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# Molecular Identification and Phylogenetic Analysis of Some Common Beetles (Coleoptera) of Jammu Region, India Using DNA Barcoding

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**ABSTRACT**

Coleoptera is a highly diverse and taxonomically important order of Class Insecta. DNA Barcoding has proven its effectiveness as an accurate molecular taxonomy tool in differentiating various species belonging to same genus. We carried out a pilot study for testing the effectiveness of mitochondrial cytochrome C oxidase sub unit 1 based DNA Barcoding for common species of beetles from Jammu region of UT of J&K. Successful generation of DNA barcodes of 04 species was achieved and the sequences were submitted to GenBank. The phylogenetic analysis was performed by using Neighbor-Joining method and Kimura-2-Parameter with 1000 bootstrap supports, gap opening penalty of 15.00 and a gap extension penalty of 6.66 in both pair-wise as well as multiple alignments.

***Key words :*** *Coleoptera, DNA Barcoding, Kimura-2-Parameter, Mitochondrial Cytochrome C Oxidase 1 gene, Phylogenetic analysis*

## Introduction

Coleoptera forms the most bio-diverse order of the animal kingdom and encloses rich fauna. In India, more than 3, 50,000 species of beetles have been identified that comprise 40% of the Insecta (Wankhade *et al.,* 2014). Beetles have their own importance in most of the ecosystems and play an important role in trophic chains (Leraut, 2003). Out of this huge order, family Scarabaeidae forms the largest group as it includes more than 3000 species in the entire world (Fincher *et al.,* 1981). Beetles are beneficial for the ecology as they control the population of various pests. For instance, larva and adults of Ladybug beetle feed on aphid colonies. Dung beetles help in reducing the populations of parasitic worms and pestilent flies breeding in the cattle dung (Brown *et al.,* 2010) and are functionally and taxonomically very important part of the terrestrial ecosystem (Kakkar and Gupta, 2009). Beetles belonging to Family- Carabidae (commonly called as ground beetles) are the predators of various insects and other arthropods like wireworms, caterpillars etc (Kromp, 1999). Scarab beetles and dermestidae, Silphidae and Tenebroinidae beetles act as scavengers. Rove beetles act as indicators of human impact on natural ecosystems (Bouchard *et al.,* 2017).

To enlist the huge diversity of beetles, naturalists carry out the classification based on the morphological characteristics and thus, it requires a community

of taxonomists and biologists for species identification which is hectic and time-consuming process. Morphology based approach of species identification has a number of shortcomings. Since the technique overlooks the morphologically cryptic taxa which are common in several groups (Knowlton, 1993; Jarman and Elliot, 2000; Bickford *et al.,* 2007; Hajibabaei *et al.,* 2006), separate keys are needed for sexually dimorphic species and even for different life stages of the same species (Tahir *et al.,* 2016). Further, expert knowledge of the taxonomic terms is required. The genetic variability and phenotypic plasticity of characters has led to incorrect identification of species as well (Hebert *et al.,* 2003).

The drastic decline in the number of taxonomists has added to the worry (Jung *et al.,* 2016). So, the researchers have shifted towards a molecular based technique that is more accurate and inarguably a better alternative to classical taxonomy and could be used by researchers who are not taxonomists. Lately, molecular data has been prioritized over morphological data for the identification of species (Hebert *et al.,* 2003). Molecular biology has driven systematics to a different direction as micro genomics identification system allows the life's distinction by the analysis of a remarkably short genomic sequence which basically is a very promising approach for the diagnosis of biodiversity (Hebert *et al.,* 2003). The approach has various advantages as it does not require expertise, is less time consuming, is more accurate and cost effective and can identify the species from any life stage. Hebert *et al.* (2003 a,b) first proposed a technique which uses a primer set for the amplification of a gene region; i.e. Mitochondrial cytochrome C oxidase 1 region which is 648 bp base pair (bp) long for ensuring the accurate and rapid identification of a wide range of biological samples (Jinbo *et al.,* 2011) and they called this technique as "DNA Barcoding**"**. So in DNA barcoding system, an agreed-upon sequence is selected based upon few criteria and is called as "DNA barcode" or "DNA tag". Genetic markers are highly beneficial as they are neutral to any stage of development (Sreedevi *et al.,* 2015) and can be used for new and unknown species.

