Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 24, No. (2) : 2022 : 296-304

ISSN-0972-3005
DOI No.: http://doi.org/10.53550/AJMBES.2022.v24i02.014

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF THE NATIVE SMALL CARPENTER BEES (*CERATINA* SPP.) OF NORTH WESTERN, INDIAN HIMALAYAS

SUNAULLAH BHAT[1], A.R.N.S. SUBBANNA[2], JOHNSON STANLEY[3], AMIT UMESH PASCHAPUR[2]*, J.P. GUPTA[4], SANDEEP KUMAR[5] AND K.K. MISHRA[6]

[1,5] Department of Zoology, Kumaun University- SSJ Campus, Almora 263 601, Uttarakhand, India
[2,3,4,6]Crop Protection Division, ICAR- Vivekananda Parvatiya Krishi Anusandhana Sansthan, Almora 263 601, Uttarakhand, India

(Received 2 January, 2022; Accepted 18 February, 2022)

Key words: *Wild bees, Ceratina species, Molecular characterization, Phylogenetic analysis, Genetic distance, Integrative taxonomy*

Abstract– Bees are a wonderful creatures on earth; contributing to 73% of pollination services provided by all animals. Although the economic value of domesticated bees is calculated through several scientific methods, but the actual value of wild bees in terms of economy, ecology and biodiversity are rarely estimated due to lack of distinct taxonomic keys. Considering the above backdrops, we decided to molecularly characterize three species of *Ceratina* native to Indian Himalayas. Three species were reported as *C. sutepensis*, *C. smaragdula* and *C. similima* based on phylogenetic analysis and comparison with the NCBI database. The COI sequence of *C. similima* was submitted to the NCBI database for the first time. Nucleotide frequency analysis showed that partial COI gene sequences were A+T biased (>75%), while the amino acid frequency analysis showed higher frequency of leucine (16.01%) and serine (11.77%). Multiple sequence alignment of COI sequences of three species illustrated the total variation in 117 SNP's. Moreover, the pairwise genetic distance analysis reported that *C. sutepensis* and *C. smaragdula* were genetically less distant when compared to*C. similima*. Suchkinds of studies are essential to analyze the biodiversity of location, distinguish cryptic species and develop distinct taxonomic tools for integrative taxonomy of bees.

INTRODUCTION

Bees are among the most important organisms on the earth both ecologically and economically due to their ability to pollinate the crops. They contribute up to 73% pollination services provided by animals in total (Michener, 2007; Abrol, 2009; Ollerton *et al.,* 2011). Apart from the ecosystem services provided by the domesticated bees like *Apis mellifera* and *Apis indica* the wild bees also contribute enormously in cross pollination of the entomophilic crops and maintenance of balance in the ecosystem (Slaa *et al.* 2006; Quezada-Euán, 2009; Brown, 2011). The wild bees stand in a clear distinction over domesticated bees for their stingless nature, less harmful to humans and animals and effective pollinators in the polyhouses (Kakutani *et al*., 1993; Heard, 1999; Del Sarto *et al*., 2005), but the major disadvantage in the study of wild bees is the lack of availability of taxonomic keys for their actual identification and lack of distinguishing morphological characters in the alpha taxonomyof bees (Weeks *et al.,* 1999; Gotelli, 2004; Packer *et al.,* 2009).

Recently several identification strategies like developing the database of prototypes and comparing the unknown specimen with the well identified specimen within the database are practiced but, with only probable accuracy (Ratnasingham and Hebert, 2007). The two such systems are Automated Bee Identification (Schröder *et al.,* 2002) and Digital Automated Identification System (DAISY) (Weeks *et al*., 1999), but they are rarely successful because the new specimen whose prototype doesn't exist in the library cannot be identified with cent per cent identity. However, the molecular characterization techniques like, DNA

barcoding with the mitochondrial cytochrome oxidase I genes has found some certainty in identification of the bee biota (Murray *et al.,* 2008; Bertsch 2009; Sheffield *et al*., 2009; Magnacca and Brown, 2010; Magnacca and Brown, 2012). Apart from mere identification, the molecular techniques also provide various other information's like identification of the sympatric and cryptic species within a locality (Packer and Taylor, 1997), association of sexes in dimorphic species classification (Gibbs, 2009; Sheffield *et al*., 2009), characterizing morphologically difficult to identify species (Gibbs, 2009; Rehan and Sheffield, 2011; Williams *et al.,* 2012) and determining the evolutionary relationship and genetic distances among the targeted insect species (Will and Rubinoff, 2004; Kekkonen and Hebert, 2014).

The Indian subcontinent is expected to house a very wide variety of bee fauna with more than 766 species under 71 genera and six families (Saini and Chandra, 2019) but, the actual number may be much higher. Even in the Indian Himalayas, the wild bees contribute to most of the pollination services over the domesticated bees, but they are the least studied because of non-availability of distinct taxonomic tools (Weeks *et al*., 1999). Nevertheless, the recent surveys and molecular characterization of Himalayan bee fauna carried out by Pakrashi *et al.* (2020) showed the huge diversity of bees in the Indian Himalayas. Out of the 156 bee species collected through extensive surveys, the bees belonging to the four major families Apidae (40 species), Halticidae (five species), Megachilidae (nine species), and Melittidae (one species) were reported to be widely distributed throughout the Himalayas. Moreover, Saini *et al.* (2020) reported a new species (*Melitta* sp.) belonging to family melittidae for the first time from Indian Himalayas, indicating the potential for study of wild bees in this undisturbed ecosystem.

