https://doi.org/10.33307/entomon.v47i1.689
Entomon 47(1): 61-70 (2022)
Article No. ent. 47107

Phylogeny of Indian Himalayan population of *Bombus haemorrhoidalis* Smith 1852 (Hymenoptera: Apidae) inferred from mitochondrial DNA sequences

Sunaullah Bhat[1], A. R. N. S. Subbanna[2], Amit Paschapur[2*], J. Stanley[2], Sandeep Kumar[1] and Jai Prakash Gupta[2]

[1]Department of Zoology, Kumaun University- SSJ Campus, Almora, Uttarakhand 263601, India.
[2]ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora, Uttarakhand 263601, India.
Email: amitp3929@gmail.com; bhatsunaullahijt@gmail.com; Avupati.Subbanna@icar.gov.in; mjstanley1980@gmail.com; sandeepento@gmail.com; guptajp80@gmail.com

ABSTRACT: Molecular variation and phylogenetic relationship of *Bombus haemorrhoidalis* Smith 1852 was studied using partial cytochrome oxidase I (COI) and cytochrome *b* (cyt *b*) sequences of mitochondrial genome. The COI and cyt *b* sequences were compared with available sequences of *B. haemorrhoidalis* and other *Bombus* species belonging to different subgenera to avail divergence within and between species. The BLASTn analysis of obtained COI sequence had cent percent identity to a hymenopteran species BOLD deposit AAC6447 (MAHYM005-10.COI-5P) which is a *Bombus* species from Pakistan. Both these species formed a separate cluster amongst the tested *B. haemorrhoidalis* species in phylogeny with pair wise genetic distance of 0.003. Moreover, the minimum evolution tree between the species revealed *B. haemorrhoidalis* is phylogenetically close with *B. funerarius* and the pair wise genetic distance between these two species was 0.102. Interestingly, *B. haemorrhoidalis* and *B. funerarius* formed separate minor cluster amongst the *Bombus* species tested for phylogeny. Additionally, both COI and cyt *b* genes were A+T biased and showed single nucleotide polymorphisms between and within species. The phylogenetic relationship of COI sequences also revealed single species status of *B. haemorrhoidalis* in the Indian Himalayan region and was evolutionarily associated with *B. funerarius* which is an another species in subgenera, Orientalibombus. The phylogeny of cyt *b* sequences showed that the *B. haemorrhoidalis* is evolutionarily close to pennsylvanicus group species. The study illustrates a complex genetic variation coupled with highly structured evolutionary divergences between and within species and provides the first report of cyt *b* sequence of *B. haemorrhoidalis*.

KEYWORDS: *Bombus* species, phylogenetic analysis, genetic variation, evolutionary divergence, COI sequences

INTRODUCTION

The members of family Apidae (Hymenoptera) are a highly divergent group of social, eusocial and semi social insects including bumble bees, euglossines (orcid bees), honeybees, stingless bees (*Melipona*) contributing to majority of worlds pollination services. Bumble bees are considered as versatile pollinators due to their more working hours and buzz pollination of crops (Chauhan *et al.,* 2016). Other

* Author for correspondence

important attributes like short flight range, cold hardiness and high elevation adaptation make them important pollinators of wild and agricultural flowering plants and crops with both ecological and economic importance (Streinzer *et al.,* 2019). In natural plant–pollinator interaction, bumble bees are often considered as keystone species due to generalist pollination services, thereby assisting plant community diversity by visiting both rare and abundant plant species (Memmott *et al.,* 2004; Goulson *et al.,* 2008; Burkle *et al.,* 2013; Cusser and Goodell, 2013). In some instances, bumble bees are the only efficient pollinators and the crop productivity is completely dependent on the ecological services provided by these insects (Sinu *et al.,* 2011; Streinzer *et al.,* 2019).

Bumble bees are highly conspicuous and abundant in cold and temperate regions. Due to their important ecosystem services, the group has become an interesting target for early naturalists and entomologists. This has led to identification of around 260 species (Williams, 1998; updated online at http://www.nhm.ac.uk/researchcuration/ research/ projects/bombus/ index.html). The current global efforts on bumble bee taxonomy are targeted towards the species distribution and subgeneric variations (Streinzer *et al.,* 2019). Decline in the population of bumble bees (Cameron *et al.*, 2011; Bartomeus *et al.,* 2013) might be due to climate change, heat stress, spill-over of pathogens, changes in agricultural practices and deforestation (Hoiss *et al.,* 2012; Kerr *et al.,* 2015; Rasmont *et al.,* 2015; Jacobson *et al.,* 2018).