The concept of considering DNA Barcoding as a substitute for traditional taxonomy is wrong; rather it is a bio-identification system that complements the traditional taxonomy (Sreedevi *et al.,* 2015) and thus, various researchers suggest DNA barcoding as a technique for committing to rapid species identification and illustration to address the present crisis in biodiversity (Hebert *et al.,* 2003 a,b). Since the molecular taxonomy itself is not a full-fledged taxonomic approach but it complements the traditional taxonomy, therefore a term "Integrative Taxonomy" has been coined by Will *et al.* (2005) which includes and links both molecular and morphological data through an integrative approach (Yeates *et al.,* 2011). Integrative taxonomy is being used for unraveling the cryptic species (Schlick-Steiner *et al.,* 2006) and for describing the species based on morphological data and sequence data (Fisher and Smith, 2008). In the study under reference, we have applied integrative taxonomy approach to identify and characterize common beetle species from Jammu region.

## Materials and Methods

### Site selection, taxonomical collection, preservation and identification

Coleopteran specimens was collected over a period of one year (October 2018- October 2019) from selected geographical areas of Jammu by standard collecting methods, *viz*. hand picking, net-sweeping, light traps and beating methods. The collected specimens were then brought to Department of Zoology, University of Jammu and were killed by using killing bottles. Insects were then segregated on the basis of their morphological characters by visualising the specimens under the stereomicroscope. Thereafter, the collected specimens were preserved in 70% ethanol at 4 $^{o}$C till DNA isolation was carried out. Identification of the specimen was done by studying the morphological characters based on the consultancy of the taxonomic keys-Klimaszewski and Watt (1997) and Crowson (1956). Specimen authentication was also performed by comparing the data with the previously available literature on the beetles' diversity in Department of Zoology, University of Jammu. Identification was further confirmed by using online portals.

### Amplification and DNA sequencing of COI gene

Ethanol preserved specimens were rinsed thoroughly with distilled water to remove other contaminations. DNA was extracted from the somatic tissue (rich in mitochondria), i.e., legs (one leg for larger specimen and two legs for smaller specimen) of specimens by using Qiagen DNAeasy® Blood and Tissue Kit, following manufacturer's protocol.

The remaining parts of the insect specimen were kept as voucher specimens. The mitochondrial COI gene amplification was carried out with the help of the primers LCO1490 5' -GGTCAACAAATCA TAAA GATATTGG-3' and HCO2198 5'-TAAACTT CAGGGTGACCAAAAAATCA-3'(Folmer *et al.*, 1994). A 50µl PCR reaction containing 5.0µl of 10X PCR buffer, 3.0µl of 25mM $MgCl^2$, 1.0 µl of 10 mM dNTPs, 1.0µl of each primer (10 µM each), 0.2µl of Taq polymerase, 3.0 µl of template DNA and 36.8 µl of nuclease free water was set up. The PCR conditions used for amplification of the COX 1 gene were initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute and at last a final extension was performed at 72°C for 7 minutes. These reactions were carried out in Applied Biosystems Veriti 96 Well Thermal Cycler. The amplified products were visualised under UV transilluminator after carrying out Agarose Gel Electrophoresis in 1.5% gel. The PCR products were then sent to Eurofins Genomic India Pvt. Ltd. #540/1, Doddanakundi Industrial Area 2 Hoodi, Whitefield, Bengaluru-560048 Karnataka, India for bidirectional Chain termination Sanger sequencing. Insect specimens were sequenced both in forward and reverse directions i.e. bidirectionally sequenced for the universal primers LCO1490 and HCO2198.

**Sequence annotation and analysis**

The mitochondrial cytochrome C oxidase subunit 1 sequences such obtained were subjected to chromatogram analysis in Finch Tv software. The sequences were assembled and edited in MEGA X and were aligned by using Clustal W for verifying the presence of stop codons to avoid the NUMT (Nuclear Mitochondrial pseudogenes) before subjecting the sequences for translations. NCBI-BLASTn (Basic Local Alignment Search Tool) was used for the sequence similarity search and sequences with more than 98% of homology were considered as the same species (Hebert *et al.*, 2004), however the sequences showing the homology less than 98% were submitted as new species to the databases. The sequences were submitted to the GenBank databases through Bio Edit; an online submission portal and with Gen Bank accession numbers written in Table 2.

