrial cytochrome oxidase subunit I (COI) gene by using primers and conditions as shown in Table 1. The PCR products were sent to Biologia Research India Pvt. Ltd. for the sanger sequencing. The generated forward and reverse chromatogram files for each specimen were checked in MEGA X[16] . The generated sequences were further screened in BLASTn (Basic Local Alignment Search Tool) for the available similar sequences in NCBI (National Centre for Biological Information) and 10 similar database sequences of each species are obtained from NCBI and are used for data curation. ClustalW software[17] has been used for pairwise and multiple alignments by using a Gap opening penalty of 15.00 and a Gap extension penalty of 6.66 to obtain the final sequence for submission. The final dataset of 22 sequences from 18 different species was submitted to the NCBI Genbank through BioEdit; an online submission portal and the GenBank accession numbers were generated for them, written in Table 2. The A, T, G, C, AT, and GC content of all the sequences were obtained by using science buddies, and online software and their percentages present in sequence are depicted in Table 3.

Genetic divergence and phylogenetic tree interpretation

The pairwise genetic distance was analysed using the Kimura 2-Parameter (K2P) model in MEGA X[16, 18]. The number of base substitutions per site was analysed between all 22

sequences. Codon positions included were 1st+ 2nd+ 3rd+non-coding. All ambiguous positions were removed for each sequence pair, and a total of 538 positions were present in the final dataset. The evolutionary relationship was inferred using the Neighbor-Joining (NJ) method and Kimura 2-parameter model with 1000 bootstrap replications in MEGAX[16, 18, 19, 20]. The database sequence of *Eristalis tenax* (ON210045) under order Diptera was used as an out-group in the phylogenetic study.

RESULTS AND DISCUSSION

Morphological identification

The hymenopteran species were identified by consulting the available keys in the literature by Archer (2014), Bingham (1897), Carpenter and Thi Nguyen (2003), Das and Gupta (1989), Goulet and Huber (1993), Kumar and Carpenter (2013), Kumar and Srinivasan (2010), Niup and Dorji (2016), Saini et al. (2011), Williams (1998), other relevant literature and identification keys[21-30]. After morphological identification, the collected specimens were identified as18 different species belonging to 5 families, namely Apidae, Formicidae, Vespidae, Halictidae, and Crabronid. The details of identified species, their collection date, the site of collection & their coordinates have been listed in Table 2.

Molecular characterisation

Of the 53 collected specimens, DNA was successfully isolated from 45 samples, and successful amplification of the COI region of 35 was carried out. Of which, sequences of 22 samples have been generated, which can be accessed by the NCBI with accession numbers (ON254654; ON259314-ON259316; ON259323; ON259474; ON259531-ON259532; ON306317-ON306330). The results of the similarity search in the NCBI database showed 99-100% identical matches with the same species except for eight species (*Tachytes sp., Bombus trifasciatus, Rhynchium carnaticum, Ropalidia brevita, Lasioglossum marginatum, Camponotus pennsylvanicus, Tapinoma melanocephalum, Formica rufibarbis*). Hence, the present study contributed eight new sequences of hymenopteran species from the Jammu region to the database. The COI sequences were found to have less GC% (23-33%) and more AT% (67-77%), as depicted in table 3. The minimum GC% was found in Apidae and the maximum in Formicidae of the studied families of Hymenoptera. The findings are following the previous studies, which reported 22-41% GC content in the hymenopteran COI region with 22-29% in Apidae and 24-41% in formicidae[31, 32]. The phylogenetic tree was constructed using the NJ method and the K2P model along with 1000