Among the different agroecological regions of India, the north western Himalayan region has its own diverse fauna of bumble bees. In the mid hills of Uttarakhand Himalayas, *B. haemorrhoidalis* is one of the key bumble bee species visiting many of the cultivated crop plants and wild plantations. Moreover, this is the only species of pollinator found during low temperature regimes in the region due to their thermoregulatory ability (Corbet *et al.,* 1993) and actively visits the flowering plants throughout the day.

Amongst different markers, partial sequences of mitochondrial genes especially cytochrome oxidase I (cox 1) and cytochrome *b* (cyt *b*) are well suited to resolve the phylogenetic issues of a wide range of hierarchical levels in insects (Simmons and Weller, 2001; Bertsch *et al.,* 2010). High mutation rate, maternal inherence and evolutionary memory made these two genes particularly important in any phylogeny related studies (Simmons and Weller, 2001). Besides, DNA sequence based approaches are independent of insect developmental stages but within species variation is the only ambiguous issue (Ahrens *et al.,* 2007) which can also be well documented using these mitochondrial markers. In the present study, both cox 1 and cyt *b* genes from the population of *B. haemorrhoidalis* native to Uttarakhand Himalayas, India were amplified. The sequence variation in these two genes was used to establish the genetic divergence and phylogenetic relationship with other *Bombus* species.

MATERIALS AND METHODS

Test insects: Specimens of *B. haemorrhoidalis* for a phylogenetic study were collected during March to December 2020 from toad flax (*Linaria vulgaris*) and ornamental poppy (*Papaver orientale*) in the premises of agricultural fields of the Vivekananda Hill Agriculture Institute, farm of ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora located at 29°372 N, 79°402 E with an altitude of 1310 msl in Uttarakhand state of North Western (NW) Himalayan Region, India

DNA extraction: Henry *et al.* (1990) method was followed for the extraction of genomic DNA. The legs of the target specimens were ground in the liquid nitrogen in micro-centrifuge tube with the help of blunted tip of the pipette. Sample obtained were washed with TENT buffer (10 mM Tris- Cl (pH 7.4), 5 mM EDTA, 10 mMNaCl, 0.5 per cent Triton X-100). Sample were centrifuged for 10 min at 10000 rpm, pellet was suspended in TEN buffer (TENT without Triton X-100) comprising 1 per cent sodium dodecyl sulphate and 1 mg/ml of proteinase K. After incubation at 37 ° C for 4 h, 1/10 th volume of 5 M NaCl were added and thoroughly mixed. The genomic DNA was extracted twice from phenol/chloroform, isopropanol was used to precipitate DNA and obtained DNA was suspended in 100 µl of TE buffer. RNA residues were removed

by incubation with DNase-free RNase A for 1 h at 37°C. To visualize intact genomic DNA 0.8 per cent agarose gel was used and the genomic DNA was diluted to get working solution of 20–25 ng/µl.

PCR protocol: PCR reaction mix was as follows: 50 ng of DNA template, 200nM of dNTPs, 1mM of each primer (Table 1), 2.5 units of Taq DNA polymerase and 5µl of PCR reaction buffer to make a final volume of 50 µl. Entire reactions were performed in the thermal cycler (Biorad) with an initial 5 min denaturation step at 92°C, accompanied by 35 amplification cycles consisting of 1 min denaturation at 92°C, 45 s annealing at 48°C and 2 min extension at 72°C with an additional final 10 min extension step at 72°C. The amplification was visualized and confirmed in the gel documentation system (Alpha Image Analyzer, Alpha Innotech Corporation) by 1 per cent agarose-EtBr gel electrophoresis of 10µl PCR product. Negative PCR controls were carried out to reduce cross-contamination.

Sequencing and data analysis: The amplified products of the particular gene were purified through gel elution columns (Sigma), sequenced directly on an automated DNA sequencer (ABI 377) with the help of the Big Dye terminator kit (Applied Biosystems) as per manufacturer's guidelines. Nucleotide sequences were aligned with the Clustal Omega (1.2.1) multiple sequence alignment (McWilliam *et al.,* 2013) and phylogenetic and molecular evolutionary analyses were done utilizing the software MEGAX (Molecular Evolutionary Genetic Analysis version 4) (Kumar *et al.,* 2018). The phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) utilizing the distance matrix from the alignment. The nucleotide sequences were also converted into amino acid sequences with the help of invertebrate mitochondrial genetic code (Gasteiger *et al.,* 2003) and were aligned using Clustal omega software (McWilliam *et al.,* 2013). NCBI accession numbers of other sequences COI and cyt b of *B. haemorrhoidalis*, *Bombus* species were presented along with results. The obtained sequences of COI and cyt b were submitted to NCBI Gene Bank nucleotide sequence databases (accession number ON073847).