**Phylogenetic Analysis**

MEGA X was used for the phylogenetic analysis of the beetle sequences. Fasta files of the sequences were aligned and pair-wise estimation was done by using ClustalW tool of MEGA X software by following the default settings and, thereafter, aligned sequences were exported in Mega format. The Phylogenetic analysis was performed by inferring the use of Neighbor-Joining Method and Kimura-2- Parameter and was performed with 1000 bootstrap supports. With 1000 bootstraps support, gap opening penalty of 15.00 and a gap extension penalty of 6.66 in both pair wise as well as multiple alignments, the phylogenetic analysis was performed. In the study under reference here, Neighbour – Joining Method, K2P and Bootstrap method was applied for finding out the evolutionary history of four beetle species. After calculating the sequence divergence, a graphical representation of the various families and interspecific divergences was created by using Neighbor-Joining tree (Tamura *et al.*, 2013). Number of substitutions per site were analysed in between all the sequences and all the positions containing the missing data and the gaps were eliminated from the data.

## Results

The twenty samples collected from the agro-ecosystems of Jammu region (the details of collection sites are shown in Table 1 and Figure 1) belonged to four families of order- Coleoptera viz., Scarabaeidae, Carabidae, Meloidae and Tenebrionidae.After successful DNA isolation, PCR was carried out for all 20 samples, out of which we got visible amplified products at 658bp for 12 samples. Out of the 12 samples, clear sequences were generated for four of

**Table 1.** Showing details of collection sites.

| Sample ID | Country | State/UT | Region | Latitude | Longitude |
|---|---|---|---|---|---|
| JCO2 | India | J&K | Jammu district | 32.7194 N | 74.8681E |
| JCO7 | India | J&K | Udhampur district | 33.04225 N | 75.1375 E |
| JCO10 | India | J&K | Udhampur district | 32.8381N | 75.1399 E |
| JCO11 | India | J&K | Udhampur district | 33.0423 N | 75.1138 E |

them. Manual annotation of these four sequences (.fasta files) was carried out in MEGA X and these sequences were aligned and phylogenetic trees of the same were generated in the same software. Good-quality sequences were assembled and edited in MEGA X and were aligned by using Clustal W before observing the translations of the sequences for verifying the presence of stop codons in between them and to avoid the nuclear mitochondrial pseudogenes (NUMTs). Afterwards the sequences were subjected to similarity search using NCBI BLASTn algorithm (Altschul *et al.*, 1990). The sequences were also subjected to translation to avoid the submission of low-quality sequences and pseudo-genes. After carrying out BLAST analysis, sequences were submitted to the online GenBank database (GenBank Overview (nih.gov)) by using an online submission portal named Bio Edit (of NCBI). All the sequences were thoroughly analyzed for high AT and GC content as shown in Table 3. These sequences were found to contain high AT than GC. Species whose sequences were submitted to the database are *Clinteria klugi* (Figure 2), *Onitis philemon* (Figure 3), *Xylotrupes mniszechi* (Figure 4), and