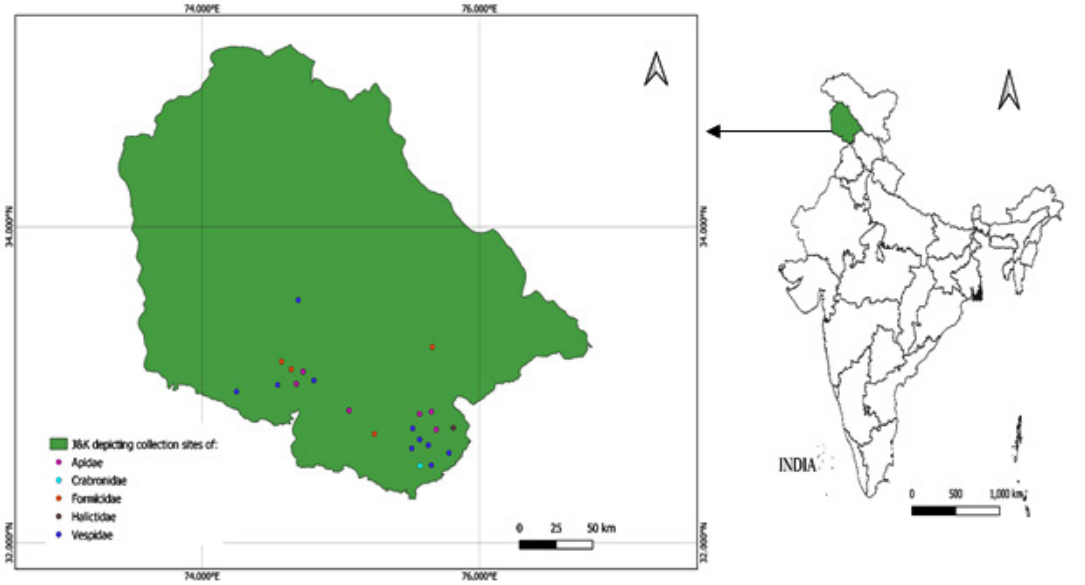

Fig. 1. Map depicting collection sites

Table 1. Primer sequences used

Gene Fragment	Fragment size	Direction	Published name	Sequence 5'- 3'	References[13, 50]	Used concentration of PCR components	Reaction conditions
COI	650bp	Forward Reverse	LCO 1490 HCO 2198	5'-GGTCAACAAATCATAAAGATATTGG-3' 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer et al., 1994	1x Dream Taq buffer, 2.5mM MgCl2, 0 .2mMdNTP mix, 0.2µM each forward and reverse primer, 1.5U Dream Taq DNA polymerase.	Initial denaturation at 94°C for 4 minutes. Denaturation at 94°C for 30 s. Annealing at 47°C for 45 s. Extension at 72°C for 45 s. No. of cycles= 35. Final extension at 72°C for 20 minutes.
		Forward Reverse	Lep-F1 Lep-R1	5'-ATTCAACCAATCATAAAGA TAT-3' 5'-TAAACTTCTGGATGTCCAA AAA-3'	Hebert et al., 2004a	0,9x Buffer, 2mM MgCl2, 0.2mM dNTP mix, 0.2mM each forward and reverse primer, 1.5U Taq polymerase	Initial denaturation at 94°C for 4 minutes. Denaturation at 94°C for 30 s. Annealing at 47°C for 45 s. Extension at 72°C for 45 s. No. of cycles= 35. Final extension at 72°C for 20 minutes.