Keeping in mind the above developments and opportunities, we decided to molecularly characterize the three species of small carpenter bees belonging to genus *Ceratina*, and we could surprisingly find them as entirely different species; with *C. similima* being reported from the Indian Himalayas for the first and its partial mitochondrial COI sequence submitted to the NCBI database for the first time. Moreover the comparison of genetic diversity between these three species showed very huge variation. This study could help the taxonomists in future to develop distinct taxonomic keys for identification of three *Ceratina* species and provide the suitable database to study bee diversity of small carpenter bees in the North Western Himalayan region.

MATERIALS AND METHODS

Test insects

The specimens of three *Ceratina* species (*Ceratinasutepensis, C. similima and C. smargdula*) were collected from the bee hotels (Plate 1-4) established at the Experimental Farm, Hawalbagh of ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora situated at 29°372 N and 79°402E with an altitude of 1310 m in Uttarakhand state of North Western (NW) Himalayan Region, India. The *Ceratina* species were allowed to harbor in the twigs of mulberry (*Morusalba*) during peak flowering periods of May and June, 2020 and one adult bee of each species was collected manually with a test tube for molecular characterization and the second specimen of respective species was dry mounted as a voucher specimen.

DNA extraction

Plate 1-4. Different species of small carpenter bees and bee hotels established

P1. *Ceratina smaragdula* P2.*C. sutepensis* P3.*C. similima* P4.Bee hotels

The standard modified CTAB method described by Subbanna *et al.* (2019) was followed for extraction of genomic DNA from the insect specimens. The three pairs of legs were ground in liquid nitrogen by autoclaved pestle and mortar and the crushed material was transferred to a micro-centrifuge tube. 600 µl of CTAB solution (20 parts of 1M Tris-HCl, 8 parts of 0.5 M EDTA, 56 parts of 5M NaCl and 4 parts of CTAB and the final volume made to 1000ml and the pH adjusted to 8. Added 0.4 parts of ß-mercaptaethanol after autoclaving the contents) along with 1 mg/ml of proteinase K was transferred to the tube and incubated in a water bath at 57 ^{0}C for 3 hours. The contents were vertexed manually after every 20 minutes for thorough degradation of the tissues. The degraded material was treated twice with phenol-chloroform-isoamyl solution (25:24:1) to extract the genomic DNA and chilled isopropyl alcohol was used to precipitate the DNA at -20 ^{0}C for 30 minutes. The DNA pellet obtained after centrifugation at 10000rpm for 7 minutes was washed with 70% chilled ethyl alcoholto remove the excess salts and was suspended in 40 µl of TE buffer. DNase-free RNase A treatment was followed for 1 h at 37^{o}C to remove the RNA residues. Electrophoresis with 0.8% agarose gel was carried out to visualize intact genomic DNA and the DNA samples were diluted if required to obtain a working solution of 20-25 ng/µl.

PCR protocol

The insect specific universal mitochondrial cytochrome oxidase I primers (JM 76 (5-GAGCTGAATTAGG(G/A)ACTCCAGG-3) and JM 77 (5-ATCACCTCC(A/T)CCTGCAGGATC-3)) were used for amplification of the target region from three *Ceratina* species. The PCR reaction mix used for the study was as follows, 50 ng of DNA template, 200nM of dNTPs, 1mM of each primer, 2.5 units of TaqDNA polymerase and 5µl of PCR reaction buffer was added to make the make a final volume of 50 µl. The PCR reactions were performed in a thermal cycler (Biorad) with an initial 3 min denaturation step at 95 ^{0}C, followed by 35 amplification cycles consisting of 1 min denaturation at 95 ^{o}C, 45 seconds annealing at 52 ^{o}C and 1 min extension at 72 ^{o}C with an additional final step of extension for 10 min at 72 ^{0}C. The presence of amplified PCR product was visualized and confirmed in the gel documentation system (Alpha Image Analyzer, Alpha Innotech Corporation) by 1.2% agarose-EtBr10mg/ml gel electrophoresis with 2.5 µl PCR product.

Sequencing and data analysis

Gel elution columns (Sigma) were used for purification of the amplified products of the target gene. The purified products were sequenced directly by an automated DNA sequencer (ABI 377) following manufacturers guidelines for the Big Dye terminator kit (Applied Biosystems). The sequence thus obtained were aligned with Clustal Omega (1.2.2) multiple sequence alignment (Sievers and Higgins, 2018). Further analysis on phylogenetic and molecular evolutionary analyses, pairwise genetic distance among three species, variation in nucleotide sequences as well as transition/ transversion rate ratios were calculated by comparing with the CO1 sequences of other closely related *Ceratina* spp. in the NCBI GenBank database by BLASTN. The MEGA X 10.0.5software (Molecular Evolutionary Genetic Analysis version X) (Kumar *et al.*, 2018) was used for the construction of Minimum evolution tree (Saitou and Nei, 1987) utilizing the distance matrix from the alignment. The confidence level of each branch was tested by bootstrapping 1000 replicates generated with random seed. The nucleotide sequences were translated into amino acid sequences with the help of invertebrate mitochondrial genetic code through the ExPASy translate: SIB bioinformatics resource portal (Artimo *et al*., 2012) and were aligned using Clustal omega software (Sievers and Higgins, 2018). The variation in the amino acid concentration within and among the species was estimated by computing the amino acid composition by MEGA X 10.0.5 software. The generated sequences of partial mitochondrial COI region were further submitted to NCBI GenBank database through Bankit submission tool (https://www.ncbi.nlm.nih.gov/WebSub/ ?tool=genbank) to acquire the individual accession number (Yet to receive the accession numbers).