RESULTS AND DISCUSSION

The BLASTn analysis of obtained COI sequence had cent percent identity to a hymenopteran species BOLD deposit AAC6447 (MAHYM005-10.COI-5P) which is a *Bombus* species from Pakistan. Both these species formed a separate cluster amongst the tested *B. haemorrhoidalis* species for phylogeny (Fig. 1) with Pair wise genetic distance of 0.003 (Table 2). As the entire mitochondrial DNA sequence was used for the construction of phylogeny, the Cox I and cyt b region proves to be highly conserved region, through which molecular characterization of insects can be taken up with higher degrees of specificity. When the phylogenetic tree was constructed with the MEGA X 10.0.5 software (Fig. 1), it was observed that the *B. haemorrhoidalis* species native to Indian Himalayas formed separate group with hymenopteran species BOLD deposit AAC6447 (MAHYM005-10.COI-5P) in the maximum likelihood evolution tree. The node support estimated using 1000 bootstrap pseudo-replicates, showed that these species evolved together as they showed 99% node value.

However, the intra-species diversity (n=10) in 683 bp nucleotide sequence region of Cox1 was manifested in the form of 11 single nucleotide polymorphism (SNPs) by utilizing CLUSTAL Omega (1.2.4) multiple sequence alignment software (Supplementary Fig. 1 and 2). The mean number nucleotide frequency amongst the *B. haemorrhoidalis* was also examined and it was observed that the COI sequences were usually A+T biased with the concentration of A+T exceeding 76.95%, while the concentration of G+C was well below 23.05% in which the concentration of thymine was highest (42.15%). The average nucleotide frequencies in *B. haemorrhoidalis* are 34.80 (A), 42.15 (T), 12.83 (C), and 10.23 per cent (G). Besides, the transition/transversion rate ratios are k_1 = 6.526 (purines) and k_2 = 2.026 (pyrimidines). The overall transition/transversion bias is R = 1.381, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$. MCL estimate of nucleotide substitutions also showed maximum base substitution as A to G and vice versa with a value of 37.52

Table 1. Primers used in the study

Gene	Primer	Sequence (5'-3')	Reference
Cytb	cb1	5^2 -TATGTACTACCATGAGGACAAATATC-3^2	Schwarz *et al.* (2003)
	cb2	5^2 -ATTACACCTCCTAATTTATTAGGAAT-3^2	
COI	C1-J-2195	TTGATTTTTTGGTCATCCAGAAGT	Simon *et al.* (1994)
	AAMT3038R	TCCATTGCACTAATCTGCCATATTAG	Muraji *et al.* (2008)

Table 2. Pair wise genetic distance for partial COI gene sequences of *B. haemorrhoidalis* isolates with *B. funerarius*

Species	1	2	3	4	5	6	7	8	9	10
1. *B. haemorrhoidalis* Almora										
2. JF865997 Hymenoptera sp. BOLD:AAC6447	0.003									
3. MF582625 *B. haemorrhoidalis* DPK-B02	0.014	0.011								
4. MF582610 *B. haemorrhoidalis* DT-B01	0.014	0.011	0.000							
5. MF582604 *B. haemorrhoidalis* DAK-B12	0.014	0.011	0.000	0.000						
6. MF582600 *B. haemorrhoidalis* DMNg-B11	0.014	0.011	0.000	0.000	0.000					
7. MF582607 *B. haemorrhoidalis* DAK-B10	0.015	0.012	0.002	0.002	0.002	0.002				
8. MF582592 *B. haemorrhoidalis* DS1-B41	0.015	0.012	0.002	0.002	0.002	0.002	0.003			
9. KT334307 *B. haemorrhoidalis* voucher 4746F01	0.015	0.012	0.002	0.002	0.002	0.002	0.000	0.003		
10. MF582621 *B. haemorrhoidalis* DI2-B03	0.017	0.014	0.003	0.003	0.003	0.003	0.005	0.002	0.005	
11. MT906010 *B. funerarius*	0.102	0.097	0.094	0.094	0.094	0.094	0.096	0.096	0.096	0.097

(Table 3). The minimum evolution tree between the species revealed *B. haemorrhoidalis* is phylogenetically close with *B. funerarius*. The pair wise genetic distance between these two species was 0.102 (Table 2). Interestingly, *B. haemorrhoidalis* and *B. funerarius* formed separate minor cluster amongst the *Bombus* species tested for phylogeny.