Table 2. Information about studied samples

Order	Family	Identified species name	Voucher specimen	Date of collection	Place of collection	Latitude	Longitude	Elevation (metres)	Genbenk Accession number generated (COI)
Hymenoptera	Apidae	*Apis cerana*	RHY10	29/08/2019	Hostel,University of Jammu	32.7180N	74.8702E	319	ON259474
		Apis dorsata	RHY12	29/08/2019	Hostel, University of Jammu	32.7180N	74.8702E	320	ON259323
		Apis dorsata	RHY51	26/12/2020	Kathua	32.7329N	74.8642E	352	ON259531
		Apis mellifera	CH9	6/11/2021	Vijaypur	32.7053N	74.8800E	341	ON259532
		Bombus trifasciatus	RHY7	25/08/2019	Ramkot	32.6042N	75.2881E	516	ON306321
		Bombus trifasciatus	RB9A	23/06/2019	Ramkot	32.6101N	75.2822E	505	ON306319
	Formicidae	*Camponotus pennslyvanicus*	ANT 0	27/12/2021	Department of Zoology, University of Jammu.	32.7242N	74.8666E	316	ON259314
		Tapinoma melanocephalum	ANT1	30/12/2021	Hostel, University of Jammu	32.7180N	74.8702E	319	ON259315
		Formica rufibarbis	ANT3	10/10/2021	Doda	33.1493N	75.5477E	1200	ON259316
		Monomorium indicum	CH6	29/09/2021	Ghaghwal	32.5092N	75.1863E	366	ON254654
	Vespidae	*Ropalidia brevita*	RHY2	25/08/2019	Ramkot	32.6042N	75.2881E	511	ON306323
		Polistes olivaceus	RHY4	25/08/2019	Ramkot	32.6042N	75.2881E	511	ON306320
		Delta pyriforme pyriforme	RHY24	24/10/2019	Ramkot	32.6081N	75.2786E	453	ON306325
		Vespa basalis	RHY30	4/11/2019	Domael, Katra	32.9395N	74.9499E	715	ON306327
		Antodynerus limbatus	RHY37	7/7/2020	Ramkot	32.6103N	75.2823E	500	ON306328
		Vespa tropica	RHY40	7/7/2020	Ramkot	32.6103N	75.2823E	500	ON306329
		Polistes watti	RHY49	24/12/2020	Campus, University of Jammu	32.7194N	74.8681E	318	ON306330
		Rhynchium carnaticum	RHY50	20/12/2020	R.S. Pura	32.7329N	74.8642E	351	ON306322
		Vespa basalis	RV4	7/4/2019	Hostel, University of Jammu	32.7180N	74.8702E	319	ON306318
		Antodynerus limbatus	RHY22	24/10/2019	Ramkot	32.6081N	75.2786E	449	ON306328
	Halictidae	*Lasioglossum marginatum*	RHY 26	24/08/2019	Ramkot	32.6081N	75.2786E	447	ON306326
	Crabronidae	*Tachytes sp.*	RHY 1	25/08/2019	Ramkot	32.6042N	75.2881E	521	ON306317

bootstrap support values because the maximum literature reviewed for this research work was found to have used the NJ tree with the K2P model with supporting bootstrap values. The phylogenetic tree constructed depicts eighteen distinct lineages of twenty-two hymenopteran species belonging to five clades corresponding to the five families (Figure 2). Family Formicidae is found to be more closely related to Vespidae, while the family Apidae is found to be more closely related to the family Halictidae. The family Crabronidae is distantly related to all the four studied families; namely, apidae, formicidae, halictidae and Vespidae, as clearly depicted in the phylogenetic tree. The phylogeny constructed using COI sequences has been found as per the previous studies based on the morphological as well as molecular approach. Brothers in 1999 studied the phylogeny and evolutionary relationship of the wasps, ants and bees by using a morphological system and their results depict that ants and wasps are more closely related to each other than their relatedness to bees[33]. Relatedness between Formicidae and Vespidae has been reported in the studies using different molecular markers[34]. There is much evidence from the literature which supports the use of molecular markers for species identification and phylogenetic studies. The successful use of molecular markers especially COI for the identification and delimitation of the species was seen in *Vespa* species identification, Trissolcus species identification, new species of *Zaischnopsis*, trichogrammatids, hymenopteran parasitoids, and Egyptian wasps are some of the examples from the literature[35-41]. The molecular data has also been found to be useful for species delimitation as depicted by the studies of Chen and coworkers; Parslow and associates; Benavides and associates and Siddiqui and coworkers[42-45]. Kwon and associates in their study constructed the phylogenetic tree for the species of genus *Osmia* using COI gene data and found that the results were satisfactory and in accordance with the traditional approach[46]. Similarly, molecular characters including COI, 16S rDNA, and 28S rDNA were used to study the evolutionary