RESULTS

The partial mitochondrial COI regions of 684 bp of *C.sutepnsis* and 686 bp each of *C. similima* and *C. smargdula* respectively, were amplified. The three wild bee *Ceratina* species native to Uttarakhand Himalayas were morphologically characterized but to clear the ambiguity at molecular level, the molecular characterization with partial mitochondrial Cox 1 gene were carried out. Through BLASTn analysis of the obtained sequences, it was observed that our specimen

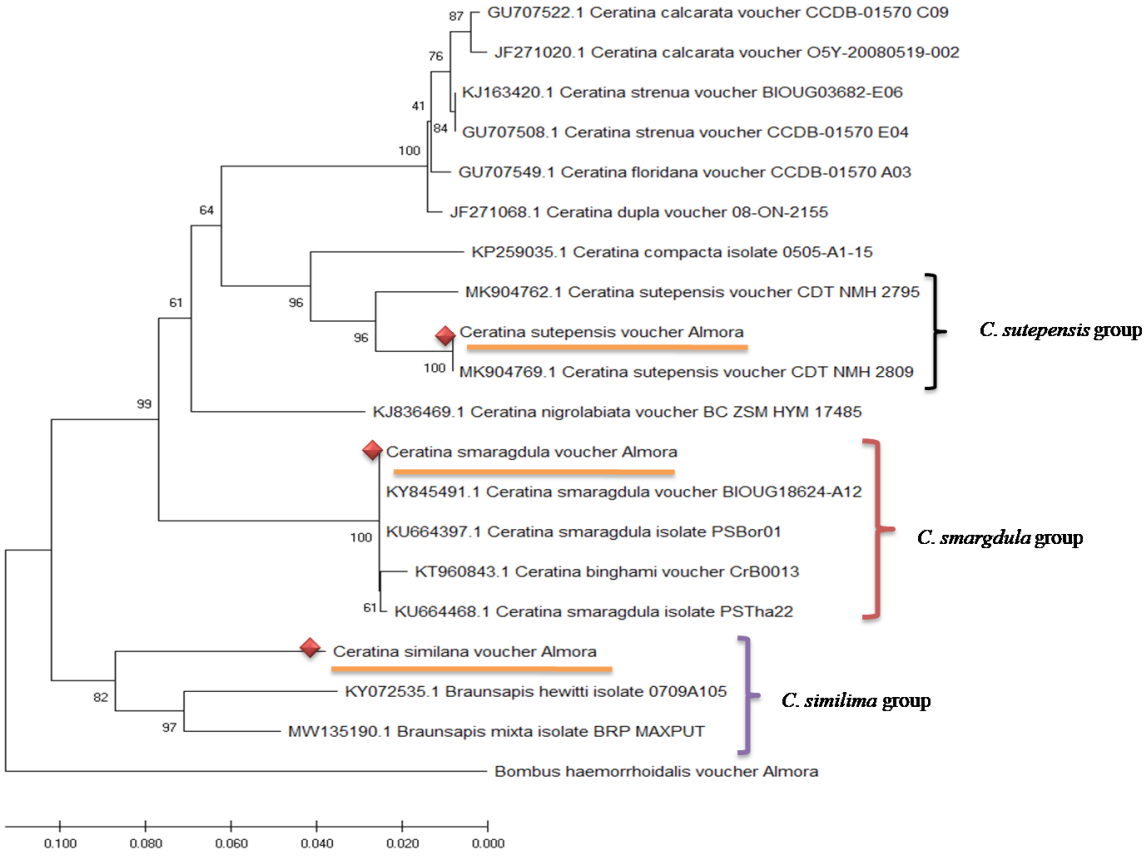

Fig. 1. Minimum Evolution tree with bootstrap support (1000 replicates) showing clustering of different species of *Ceratina*, constructed using partial COI sequences