The sequenced 449 bp region of cyt *b* of *B. haemorrhoidalis* spans between 436 to 884 bp region of total cyt *b* sequence (1151 bp) of *A. mellifera*. The NCBI blast search of the

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution in COI sequences of ten (as given in phylogeny tree) *B. haemorrhoidalis* species

	A	T	C	G
A	-	6.96	2.12	**11.03**
T	5.75	-	**4.29**	1.69
C	5.75	**14.11**	-	1.69
G	37.52	6.96	2.12	-

Rates of transitional substitutions were shown in bold and those of transversional substitutions in italics

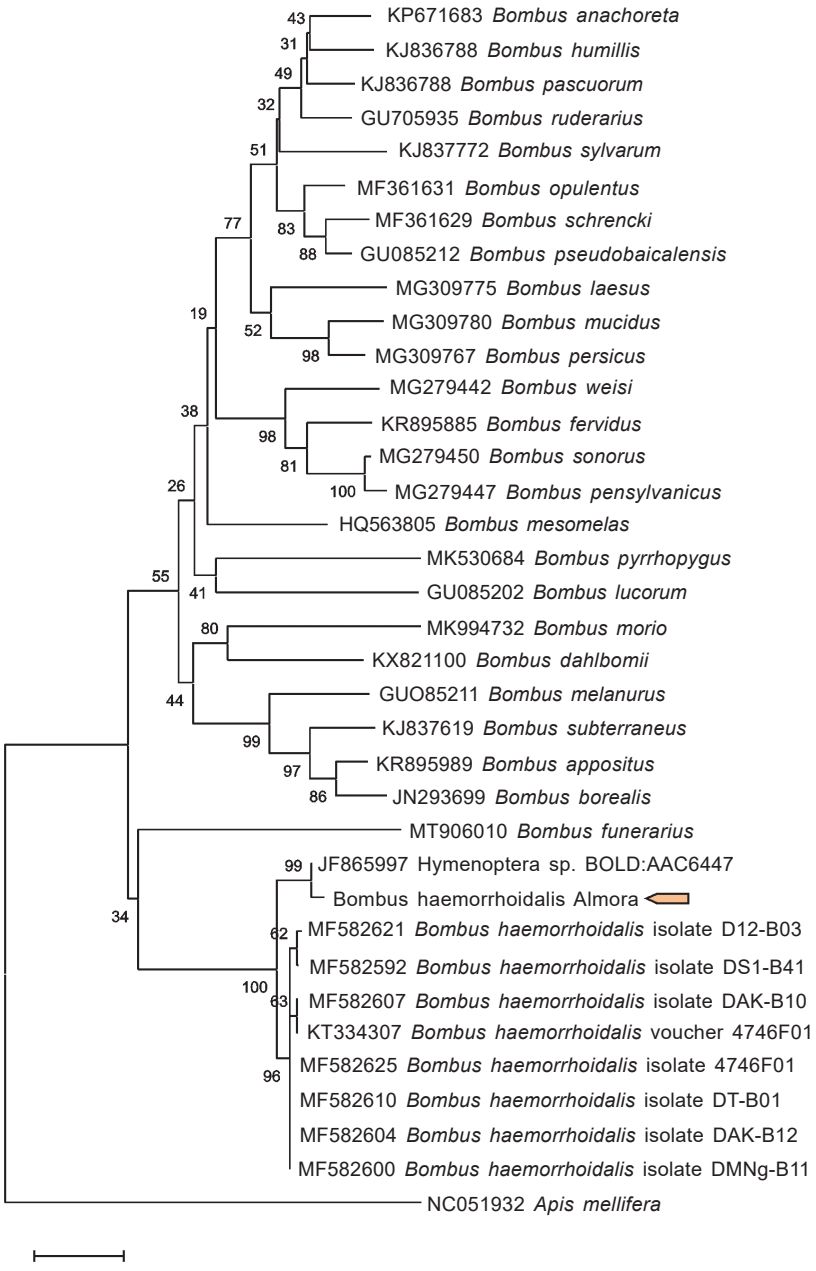

Fig. 1 ME tree with bootstrap support showing clustering of different species of *Bombus* (starts with accession numbers) constructed using partial COI sequences