Table 3. ATGC content of all the studied Samples

Sample Name	All base count	A (%)	T (%)	G (%)	C (%)	GC (%)	AT (%)
Apis cerana	538	179	222	57	80	25.5	74.5
Apis dorsata	580	189	249	59	83	24.5	75.5
Apis dorsata	538	178	227	57	76	24.7	75.3
Apis mellifera	578	189	241	60	88	25.6	74.4
Bombus trifasciatus	564	183	246	56	79	23.9	76.1
Bombus trifasciatus	672	228	285	71	88	23.7	76.3
Tachytes sp.	598	202	248	77	71	24.7	75.3
Camponotus pennsylvanicus	637	191	268	71	107	27.9	72.1
Tapinoma melanocephalum	654	194	246	86	128	32.7	67.3
Formica rufibarbis	660	204	259	75	122	29.8	70.2
Monomorium indicum	636	192	250	81	113	30.5	69.5
Lasioglossum marginatum	621	197	251	70	103	27.9	72.1
Ropalidia brevita	604	187	240	76	101	29.3	70.7
Polistes olivaceus	672	223	262	78	109	27.8	72.2
Delta pyriforme pyriforme	604	211	228	73	92	27.3	72.7
Vespa basilis	560	178	217	68	97	29.5	70.5
Antodynerus limbatus	604	185	246	79	94	28.6	71.4
Vespa tropica	604	179	234	76	115	31.6	68.4
Polistes watti	604	189	252	73	90	27	73
Rhynchium carnaticum	598	172	244	78	104	30.4	69.6
Vespa basalis	672	206	273	81	112	28.7	71.3
Antodynerus limbatus	604	185	245	79	95	28.8	71.2

Table 4. Genetic distance among the studied species

Species	Mean inter-specific distance (%) as calculated using K2P model of genetic divergence calculation																				
Tachytes species																					
Vespa basalis 1	26.49																				
Bombus trifasciatus 1	24.91	31.41																			
Polistes olivaceus	24.13	19.10	30.88																		
Bombus trifasciatus 2	24.72	31.98	0.58	31.72																	
Rhynchium carnaticum	27.00	22.72	30.27	26.05	30.21																
Ropalidia brevita	27.27	21.77	33.70	19.22	34.94	23.72															
Antodynerus limbatus	25.16	19.42	29.45	22.63	29.93	20.90	21.15														
Delta pyriforme pyriforme	24.90	20.88	27.31	19.65	28.28	24.93	21.12	20.88													
Lasioglossum marginatum	29.21	28.65	23.88	24.90	24.44	27.29	28.62	28.35	27.54												
Vespa basalis 2	26.49	0.37	31.70	19.58	32.27	23.00	21.79	19.90	20.89	28.92											
Antodynerus limbatus 1	24.90	19.66	29.45	22.37	29.93	21.15	20.90	0.19	20.63	28.08	20.15										
Vespa tropica	27.56	16.69	31.97	20.15	33.13	23.52	19.42	19.43	23.38	28.35	16.92	19.18									
Polistes watti	23.89	18.99	30.88	13.42	31.44	23.93	21.43	23.36	21.61	26.74	19.00	23.11	20.17								
Camponotus pennsylvanicus	31.11	27.54	33.22	33.70	33.83	32.82	31.40	30.01	33.11	33.14	28.08	30.28	31.17	30.27							
Tapinoma melanocephalum	29.46	31.03	35.75	28.13	36.15	30.08	27.44	29.74	30.55	30.83	31.30	29.46	27.32	29.25	29.63						
Formica rufibarbis	27.85	29.01	32.25	30.91	32.53	29.78	30.60	29.57	30.55	30.55	29.00	29.28	30.75	30.39	24.22	23.61					
Monomorium indicum	31.96	35.46	33.45	33.69	34.97	31.40	27.81	31.68	32.27	34.07	35.46	31.40	32.25	30.84	22.61	25.78	20.65				
Apis cerana	27.07	31.68	14.97	29.74	15.45	34.57	32.56	30.56	27.87	26.74	31.96	30.28	30.58	30.62	32.87	36.36	33.43	37.69			
Apis dorsata 1	24.49	32.25	15.20	29.27	15.69	32.24	32.59	30.00	28.73	22.12	32.54	29.73	30.00	30.94	33.46	34.58	31.41	35.03	8.67		
Apis dorsata 2	24.49	32.25	15.43	29.27	15.93	32.53	32.59	30.00	28.99	22.36	32.54	29.73	30.00	30.94	33.46	34.58	31.41	35.03	8.88	0.19	
Apis mellifera	27.88	31.68	15.66	30.31	15.70	31.39	33.11	30.85	29.23	23.12	31.96	30.58	29.75	30.88	33.42	33.40	33.44	35.53	10.57	9.51	9.72