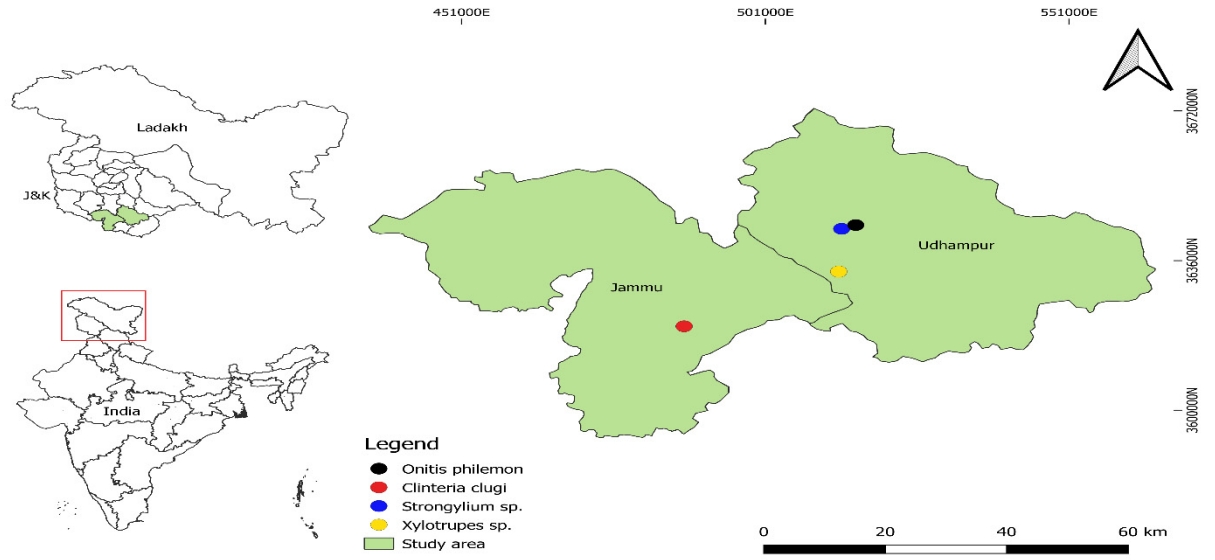


**Fig. 1**. Map depicting collection sites

**Table 2.** Showing classification of the identified insects.

| Sample ID | Phylum | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|---|
| JCO2 | Arthropoda | Insecta | Coleoptera | Scarabaeidae | *Clinteria* | *kluge* |
| JCO7 | Arthropoda | Insecta | Coleoptera | Scarabaeidae | *Onitis* | *philemon* |
| JCO10 | Arthropoda | Insecta | Coleoptera | Scarabaeidae | *Xylotrupes* | *mniszechi* |
| JCO11 | Arthropoda | Insecta | Coleoptera | Tenebrionidae | *Strongylium* | *species* |

**Table 3.** Showing ATGC Content of the various sequences submitted to database.

| ATGC Content | *Clinteria klugi* | *Strongylium* species | *Onitis philemon* | *Xylotrupes mniszechi* |
|---|---|---|---|---|
| GenBank ID | MW143316 | MW143317 | MW143318 | MW143319 |
| Total Count | 448 | 445 | 445 | 445 |
| Adenine(A) Count | 134 | 147 | 125 | 125 |
| Thymine(T) Count | 157 | 124 | 168 | 163 |
| Guanine(G) Count | 67 | 70 | 68 | 71 |
| Cytosine(C) Count | 90 | 104 | 84 | 86 |
| %GC Content | 35% | 39.1% | 34.2% | 35.3% |
| %AT Content | 64.9% | 60.8% | 65.8% | 64.7% |

*Strongylium* species (Figure 5). Their classification is shown in Table 2. *Xylotrupes mniszechi* showed 100% homology with the database while the remaining three were reportedly new sequences.


**Fig. 2.** *Clinteria kluge*


**Fig. 3.** *Onitis philemon*


**Fig. 4.** *Xylotrupes mniszechi*


**Fig. 5.** *Strongylium species*

**Phylogenetic analysis**

We used "Bootstrapping method" which is a statistical method for gaining confidence in the evolutionary studies. The probability of inference of clades by bootstrap method is considered to be highly accurate as it works on probability. The evolutionary distances were calculated by using Kimura-2-Parameters (Kimura, 1980). For constructing the phylogenetic trees, we used Neighbor-Joining approach as it has the efficiency of building a correct un-rooted evolutionary tree (Saitou and Nei, 1987). Complete phylogenetic analysis was carried out in MEGA X as it has acquired new multiple computing cores which help in inferring the molecular evolutionary analysis (Kumar *et al.,* 2018). The phylogenetic history was inferred by using the Neighbor-Joining method (Saitou and Nei, 1987). The sum of the branch length of the optimal tree was found out to be 0.50421985. The bootstrap test (1000 replicates) clustered the associated taxa together and the percentage of each replicate tree is written next to each branch (Felsenstein, 1985). Evolutionary distances were measured by using Kimura-2-parameter method and all the ambiguous positions were deleted by using pair wise-deletion option (Kimura, 1980). The present analysis contains 4 nucleotide sequences; a total of 448 positions in the final dataset. This evolutionary analysis was done by using MEGA X (Kumar *et al.,* 2018). It can clearly be inferred from the figure 6 that the species belonging to family Scarabaeidae are closely associated and the *Strongylium species* lies distanced from the other three species.