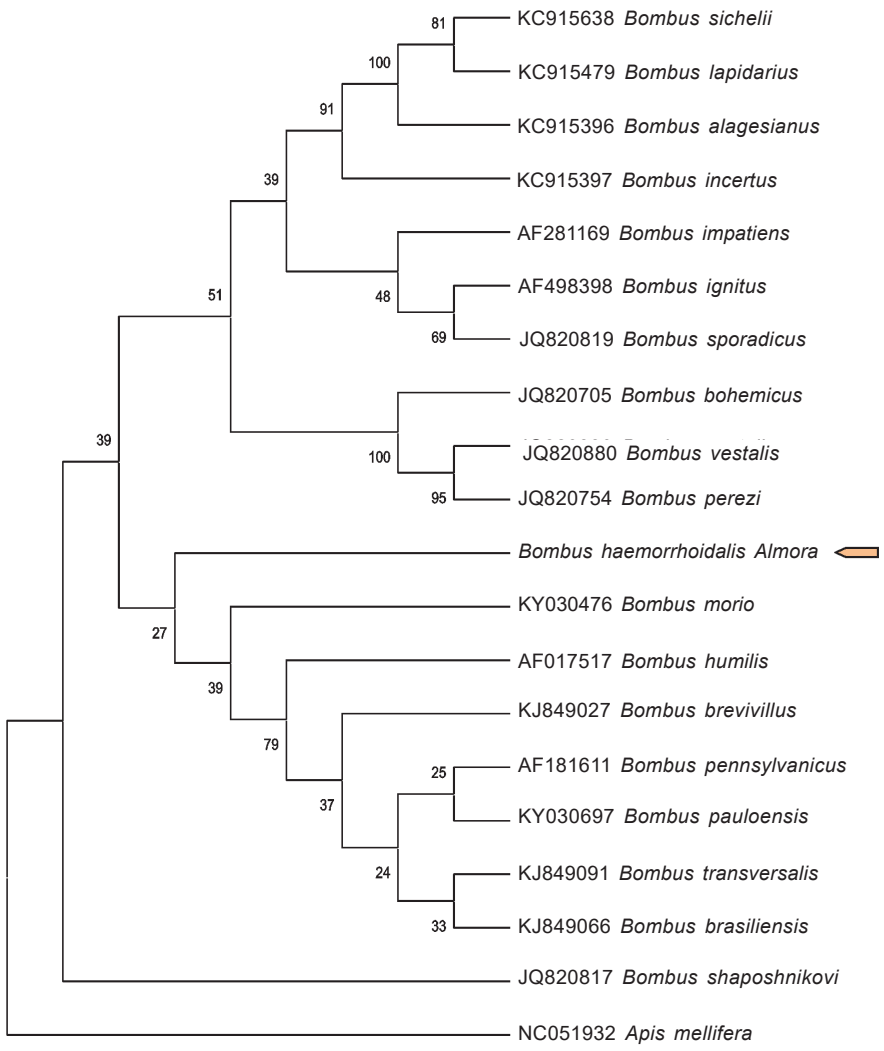

Fig. 2.ME tree with bootstrap support showing clustering of different species of *Bombus* (starts with accession numbers) constructed using partial cyt *b* sequences

sequence revealed no submissions of cyt *b* sequence for *B. haemorrhoidalis* so the study submits the first ever sequence of the species to NCBI (Accession No. ON073847). However, other *Bombus* species cyt *b* sequences are available for the sequenced region and were used in phylogeny construction and further analysis. The BLASTn search of the cyt *b* sequence revealed a proximal cyt *b*-like sequence of *B. fragrans* (88.99 percent identity) and other *Bombus* species. However, these sequences have double and triple nucleotide insertions and so are not included in phylogeny. Minimum evolution phylogeny tree constructed using 19 cyt *b* sequences of *Bombus* species clearly differentiated two sub groups where *B. haemorrhoidalis* was in evolutionary association with other seven species (Fig. 2). Interestingly, *B. shaposhnikovi* formed a separate clade. Pairwise genetic distance analysis of these eight species (including *B. haemorrhoidalis*) showed *B. haemorrhoidalis* is closely related with *B. pennsylvanicus* with 0.121 (Table 4). The mean number nucleotide frequency amongst the *B. haemorrhoidalis* was also examined and it was observed that the cyt *b* sequences were usually A+T biased with the concentration of A+T

Table 4. Pair wise genetic distance for partial cyt *b* gene sequences of *Bombus* species

S. No &Species	1	2	3	4	5	6	7	8	9
1: *B. haemorrhoidalis* Almora									
2: AF181611 *B. pennsylvanicus*	0.121								
3: KJ849091 *B. transversalis*	0.130	0.050							
4: KJ848955 *B. pauloensis*	0.139	0.050	0.057						
5: KJ849066 *B. brasiliensis*	0.142	0.047	0.052	0.062					
6: KJ849027 *B. brevivillus*	0.148	0.053	0.073	0.078	0.065				
7: JQ820817 *B. shaposhnikovi*	0.157	0.110	0.117	0.135	0.132	0.123			
8: AF017517 *B. humilis*	0.143	0.066	0.079	0.094	0.088	0.091	0.138		
9: KY030476 *B. morio*	0.172	0.118	0.114	0.121	0.130	0.127	0.132	0.139	