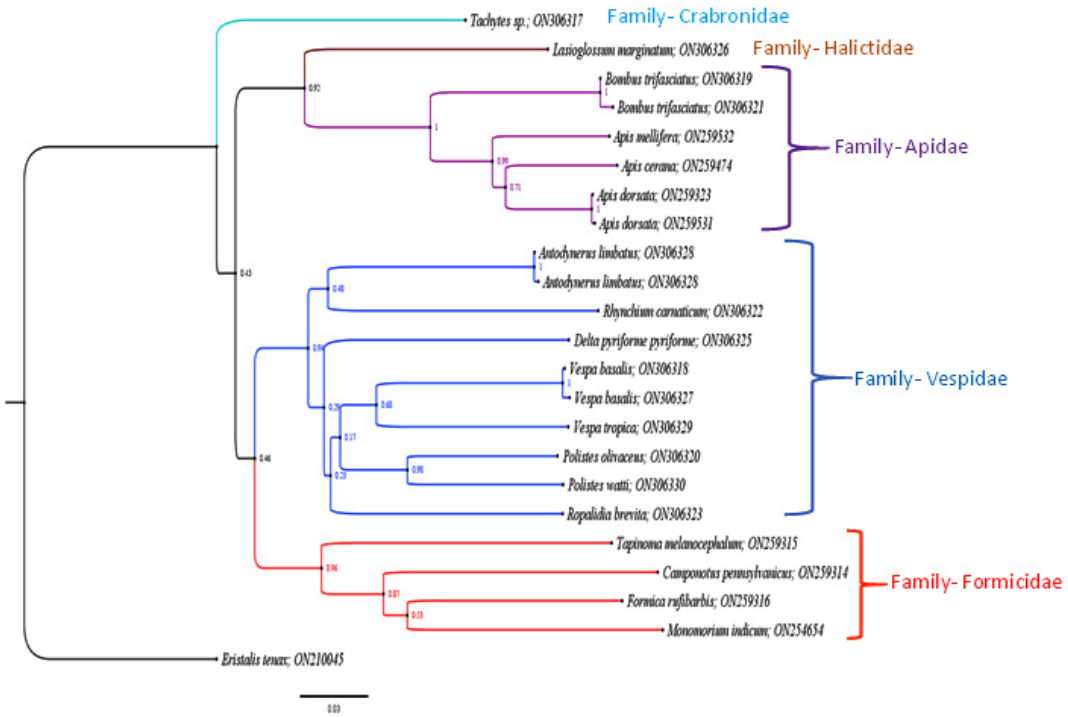

Fig. 2. NJ tree (kimura-2-Parameter)

transitions in parasitic wasps belonging to Apocrita in recent times[47]. Aman-zuki and coworkers through their work also suggested the usefulness of molecular markers in resolving the phylogeny of species belonging to Microgastrine wasps[48]. The studies of Niehuis and coworkers with the Euchroeus group of tribe Chrysidini have detected the inappropriate rooting of the group with the help of molecular data. Their study provides the insights that the molecular data not only supports traditional taxonomic views but also helps in resolving the phylogeny of various groups where the morphological characters have given erroneous interpretations[49]. Hence, the study supports the use of COI gene sequences (molecular markers) for inferring the phylogenetic relationship among the species in addition to species identification and delimitation. The genetic relatedness among the species was also calculated with the generated sequence data. The overall mean genetic distance among the species in the present dataset was found to be 26.9%. The intraspecific genetic distance ranges from 0.19% (*Apis cerana*) to 0.58% (*Bombus trifasciatus*). However, the highest inter-specific genetic distance (37.69%) was observed between Apis cerana and Monomorium indicum, and the lowest inter-specific genetic distance (8.67%) was observed between *Apis cerana* and *Apis dorsata*. The values are clearly depicted in Table 4.