Ceratina sutepensis voucherspecimen_Almora showed 99.83% identity to *Ceratina sutepensis* voucher CDT_NMH_2809 (NCBI accession number MK904769) reported earlier from Indian Himalayas by Pakrashi *et al.* (2020). While, the *Ceratina smargdula* voucherspecimen_Almora recorded 99.83% identity with the *Ceratina smargdula* isolate PSBor01 specimen (NCBI accession number KU664397) which was introduced into Hawaiian archipelago for range expansion and competitive exclusion of yellow masked bees (*Hylaeus* spp.) (Shell and Rehan, 2016). However, the *Ceratina similima* voucher specimen_Almora showed only 92.03% identity to *Braunsapismixta* isolate BRP_MAXPUT (NCBI accession number MW135190) that was reported to pollinate cashew flowers in the southern parts of India (Ashika *et al*., 2020 unpublished). Although both *C. similima* and *B. mixta* belong to same family Apidae and sub-family Xylocopinae, their classification at the tribe level varies, wherein the *Ceratina* species belongs to tribe Ceratinini and *Braunsapis* belongs to tribe Allodapini. When the phylogenetic tree was constructed with the MEGA X 10.0.5 software (Fig. 1), it was observed that the three species of *Ceratina* native to Indian Himalayas formed three separate groups in the minimum evolution tree. The node support estimated using 1000 bootstrap pseudoreplicates, showed that *C. sutepensis* and *C. smargdula* species evolved together as they showed 100% node value. However, as no sequences of *C. similima* were obtained from the NCBI database, the phylogenetic analysis was carried out with the available closely related species of *Braunsapis*. Theresults showed that the *C. similima* species showed only 82% relatedness to *Braunsapis* species during the evolutionary analysis.

The pairwise genetic distance analysis carried out between the three *Ceratina* species, recorded that *C. sutepensis* and *C. smargdula* had least genetic distance of 0.114 while the genetic distance between *C. sutepensis* and *C. similima* was the highest with the value 0.169.

Further to understand the inter-specific diversity

```
similima        GGTATAATTGGGATCTTCAATAAGATTAATTATTCGTATAGAATTAGGAACACCAGGAAG    60
sutepensis      GGAATAATTGGGTGCATCAATAAGTTTAATTATTCGAATAGAATTAAGAATTCCTGGTAA    60
smaragdula      GGAATAATTGGGTGCATCTATAAGATTAATTATTCGAATAGAGTTAAGAATTCCTGGAAA    60
                ** *********  * ** ***** ********** ***** *** ***  ** ** *

similima        ATGAATTAATAATGATCAGATCTATAATTCTATAGTAACTTCCCATGCATTTCTAATAAT    120
sutepensis      TTGAATTAGAAATGATCAAATTTATAATTCTCTAGTTACTGCCCATGCTTTTTTAATAAT    120
smaragdula      TTGAATTAATAATGATCAAATTTATAATTCTTTAGTTACAGCTCATGCTTTTTTAATAAT    120
                 *******  ******** ** ********* **** **  * ***** *** *******

similima        TTTTTTTATAGTTATACCATTTATAATTGGAGGATTTGGTAATTGACTTATTCCATTAAT    180
sutepensis      TTTTTTTATAGTTATACCATTTATAATTGGTGGATTTGGAAATTGATTAATTCCATTAAT    180
smaragdula      TTTTTTTATAGTAATACCTTTTATAATTGGAGGATTTGGTAATTGATTAATTCCATTAAT    180
                ************ ***** ********** ******** ****** * **********

similima        ATTAGGATCACCTGATATAGCTTTTCCACGAATAAATAATATTAGATTTTGATTACTTCC    240
sutepensis      ACTAGGATCTCCTGATATATCATTTCCTCGATTAAATAATATTAGATTTTGATTATTACC    240
smaragdula      ATTAGGTTCTCCAGATATATCTTTTCCTCGATTAAATAATATTAGATTTTGATTATTACC    240
                * **** ** ** ****** * ***** *** ********************** * **

similima        CCCATCATTATTATTATTATTAAGTAATTTATTTAATCCAAGACCTGGTACAGGTTG    300
sutepensis      TCCATCACTATTATTACTTTTATCAAGAAATTTATTTTCTATAAGACCTGGAACTGGATG    300
smaragdula      ACCTTCTTTATTATTATTATTATCAAGAAATTTATTTACTTTAAGTCCAGGAACTGGTTG    300
                 ** **  ******** * **** *** ********  *  *** ** ** ** ** **

similima        AACTGTATATCCTCCTTTATCTTCATATATATTTCATTCATCTCCTTCAGTTGATTTAAC    360
sutepensis      AACAATTTATCCACCTCTATCATCTTATTTATTTCATTCCTCTCCATCAGTTGATTTAGC    360
smaragdula      AACTGTATATCCACCATTATCATTATATTTATATCATTCATCTCCTTCAGTTGATTTAAC    360
                ***  * ***** **  **** *  *** *** ****** ***** ************ *

similima        AATTTTTTCTTTACATATATCAGGTATTTCTTCAATCTTAGGAGCAATAAATTTTATAGT    420
sutepensis      TATTTTTTCATTACATATATCAGGAATTTCATCAATTTTAGGGGCTATTAATTTTATAGT    420
smaragdula      TATTTTTTCTTTACATATATCTGGTATTTCATCAATTTTAGGTGCTATTAATTTTATAGT    420
                 ******** *********** ** ***** ***** ***** ** ** ***********

similima        TACTATTATAATAATAAAAAATTTATCTCTAAATTATGATTATATTACTCTATTTTCTTG    480
sutepensis      TACAATTATAATAATAAAAAATATTTCTTTAAATTATGATAATATTCCATTATTTTCTTG    480
smaragdula      AACTATTATAATAATAAAAAATATTTCTATTAATTATGATAATATTAGATTATTTTCTTG    480
                 ** ****************** * *** * ********* *****    **********

similima        ATCAGTATTTATTACTGCTATTTTATTATTATTATCATTACCTGTATTAGCTGGAGCTAT    540
sutepensis      ATCAGTATTTATTACTGCAATTTTATTATTACTTTCATTACCAGTTTTAGCTGGTGCTAT    540
smaragdula      ATCAGTATTTATTACAGCTATTTTATTATTATTATCTTTACCTGTATTAGCAGGTGCTAT    540
                ************** ** *********** * ** ***** ** ***** ** *****

similima        TACTATACTATTATTTGATCGTAATTTTAATACATCTTTTTTTGACCCAATAGGGGGAGG    600
sutepensis      TACTATATTATTATTTGATCGTAATTTAAATACATCATTTTTTGATCCAATAGGTGGTGG    600
smaragdula      TACTATATTATTATTTGATCGTAATTTAAATACATCATTTTTTGATCCAATAGGAGGAGG    600
                ******* ****************** ******** ******** ******** ** **

similima        AGATCCAATTTTGTATCAACATTTATTTTGATTTTTTGGTCACCCTGGAAAGTTAAAAT-    659
sutepensis      AGATCCAATTCTATTTCAACATCTATTTTGATTTTTTGGTCACCCTGAAAGTTATAAAT-    659
smaragdula      AGATCCTGTTTTATATCAACATTTATTTTGATTTTTTGGTCACCCTGGAAGTTAAAATTT    660
                ******  ** * * ******* ************************ **  *  ** *
```