The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Tajima-Nei model

Table 5. Maximum composite likelihood estimate of the pattern of nucleotide substitution in cyt *b* sequences of *Bombus* species

	A	T	C	G
A	-	10.93	3.1	**2.85**
T	8.91	-	**7.4**	1.78
C	8.91	**26.06**	-	1.78
G	**14.25**	10.93	3.1	-

Rates of transitional substitutions were shown in bold and those of transversional substitutions in italics

exceeding 80.24 per cent, while the concentration of G+C was well below 19.76 per cent in which the concentration of thymine/uracil was highest (44.19%). MCL estimate nucleotide substitutions of these eight sequences showed nucleotide frequencies of 36.05 per cent (A), 44.19 per cent (T/U), 12.54 per cent (C), and 7.21 per cent (G), respectively. Amongst the species maximum base substitution was observed between C to T and vice versa with 26.06 (Table 5).

The genus *Bombus* comprises 250 known species under 38 subgenera with distribution all over the world (Cameron *et al.,* 2007) which was reviewed and reduced to 15 subgenera by Williams *et al.* (2008) (https://www.nhm.ac.uk/researchcuration/research/projects/bombus/ groups.html). Besides, they are highly vulnerable species to climate change and continents like Europe, North America, and Asia are already experiencing decline in bumble bee population (Williams and Osborne 2009). India is home for around 48 species of bumble bees out of which 37 species are recorded from North West Himalayas (Saini *et al.,* 2012) and 21 species from Arunachal Pradesh (Streinzer *et al.,* 2019). The distribution of these species spanning from an altitude of 230-2990 m. Keeping in view of the ecological and economic importance of the genus *Bombus,* both biological and molecular studies are important for their conservation and successful utilization as pollinators.

In the study area, the Uttarakhand Himalayas, *B. haemorrhoidalis* is distributed bumble bee found all over the flowering crop plants. The species belongs to subgenus Orientalibombus Richards which also contains *B. braccatus* and *B. funerarius* (https://www.nhm.ac.uk/research curation/research/projects/bombus/ or.html#haemorrhoidals). The COI based phylogenetic tree clearly differentiated both *B. haemorrhoidalis* and *B. funerarius* into a separate clade. This is in agreement with the phylogeny study by Cameron *et al.* (2007) and also confirms the COI based identity of *B. haemorrhoidalis* from Uttarakhand Himalayas. The high bootstrap values also support the single

species status of the populations (Subbanna *et al.,* 2016). The number of base substitutions per site between these two species is around 0.1. Whereas for the third species under the subgenus, *B. braccatus* no COI sequence is available in NCBI data base. The phylogeny with in the species also showed significant variation with pairwise genetic variation ranging from 0.14-0.17 which are the geographic isolates from Thailand. The geographic isolate from Pakistan showed pairwise genetic variation of only 0.03. This type of geography dependent intraspecies variations (Streinzer *et al.,* 2019) and body color phenotypes are common in *Bombus* species (Hines and Williams 2012; Koch *et al.,* 2018). Streinzer *et al.* (2019) also reported *Bombus* species are one of the suitable taxa to study potential adaptations to specific climatic conditions at the individual and as well as population level. *De novo* sequencing of genomes of 17 species, representing all 15 subgenera of *Bombus* revealed dynamically evolving gene families in response to positive selection points linked to foraging, diet, detoxification, immunity to invading pathogens and adaptations to high altitudes (Sun *et al.,* 2021). Present study also shows an evidence of ecological and behavioral traits driven intraspecies variation in COI gene of *B. haemorrhoidalis*.

The high rates of nucleotide substitutions in mitochondrial genes helped molecular entomologists to estimate phylogenetic relationships among different species and populations of single species (e.g. Hebert *et al.*, 2003; Pons *et al.*, 2004; Havill *et al.*, 2007). Clustal comparison of cyt *b* sequences between the species showed variation as SNPs and unequal nucleotide frequencies. In general, all the sequences of the species are rich in AT region. Due to unavailability of cyt *b* region of different species in NCBI data, a phylogeny was prepared using available ones. The cyt *b* derived phylogenetic tree showed evolutionary association of *B. haemorrhoidalis* (belongs to Orientalibombus group) species with Morio (*B. morio*), Muscorum (*B. humilis*) and Pennsylvanicus group (*B. pennsylvanicus*, *B. pauloensis*, *B. transversalis* and *B. brasiliensis*) of *Bombus* species. The pairwise genetic distance using Tajima-Nei model also showed *B. haemorrhoidalis* was close to *pennsylvanicus* group species with an average value of 0.136. However, this grouping can't be considered as appropriate due to the use of limited available. Studies also reported that mitochondrial cyt *b* sequences also provide an accurate, rapid, and economic technique for separation of various insect pests in different orders (Jermiin and Crozier, 1994; Simmons and Weller, 2001) and found to have the same level of sequence variation and AT bias as COI (Simmons and Weller, 2001).