CONCLUSION

The present study was the first approach for carrying out molecular characterisation and phylogenetic analysis of Hymenoptera from the Jammu region. The study contributed 22 barcode sequences including 8 novel sequences belonging to the insects of order Hymenoptera to the database. The study also provided evidence in support of the use of molecular markers for phylogenetic analyses as the results are in concordance with the morphological available evidence. The current study also helps future workers, especially non-taxonomists, to identify the species based on the generated barcodes of the species to the online database.

ACKNOWLEDGEMENT

The authors are grateful to the Head, Department of Zoology, the University of Jammu, for providing necessary facilities and equipment availability (purchased out of RUSA/ PURSE/ FIST grants). One of the authors, Charul, also acknowledges the financial support from The Department of Science and Technology, Government of India, INSPIRE Fellowship under sanction no.: DST/INSPIRE Fellowship/2018/ IF180760.

Authors contribution

Charul carried out the extensive field work, lab work and data compilation; Sunali Bandral and Shivalika also performed the lab work; Mohd Feroz and Umer Bin Farooq identified the Hymenopteran insect species; Vikas Dogra and Rakesh K. Panjaliya has framed the work plan, provide guidance at every step and reviewed the article.

REFERENCES

1. Agular A.P., Deans A.R., Engel M.S., Forshage M., Huber J.T., Jennings J.T., Johnson N.F., Lelej A.S., Longino J.T., Lohrmann V., et al. Order Hymenoptera. *Zootaxa*. 2013; 3703:51-62.
2. Grimaldi D.A., Engel M.S. Evolution of the Insects (Cambridge University Press). 2005.
3. Quicke D.L.J. Parasitic Wasps (Chapman & Hall). 1997.
4. Toler T.R., Evans E.W., Tepedino V. J. Pan-trapping for bees (Hymenoptera: Apiformes) in Utah's West Desert: the importance of color diversity. *Pan Pacific Entomologist* 2005; 81(3-4):103-113.
5. Free J.B. Insect pollination of crops. 2nd ed. Academic press.1993; 1-684.
6. Raj H., Mattu V.K., Thakur M.L. Pollinator diversity and relative abundance of insect visitors on apple crop in Shimla hills of western Himalaya, India. *International Journal of Science and Nature*. 2012; 3(3):507-513.
7. Bennett F.D., Yaseen M., Beg M.N., Sommeijer M.J. *Anastrepha* spp.- investigations on their natural enemies and establishment of Biosteres longicaudatus in Trinidad, West Indies. *CIBC Tech. Bull*. 1997; 18:1-12.
8. Wong T.T.Y., Ramadan M.M., Mclnnis D.O., Mochizuki N. Influence of cohort age and host age on oviposition activity and offspring sex-ratio of Biosteres tryoni (Hymenoptera: Braconidae), a larval parasitoid of Ceratitis capitate (Diptera: Tephritidae). *J. Econ. Entomol.* 1990; 83: 779-783.s
9. Wharton R.A. Classical biological control of fruit infesting Tephritidae. In: Robinson, A.S., Hooper, G. (Eds.), Fruit Flies: Their Biology, Natural Enemies, and Control. *Elsevier, Amsterdam.* 1989; pp. 303-313.
10. Suryanto D.W.I. A survey of ants as candidates for potential biology control of pear psylla (Cacopsylla pyricola Foerster) in Michigan (Michigan State University) Dissertation Abstracts. 1993. MAI32/03.
11. Patkar N.B., Chavan R.J. Diversity of Ants (Hymenoptera: Formicidae) from Undisturbed and disturbed habitats of Great Indian Bustard Wildlife Sanctuary, (M.S.), India. *Interntional Journal of Scientific Research.* 2014; 3(12):398-401.
12. Hebert P.D.N., Cywinska A., Ball S.L., deWaard J.R. Biological identifications through DNA barcodes. The Royal Society of London. 2003; 270:313-321.
13. Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H., Hallwachs W. Ten species in one: DNA Barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*. 2004a; 101:14812-14817.
14. Ward R.D., Zemlak T.S., Innes B.H., Last P.R., Hebert P.D.N. DNA Barcoding Australia's fish species. Philosophical Transactions of the Royal Society of London. *Series B, Biological Sciences*. 2005; 360:1847-1857.
15. Smith D. Biological control of Ceroplastes rubens Maskell, by the introduced parasitoid Anicetus benefices Ishii and Yasumatsu. *Queensland Journal of Agricultural and Animal Sciences*. 1986; 43, 101-105.
16. Kumar S., Stecher G., Li M., Knyaz C., Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution.* 2018; 35:1547-1549.
17. Higgins D., Thompson J., Gibson T., Thompson J.D., Higgins D.G., Gibson T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994; 22:4673-4680.
18. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution.* 1980; 16:111-120.
19. Felsenstein J. Confidence limits on phylogenies:

An approach using the bootstrap. *Evolution*. 1985; 39:783-791.

20. Saitou N., Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* 1987; 4:406-425.
21. Archer M. An alternative to the males of the *Vespa* species (Hymenoptera: Vespidae). Entomologist's Monthly Magazine. 2014; 151: 57-62.
22. Bingham C. T. The Fauna of British India, Including Ceylon and Burma, Hymenoptera, Vol. I, London, UK (1897).
23. Carpenter J.M. and Thi Nguyen L.P. Keys to the genera of social wasps of South-East Asia (Hymnoptera: Vespiae). *Entomological Science*. 2003; 6: 183-192.
24. Das B.P. and Gupta V.K. The social wasps of India and adjacent countries. Oriental Insects Monograph. 1989; 11:1-292.
25. Goulet H., Huber J.T. Hymenoptera of the world: An identification guide to families. *Centre for Land and Biological Resources Research Ottawa, Ontario*. 1993; 1894-1993.
26. Kumar G.P. and Carpenter J.M. A taxonomic review of the genus Antodynerus de Saussure, 1855 (Hymenoptera: Vespidae: Eumeninae) from the Indian subcontinent. *Zootaxa*. 2013; 3731 (2): 267-278.
27. Kumar P.G. and Srinivasan G. Taxonomic studies of hornet wasps (Hymenoptera: Vespidae) *Vespa* Linneaus of India. Records of the Zoological Survey of India. 2010; 110(2): 57-80.
28. Nidup T. and Dorji P. The Honey Bees (Hymenoptera: Apidae) of Bhutan with a key to the *Apis* species. *Bio Bulletin*. 2016; 2(2): 01-07.
29. Saini M.S., Raina R.H., Khan Z.H. A Check list of Bumblebees (Hymenoptera: Apidae) from Indian Himalaya. Journal of Insect Science. 2011; 24:326-52.
30. Williams P. H. Bull. Nat. History Mus. London UK. *Entomol.* 1998; 67: 79.
31. Kim D.W., Yoo W.G., Park H.C., Yoo H.S., Kang D.W., Jin S.D., Min H.K., Paek W.K., Lim J. DNA barcoding of fish, insects, and shellfish in Korea. *Genomics & informatics*.2012; 10(3):206–211.
32. Elizabeth L, Clare Æ, Kevin CR, Kerr Æ, Taika E, von Ko¨nigslo¨w Æ, John J, Wilson Æ, Paul DN, Hebert. Diagnosing Mitochondrial DNA Diversity: Applications of a Sentinel Gene Approach. *J Mol Evol*. 2008; 66:362-367.
33. Brothers D.J. Phylogeny and evolution of wasps, ants and bees (Hymenoptera, Chrysidoidea, Vespoidea and Apoidea). *Zoologica Scripta.* 1999; 28: 233-250.
34. Pilgrim E.M., Von Dohlen C.D., Pitts J.P. Molecular phylogenetics of Vespoidea indicate paraphyly of the superfamily and novel relationships of its component families and subfamilies. *Zoologica Scripta*. 2008; 37:539-560.
35. Namin S.M., and Jury C. Genetic diversity of genus *Vespa* including an invaded species of *V velutina* (Hymenoptera: Vespoidea) in Korea inferred from DNA barcoding data. *Journal of Asia-Pacific Entomology*. 2020; 23: 540-545.
36. Guz N., Kocak E. and Kilner N. Molecular phylogeny of *Trissolcus* species (Hymenoptera: Scelionidae). *Biochemical systematics and ecology.* 2013; 48: 85-91.
37. Tang H., Jiang J., Wu T., Deng J. and Peng L. Six new species of *Zaischnopsis* Ashmead (Hymenoptera: Chalcidoidea: Eupelmidae) from China based on morphological and molecular data. European Journal of Taxonomy. 2022; 847: 1-27.
38. Sohn J., Cornelis V.A., Yu Y. and Kim H. Two species new to South Korea, in the genus *Apronopa* Van Achterberg (Hymenoptera : Braconidae: Alysiine). *Journal of Asia-Pacific Biodiversity.* 2021; 14: 196-199.
39. Venkatesan T., More R.P., Baskar R., Jalali S.K., Lalitha Y. and Ballal C.R. Differentiation of some indigenous and exotic trichogrammatids (Hymenoptera: Trichogrammatidae) from India based on Internal transcribed spacer-2 and cytochrome oxidase -1 markers and their phylogenetic relationship. *Biological Control*. 2016; 101:130-137.
40. Zhou Q-S., Xi Y-Q., Yu F., Zhang X., Li X-J., Liu C-L., Niu Z-Q., Zhu C-D., Qiao G-X.and Zhang Y-Z. Application of DNA barcoding to the identification of Hymenoptera parasitoids from the soybean aphid (*Aphis glycines*) in China. *Insect Science.* 2014; 21: 363-373.
41. Abd-El-Samie E.M., Elkafrawy I., Osama M. and Ageez A. Molecular phylogeny and identification of the Egyptian wasps (Hymenoptera: Vespidae) based on COI mitochondrial gene sequences. *Egyptian Journal of Biological Pest Control*. 2018; 28: 36.
42. Chen H.Y., Li H.L., Pang H., Zhu C.D., and Zhang Y.Z. Investing the parasitoid community associated with the Invasive Mealybug *Phenacoccus solenopsis* in Southern China. *Insects.* 2021; 12:290.
43. Parslow B.A., Schwarz M.P. and Stevens M.I. Molecular diversity and species delimitation in the family Gasteruptiidae. *Genoma*. 2021; 64: 253-264.
44. Benavides A.L.P, Hernandez-Baz F., Gonzalez