Fig. 2. Multiple sequence alignment of three species of *Ceratina* for analyzing the variation in single nucleotide polymorphism (SNPs)

Table 1. Pair-wise genetic distance analysis between three species of Ceratina

	C. sutepensis voucher_Almora	*C. similima* voucher_Almora	*C. smaragdula* voucher_Almora
C. sutepensis voucher_Almora	1.000		
C. similima voucher_Almora	0.169	1.000	
C. smaragdula voucher_Almora	0.114	0.150	1.000

Table 2. Average nucleotide frequency among the three species of *Ceratina*

	A	T(U)	G	C	Total
C. sutepensis voucher_Almora	32.02	43.71	10.96	13.30	100.00
C.similima voucher_Almora	31.68	43.36	11.53	13.43	100.00
C. smaragdula voucher_Almora	31.68	45.69	11.24	11.39	100.00

among the three species on the basis of 684 bp CO1 sequence, the variation in single nucleotide polymorphism (SNPs) was estimated by aligning the three sequences in CLUSTAL Omega (1.2.4) multiple sequence alignment software (Fig. 2). It was observed thata total variation in 117 SNP's were recorded, which was large enough to differentiate the species at inter-specific level.

The average nucleotide frequency among the three species of *Ceratina* was also analyzed (details in Table 2) and it was noticed that the COI sequences were usually A+T biased with the concentration of A+T exceeding 75%, while the concentration of G+C well below 25%. Besides nucleotide frequencies, the transition/transversion rate ratios were also calculated (Table 3) for the 14 species of *Ceratina* and *Braunsapis* and it was reported that the ratios were *k1* = 4.286 (purines) and *k2* = 5.698 (pyrimidines) and the overall transition/transversion bias was R=1.962, where R = [A*G*k1 +T*C*k2]/[(A+G)*(T+C)]. MCL estimate of nucleotide substitutions also showed maximum base substitution between C to T and vice versa with a maximum value of 35.96.

Table 3. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution of 14 species of *Ceratina*

	A	T	C	G
A	-	6.31	1.89	**5.93**
T	4.49	-	**10.75**	1.38
C	4.49	**35.96**	-	1.38
G	**19.23**	6.31	1.89	-

To further analyze the variation in amino acid composition among the three sequences of *Ceratina* species, the sequences were translated by ExPASy translate software and the nucleotide compositions were estimated by MEGA X software (Fig 3). The per cent amino acid composition among the three *Ceratina* species showed that Leucine and Serine were found at the highest frequencies *viz*., 16.01% and 11.77% respectively. Moreover it was observed