The phylogenetic analysis reported in the study is first of its kind with respect to *B. haemorrhoidalis* and will serve as stimulus for the *Bombus* community to initiate a more thorough evolutionary study to fill the currently existing gaps on conservation and threats of Indian bumble bees.

ACKNOWLEDGMENTS

Authors are thankful to Director, ICAR-VPKAS, Almora, Uttarakhand, India and Indian Council of Agricultural Research, New Delhi for providing facilities. The study is a part of Ph.D thesis submitted to Kumaun University, Almora, Uttarakhand, India and financially supported by DST-SERB project.

REFERENCES

Ahrens D., Monaghan M.T. and Vogler A.P. (2007) DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). Molecular Phylogenetics and evolution 44(1): 436-449.

Bartomeus I., Ascher J.S. and Gibbs J. (2013) Historical changes in northeastern US bee pollinators related to shared ecological traits. Proceedings of the National Academy of Sciences, USA 110: 4656–4660.

Bertsch A., de Angelis M.H. and Przemeck G.K. (2010) Phylogenetic relationships of the bumblebees *Bombus moderatus*, *B. albocinctus*, *B. burjaeticus*, *B. florilegus* and *B. cryptarum* based on mitochondrial DNA markers: a complex of closely related taxa with circumpolar distribution. (Hymenoptera: Apidae: *Bombus*). Beiträge zur Entomologie, Contributions to Entomology 60(1): 13-32.

Burkle L.A., Marlin J.C. and Knight T.M. (2013) Plant-pollinator interactions over 120 years: loss of species, co-occurrence, and function. Science, 339: 1611–1615.

Cameron S.A., Hines H.M. and Williams P.H. (2007) A comprehensive phylogeny of the bumble bees (*Bombus*). Biological Journal of the Linnean Society 91: 161–188.

Cameron S.A., Lozier J.D. and Strange J.P. (2011) Patterns of widespread decline in North American bumble bees. Proceedings of the National Academy of Sciences, USA 108: 662–667.

Chauhan A., Rana B.S., Katna S. and Rana K. (2016) Morphometric studies on bumble bee, *Bombus haemorrhoidalis* Smith. International Journal of Farm Sciences 6(1): 74-78.

Corbet S.A., Fussell M., Ake R., Fraser A., Gunson C., Savage A. and Smith K. (1993) Temperature and the pollinating activity of social bees. Ecological Entomology 18(1): 17-30.

Cusser S. and Goodell K. (2013) Diversity and distribution of floral resources influence the restoration of plant-pollinator networks on a reclaimed strip mine. Restoration Ecology 21: 713– 721.

Gasteiger E., Gattiker A., Hoogland C., Ivanyi I., Appel R.D. and Bairoch A. (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Research, 31: 3784-3788.

Goulson D., Lye G.C. and Darvill B. (2008) Decline and conservation of bumble bees. Annual Review of Entomology 53: 191–208.

Havill N.P., Foottit R.G. and Von Dohlen C.D. (2007) Evolution of host specialization in the Adelgidae (Insecta: Hemiptera) inferred from molecular phylogenetics. Molecular and Phylogenetic Evolution 44: 357-370.

Hebert P.D.N., Cywinska A., Ball S.L. and deWaard J.R. (2003) Biological identifications through DNA barcodes. Proceedings of Royal Society B: Biological Sciences 270: 313–321.

Henry J.M., Raina A.K. and Ridgway R.L. (1990) Isolation of high-molecular-weight DNA from insects. Analytical Biochemistry 185: 147-150.

Hines H.M. Williams P.H. (2012) Mimetic colour pattern evolution in the highly polymorphic *Bombus trifasciatus* (Hymenoptera: Apidae) species complex and its comimics. Zoological Journal of the Linnean Society 166(4): 805-826.

Hoiss B., Krauss J., Potts S G., Roberts S. and Steffan-Dewenter I. (2012) Altitude acts as an environmental filter on phylogenetic composition, traits and diversity in bee communities. Proceedings of Royal Society B: Biological Sciences 279(1746): 4447-4456.