J.M., Romero-Napoles J., Hanson P.E. and Zaldivar-Riveron A. Integrative taxonomy to assess the species richness of Chalcidoid parasitoids (Hymenoptera) associated to Bruchinae (Coleoptera: Chrysomdidae) from Mexico. Revista Mexicana de Biodiversidad. 2020; 91:e913492.

45. Siddiqui J.A., Chen Z., Li Q., Deng J., Lin X. and Hung X. DNA barcoding of aphid-associated ants (Hymenoptera, Formicidae) in a subtropical area of southern China. *Zookeys*. 2019; 879:117-136.
46. Kwon Y.J., Lee M.L., Suh S.J. Phylogenetic relationship of *Osmia* species in Korea Based on mitochondrial COI gene sequences (Hymenoptera: Megachilidae). *Journal of Asia-Pacific Entomology*. 2003; 6(1): 7-14.
47. Dowton M. and Austin A.D. Simultaneous analysis of 16S, 28S, COI and morphology in the Hymenoptera: Apocrita- evolutionary transitions among parasitic wasps. *Biological Journal of the Linnean Society.* 2008; 74(1):87-111.
48. Aman-Zuki A., Mohammed M.A., Md-Zain B.M. and Yaakop S. Phylogenetic relationships of five oriental *Apanteles* species groups (Hymenoptera: Braconidae: Microgastrine) by concatenating four molecular markers. *Journal of Asia-Pacific Entomology*. 2019; 22:341-352.
49. Niehuis O. and Waagele J.W. Phylogenetic analysis of the mitochondrial genes LSU rRNA and COI suggest early adaptive differentiation of anal teeth in Chrysidine cuckoo wasps (Hymenoptera: chrysididae). *Molecular phylogenetics and Evolution.* 2004; 30(3): 615-622.
50. Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R. DNA primers for amplification of mitochondrial Cytochrome Oxidase subunit I from diverse metazoan invertebrates. *Molecular Biology.* 1994; 3:294-299.