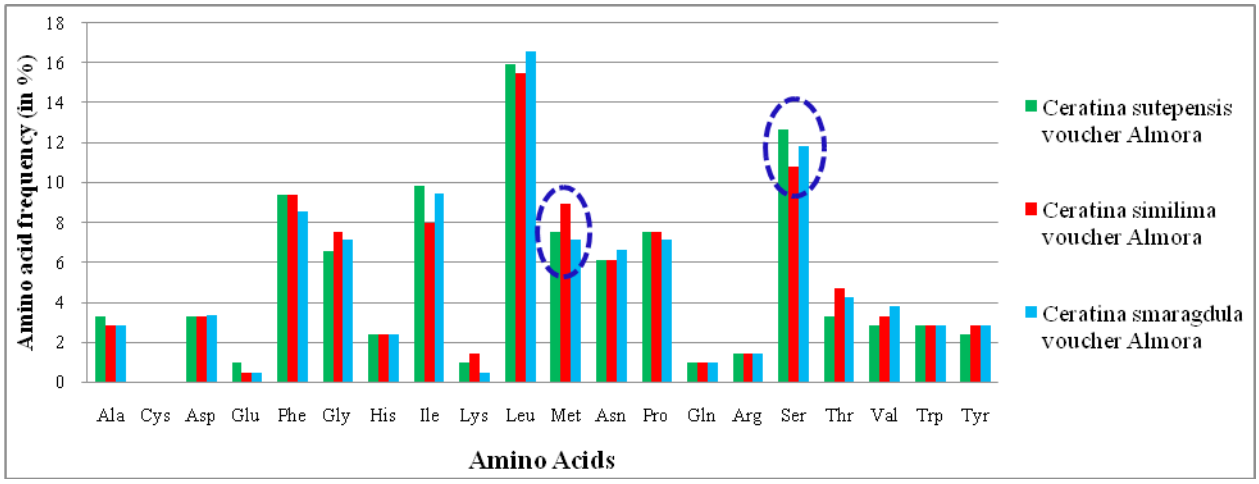

Fig. 3. Variation in amino acid composition of three *Ceratina* species native to North Western Himalayas

that the amino acid frequency among the three species was almost similar, except for metheonine and serine, whose concentration varied greatly and was found in highest concentration in *C. similima* (8.92%) and *C. sutepensis* (12.67%) respectively, these results indicate the huge variation among the three *Ceratina* species native to Indian Himalayas.

DISCUSSION

Although more than 20,355 species of domesticated and wild bees are reported worldwide (Ascher and Pickering, 2019), their identification through taxonomic tools is still in infant stages due to lack of distinguishing morphological characters in bee taxonomy (Packer *et al.,* 2009), presence of cryptic species and huge biodiversity has made identification of bees a herculean task for biologists all over the world (Hines and Williams, 2012; Vamosi *et al.,* 2017). Recently, Saini *et al.* (2019) discovered a new species of wild bee (*Melitta indica*) (Hymenoptera: Melittidae) from Western Himalayan region of Uttarakhand through molecular characterization and DNA barcoding (Hebert *et al.,* 2003) methods that has sparked the importance of molecular techniques in understanding the species delimitation of wild bees in various regions of the world (Smith *et al.,* 2008; Butcher *et al*., 2012), association of sexes in dimorphic species classification (Gibbs, 2009; Sheffield *et al.,* 2009), characterizing morphologically difficult to identify species (Gibbs 2009; Rehan and Sheffield, 2011; Williams *et al.,* 2012), proper identification of the specimens up to species level (Schmidt *et al.,* 2015), determining the evolutionary relationship and genetic distances among the targeted insect species (Kekkonen and Hebert, 2014), clarifyinglevel speciation of several species associated with various taxonomic levels (Trunz *et al*., 2016; Oh *et al.,* 2013) and cryptic species detection (Packer and Taylor, 1997). Apart from the above mentioned applications of molecular techniques in species identification, they also support the taxonomists in integrative taxonomy to decipher the biodiversity of a locality (Dayrat, 2005).

In our present study to characterize the three species of *Ceratina,* native to Indian Himalayas; molecular tools like DNA barcoding and phylogenetic analysis with the closely related *Ceratina* species obtained from NCBI data base was carried out. The results obtained showed that, the presence of *Ceratina sutepensis* in the Indian Himalayas was recently reported by Pakrashi *et al*. (2020) which closely corroborated with our results. However, the other two species *C. similima* and *C. smargdula* were reported for the first time from this region and their partial CO1 sequences were submitted to the NCBI database (Accession numbers yet to be received) which can be of prime importance to the molecular taxonomists working on wild bees in the Indian Himalayas.

Moreover the studies carried out by Oppenheimer *et al.* (2018) to molecularly analyze the Phylogeography and population genetics of the Australian small carpenter bee, *Ceratina australensis* showed the significant decrease in genetic diversity in Sothern Australia (SA). Based on differences between nucDNA and mtDNA of native ceratina species, they could conclude that *C. australensis* thought to have dispersed out of Asia, moved south and east along Australia's coast, and then headed south and west through to SA. Similar kind of study needs to be undertaken for the native Himalayan wild bees because, one of the species reported in our study (*C. smargadula*) shows very close identity to the *C. smaragdula* species introduced into Hawaiian archipelago for range expansion and competitive exclusion of yellow masked bees (*Hylaeus* spp.) (Shell and Rehan, 2016).

Similarly Rehan and Sheffield (2011) investigated the importance of molecular tools for the delineation of a new species in the *Ceratina dupla* species-group (Hymenoptera: Apidae: Xylocopinae) of eastern North America. They worked on an important aspect of verifying the taxonomic characters of wild bees in order to morphologically differentiate genetically distinct species of *Ceratina*. Their study added a new species (*C. mikmaqi*) to the *Ceratina dupla* species-group and raised another form, formerly *C. duplafloridana*, to *C. floridana* to full species status in the eastern North American region. Our study forms a close concurrence with the study of Rehan and Sheffield (2011), wherein, the two native species *C. sutepensis* and *C. similima* were earlier thought to be same species *C. similima* and their morphological differentiation was not only difficult, but was often neglected during the faunal surveys due to non-availability of crystal clear taxonomic keys for species identification. Based on our molecular characterization studies; now we can easily differentiate the two species of *Ceratina* and in turn formulate taxonomic keys to characterize the wild bee species native to Indian Himalayas.