**Phylogenetic relationship with the out-group (Hymenoptera)**

The phylogenetic history was inferred by using the Neighbor-Joining method (Saitou and Nei, 1987). The sum of the branch length of the optimal tree = 0.89181714. The bootstrap test (1000 replicates) clustered the associated taxa together and the percentage of each replicate tree is written next to each branch (Felsenstein, 1985). The present analysis contains 9 sequences- four sequence from the present studies and rest five sequences corresponding to order Hymenoptera were downloaded from NCBI-BLAST to make the comparisons and a better analysis of our results, a total of 1569 positions in the final dataset. This evolutionary analysis was done by us-

ing MEGA X (Kumar *et al.,* 2018) as shown in Fig. 7.

## Discussion

Integrative approach for the taxonomic study of insects including beetles has been used by different workers from time to time. Molecular approach in the systematic study has given satisfactory results. Many workers have used the study to generate barcode reference libraries. One such work was done by Jung *et al.,* (2016) in which they focused on preparing the barcode reference library of beetles from Korea. Mitochondrial COI gene-based study has also been done by Wang and workers (2019) where these workers worked on the evaluations of DNA barcoding and its implications on the Family-Coccinellidae of Order-Coleoptera and successfully amplified, analyzed and sequenced the amplified products for 17 species of lady bird beetle. Their results clearly showed the A+T biased results and low G+C content which signified the values concluded by the earlier studies of Hebert *et al.,* 2003. An attempt on generating a DNA Barcode library of the Lepidoptera of Pakistan has been made by Ashfaq *et al.,* (2017), wherein they generated BINs for various species of the moths as mainly the species were not identifiable. The DNA Barcode library for the ground beetles of Germany was generated by Raupach *et al.,* 2018. In this study, a total of 690 DNA barcodes consisting of 47 carabid species were generated and the mean of the individual nucleotide contents were also calculated. Various researchers have contributed in the molecular identification of various insect orders; Yoshitake *et al.,* (2010), Greenstone *et al.,* (2011) and Raupach *et al.,* (2018): on order- Coleoptera, Foottit *et al.,* (2009) and Shufran and Putreka *et al.,* (2011): on order- Hemiptera, Smith *et al.,* (2005) and Smith *et al.,* (2008): on order-