Jacobson M.M., Tucker E.M., Mathiasson M.E. and Rehan S.M. (2018) Decline of bumble bees in northeastern North America, with special focus on *Bombus terricola*. Biological Conservation 217: 437-445.

Jermiin L.S. and Crozier R.H. (1994) The cytochrome *b* region in the mitochondrial DNA of the ant, *Tetraponera rufoniger:* Sequence divergence in hymenoptera may be associated with nucleotide content. Journal of Molecular Evolution 38: 282-294.

Kerr J.T., Pindar A., Galpern P., Packer L., Potts S.G., Roberts S.M. and Pantoja A. (2015) Climate change impacts on bumblebees converge across continents. Science 349(6244): 177-180.

Koch J.B., Rodriguez J., Pitts J.P. and Strange J.P. (2018) Phylogeny and population genetic analyses reveals cryptic speciation in the *Bombus fervidus* species complex (Hymenoptera: Apidae). PloS one 13(11): e0207080.

Kranthi S., Kranthi K.R., Bharose A.A., Syed S.N., Dhawad C.S., Wadaskar R.M., Behere G.T. and Patil E.K. (2006) Cytochrome oxidase I sequence of *Helicoverpa* (Noctuidae: Lepidoptera) species in India – Its utility as a molecular tool. Indian Journal Biotechnology 5: 195-199.

Kumar S., Stecher G., Li M., Knyaz C. and Tamura K. (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35(6): 1547-1549.

McWilliam H., Li W., Uludag M., Squizzato S., Park Y.M., Buso N., Cowley A.P. and Lopez R. (2013) Analysis Tool Web Services from the EMBL-EBI. Nucleic acids research 41: W597-600.

Memmott J., Waser N.M. and Price M.V. (2004) Tolerance of pollination networks to species extinctions. Proceedings of the Royal Society B: Biological Sciences 271: 2605–2611.

Muraji M., Hirai Y., Akion T., Wakamura S. and Arakaki N. (2008) Genetic divergence among populations of the white grub beetles, *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae), distributed in the southern part of the Ryukyu island of Japan, detected from mitochondrial DNA sequences. Applied Entomology and Zoology 43: 287-292.

Pons J., Barraclough T., Theodorides K., Cardoso A. and Vogler A (2004) Using exon and intron sequences of the gene Mp20 to resolve basal relationships in Cicindela (Coleoptera: Cicindelidae). Systematic Biology 53: 554-570.

Rasmont P., Franzén M., Lecocq T., Harpke A., Roberts S.P., Biesmeijer J.C. and Schweiger O. (2015) Climatic risk and distribution atlas of European bumblebees. Vol. 10. Pensoft Publishers. pp 1-236.

Simmons R.B. and Weller S.J. (2001) Utility and evolution of Cytochrome *b* in insects. Molecular Phylogenetics and Evolution 20: 196-210.

Sinu P.A., Kuriakose G. and Shivanna KR. (2011). Is the bumblebee (*Bombus haemorrhoidalis*) the only pollinator of large cardamom in central Himalayas, India? Apidologie 42(6): 690-695.

Streinzer M., Chakravorty J., Neumayer J., Megu K., Narah J., Schmitt T., Bharti H., Spaethe J. and Brockmann A. (2019) Species composition and elevational distribution of bumble bees (Hymenoptera, Apidae, *Bombus* Latreille) in the East Himalaya, Arunachal Pradesh, India. ZooKeys 851: 71–89.

Subbanna A.R.N.S., Stanley J., Jain S.K., Bhatt J.C. and Bisht J.K. (2016) Phylogeny and Genetic Divergence of Indian Himalayan Population of *Anomala dimidiata* (Coleoptera: Scarabaeidae) Inferred from Mitochondrial DNA Sequences. Agriculture research 5(1): 64-71.

Sun C., Huang J., Wang Y., Zhao X., Su L., Thomas G.W. and Mueller R.L. (2021) Genus-wide characterization of bumblebee genomes provides insights into their evolution and variation in ecological and behavioral traits. Molecular Biology and Evolution 38(2): 486-501.

Tamura K., Dudley J., Nei M. and Kumar S. (2007) MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

Williams P.H. and Osborne J.L. (2009) Bumblebee vulnerability and conservation world-wide. Apidologie 40(3): 367-387.

Williams P.H., Cameron S.A., Hines H.M., Cederberg B. and Rasmont P. (2008) A simplified subgeneric classification of the bumblebees (genus Bombus). Apidologie 39(1): 46-74.

(Received December 04, 2021; revised ms accepted March 28, 2022; printed March 31, 2022)