CONCLUSION

The world of bees is very fascinating and highly diverse. But, their scientific classification based on morphology has not gained much importance due to lack of taxonomic tools and presence of cryptic species in a given locality. However, the recent advances in molecular taxonomy like, DNA barcoding and phylogenetic analysis has paved the path for integrative taxonomy. The databases like BOLD, DAISY and ABI have also created a ray of hope for study of wild bees in various unexplored locations. The present study not only generated the information about the wild bees, but also reported the presence of three different species of *Ceratina*. Moreover, the Indian Himalayas are undisturbed as well as unexplored ecosystems on the earth whose biodiversity analysis has not been studied extensively so far because of the hilly terrains and difficult to survey areas. Our study not only forms the point of inception for study of wild bees in Indian Himalayas, but also opens the doors of opportunities to explore the faunal diversity of the Himalayas.

ACKNOWLEDGEMENTS

The authors are thankful to Indian Council of Agricultural Research (ICAR) and ICAR-VPKAS, Almora for the financial assistance and providing facilities for conducting experiments successfully. We also thank our Senior Technical Assistant, Mr. J P Gupta for his untiring technical support during field and laboratory studies.

Conflict of Interest

All the authors have thoroughly reviewed the research article and have no conflict of interest for submission of the article to the "*Asian Journal of Microbiology, Biotechnology & Environmental Sciences*".

REFERENCES

Abrol, D.P. 2009. Plant-pollinator interactions in the context of climate change - an endangered mutualism. *Journal of Palynology*. 45 : 1–25.

Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., De Castro, E. and Stockinger, H. 2012. ExPASy: SIB bioinformatics resource portal. *Nucleic-acids Research.* 40(W1): W597-W603.

Ascher, J.S. and Pickering, J. 2019. Discover life bee species guide and world checklist (Hymenoptera: Apoidea). Available from: https://www.discoverlife.org (Accessed on June 2020)

Bertsch, A. 2009. Barcoding cryptic bumblebee taxa: *B. lucorum, B. crytarum* and *B. magnus*, a case study (Hymenoptera: Apidae: Bombus). Contributions to Entomology. 59(2):287-310.

Brown, M.J. 2011. The trouble with bumblebees. *Nature.* 469(7329) : 169-170.

Butcher, B.A., Smith, M.A., Sharkey, M.J. and Quicke, D.L.J. 2012. A turbotaxonomic study of Thai Aleiodes (Aleidodes) and Aleiodes (Arcaleiodes) (Hymenoptera: Braconidae: Rogadinae) based largely on COI barcoded specimens, with rapid description of 179 new species. *Zootaxa.* 3457 : 1–232.

Dayrat, B. 2005. Towards integrative taxonomy. *Biological Journal of the Linnean Society.* 85(3):407-417.

Del Sarto, M.C.L., Peruquetti, R.C., Campos, L.A.O. 2005. Evaluation of the neotropical stingless bee *Meliponaquadrifasciata* (Hymenoptera: Apidae) as pollinator of greenhouse tomatoes. *Journal of Economic Entomology.* 98(2) : 260-266.

Gibbs, J. 2009. Integrative taxonomy identifies new (and old) species in the *Lasioglossum* (Dialictus) tegulare (Robertson) species group (Hymenoptera, Halictidae). *Zootaxa.* 2032(1) : 1-38.

Gotelli, N.J. 2004. A taxonomic wish-list for community ecology. Philosophical Transactions of the Royal Society B: *Biological Sciences.* 359 : 585–597.

Heard, T.A. 1999. The role of stingless bees in crop pollination. *Annual Review of Entomology.* 44(1) : 183-206.

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

Kakutani, T., Inoue, T., Tezuka, T. and Maeta, Y. 1993. Pollination of strawberry by the stingless bee, *Trigonaminangkabau*, and the honey bee, *Apis mellifera*: an experimental study of fertilization efficiency. *Researches on Population Ecology.* 35(1) : 95-111.

Kekkonen, M. and Hebert, P.D. 2014. DNA barcodebased delineation of putative species: efficient start for taxonomic workflows. *Molecular Ecology Resources.* 14(4) : 706-715.

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.

Magnacca, K.N. and Brown, M.J. 2012. DNA barcoding a regional fauna: Irish solitary bees. *Molecular Ecology Resources.* 12(6) : 990-998.

Michener, C.D. 2007. *The Bees of the World,* 2nd edn. Johns Hopkins University Press, Baltimore, Maryland.

Murray, T.E., Fitzpatrick, U., Brown, M.J. and Paxton, R.J. 2008. Cryptic species diversity in a widespread bumble bee complex revealed using mitochondrial DNA RFLPs. *Conservation Genetics.* 9(3) : 653-666.

Oh, H.K., Yoon, H.J., Lee, J.Y., Park, J.S. and Kim, I. 2013. Population genetic structure of the bumblebee,

Bombusignitus (Hymenoptera: Apidae), based on mitochondrial COI gene and nuclear ribosomal ITS2 sequences. *International Journal of Industrial Entomology.* 27(1) : 142-158.

Ollerton, J., Price, V., Armbruster, W.S., Memmott, J., Watts, S., Waser, N.M. and Tarrant, S. 2012. Overplaying the role of honey bees as pollinators: a comment on Aebi and Neumann (2011). *Trends in Ecology and Evolution.* 27(3) : 141.

Oppenheimer, R.L., Shell, W.A. and Rehan, S.M. 2018. Phylogeography and population genetics of the Australian small carpenter bee, *Ceratina australensis*. *Biological Journal of the Linnean Society.* 124(4) : 747-755.

Packer, L., Gibbs, J., Sheffield, C. and Hanner, R. 2009. DNA barcoding and the mediocrity of morphology. *Molecular Ecology Resources.* 9 : 42-50.

Packer, L. and Taylor, J.S. 1997. How many hidden species are there? An application of the phylogenetic species concept to genetic data for some comparatively well known bee "species". *The Canadian Entomologist.* 129(4) : 587-594.

Pakrashi, A., Kundu, S., Saini, J., Tyagi, K., Chandra, K. and Kumar, V. 2020. Molecular identification of selected bees from the Indian Himalaya: A preliminary effort. *Journal of Asia-Pacific Entomology.* 23(4) : 883-889.

Quezada-Euán, J.J.G. 2009. Potencial de lasabejasnativas en la polinización de cultivos. *Acta Biológica Colombiana.* 14 : 169–172

Ratnasingham, S., Hebert, P.D. 2007. BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). *Molecular Ecology Notes.* 7(3) : 355-364.

Rehan, S.M. and Sheffield, C.S. 2011. Morphological and molecular delineation of a new species in the *Ceratina dupla* species-group (Hymenoptera: Apidae: Xylocopinae) of eastern North America. *Zootaxa.* 2873(1) : 35-50.

Saini, J., Chandra, K. and Kumar, H. 2020. Description of a new species of genus *Melitta* (Hymenoptera: Melittidae) from India. *Oriental Insects.* 54(3) : 411-416.

Saini, J. and Chandra, K. 2019. Current status of diversity and distribution of bee pollinators (Apoidea: Hymenoptera) of India. *ZSI ENVIS Newsletter.* 25(1-4) : 22-25.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* 4(4) : 406-425.

Schmidt, S., SchmidEgger, C., Morinière, J., Haszprunar G. and Hebert, P.D. 2015. DNA barcoding largely supports 250 years of classical taxonomy: identifications for Central European bees (Hymenoptera, Apoideapartim). *Molecular Ecology Resources.* 15(4) : 985-1000.

Schroder, S., Wittmann, D., Drescher, W., Roth, V., Steinhage, V. and Cremers, A.B. 2002. The new key to bees: automated identification by image analysis of wings. Pollinating bees–the Conservation link between Agriculture and Nature, Ministry of Environment, Brasilia. 209-218.

Sheffield, C.S., Hebert, P.D., Kevan, P.G. and Packer, L. 2009. DNA barcoding a regional bee (Hymenoptera: Apoidea) fauna and its potential for ecological studies. *Molecular Ecology Resources*. 9 : 196-207.

Shell, W.A. and Rehan, S.M. 2016. Recent and rapid diversification of the small carpenter bees in eastern North America. *Biological Journal of the Linnean Society.* 117(3) : 633-645.

Sievers, F. and Higgins, D.G. 2018.Clustal Omega for making accurate alignments of many protein sequences. *Protein Science.* 27(1) : 135-145.

Slaa, E.J., Chaves, L.A.S., Malagodi-Braga, K.S. and Hofstede, F.E. 2006. Stingless bees in applied pollination: practice and perspectives. *Apidologie.* 37(2) : 293-315.

Smith, A.M., Rodríguez, J.J., Whitfield, J.B., Deans, A.R., Janzen, D.H., Halwachs, W. and Hebert, P.D.N. 2008. Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. *PNAS.* 105 : 12359–12364.

Subbanna, A.R.N.S., Chandrashekara, C., Stanley, J., Mishra, K.K., Mishra, P.K. and Pattanayak, A. 2019. Bio-efficacy of chitinolytic *Bacillus thuringiensis* isolates native to northwestern Indian Himalayas and their synergistic toxicity with selected insecticides. *Pesticide Biochemistry and Physiology.* 158: 166-174.

Trunz, V., Packer, L., Vieu, J., Arrigo, N. and Praz, C.J. 2016. Comprehensive phylogeny, biogeography and new classification of the diverse bee tribe Megachilini: Can we use DNA barcodes in phylogenies of large genera? *Molecular Phylogenetics and Evolution.* 103 : 245-259.

Vamosi, J.C., Gong, Y.B., Adamowicz, S.J. and Packer, L. 2017. Forecasting pollination declines through DNA barcoding: the potential contributions of macroecological and macroevolutionary scales of inquiry. *New Phytologist.* 214(1):11-18.

Weeks, P.J.D., O'Neill, M.A., Gaston, K.J. and Gauld, I.D. 1999. Automating insect identifications: exploring the limitations of a prototype system. *Journal of Applied Entomology.* 123 : 1-8.

Williams, N.M., Regetz, J. and Kremen, C. 2012. Landscapescale resources promote colony growth but not reproductive performance of bumble bees. *Ecology.* 93(5) : 1049-1058.