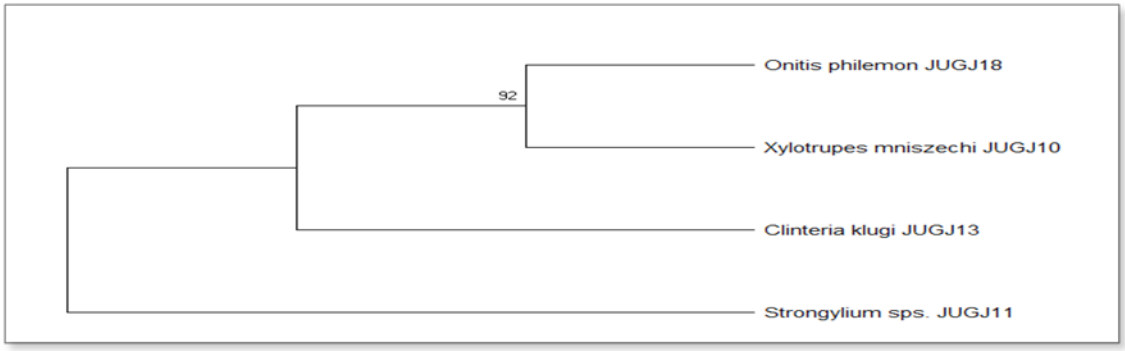


**Fig. 6.** Phylogenetic relationship (original tree) of four Coleoptera species.

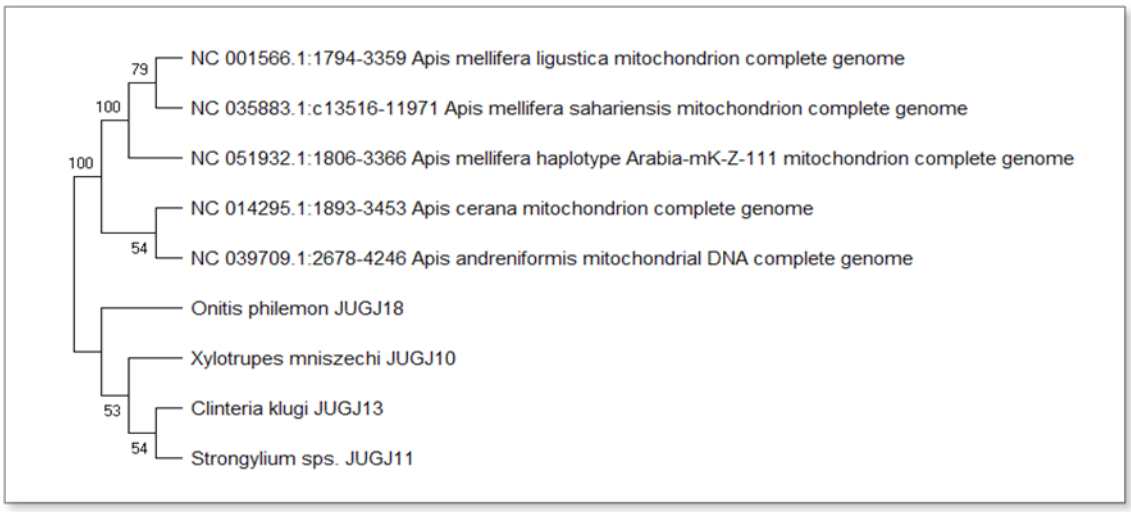


**Fig. 7.** The evolutionary relationship of the clades of order coleoptera lies completely differentiated from the clades of the sequences of order Hymenoptera.

Hymenoptera. Kumar *et al.* (2018) followed DNA barcoding technique for the identification of the geometridae moths of the Namdapha national park belonging to the eastern Himalayas. Unique barcode index numbers and unique accession numbers were acquired from the BOLD and GenBank databases by these workers.

In addition to molecular identification, DNA barcoding studies have been used by various workers for phylogenetic studies also. The evolutionary distances were calculated by using Kimura-2-Parameters (Kimura, 1980). For the phylogenetic analysis of ladybird beetles, Wang *et al.,* 2019 used Kimura-2-parameter for measuring the genetic distances on intra-specific and inter-specific level and constructed a Bayesian Inference (BI) tree. Similarly, Ashfaq and workers (2017) used the neighbor-joining tree type for analyzing the phylogeny of various Lepidoptera species from Pakistan. Likewise, Raupach *et al.,* (2018) did the phylogenetic analysis of 47 carabid beetle species by building NJ tree based on Kimura-2-Parameters along with the bootstrap support values. Neighbor-Joining tree based on the K2P distance along with the pair-wise deletion of the gaps and the missing data within the sequences of the beetles was used by Jung *et al., (*2016). Phylogenetic analysis of 42 insect species has been attempted by Jalali *et al.,* (2015), by building NJ tree using Bootstrap Test phylogeny depicting the genetic relationships of the COI sequences derived from the 42 insect species. Rodrigues and workers (2017) used Geneious software 8.1.6 for manual editing of the sequences and aligned the sequences by using the Clustal X version 2.1 for multiple alignments and also applied the Neighbor-Joining tree and Kimura-2-Parameter model as well as the bootstrap support values for the phylogenetic analysis of the sequences of *T. cruzi.* Barcodes of some common spider species were generated by Tahir *et al.,* 2016 and the sequences were also submitted to the GenBank databases for their online access to other researchers. For the phylogenetic analysis of 15 insect pest species of South India, Karthika *et al.,* 2016 used the Neighbor-Joining Tree formed by the GTR+G model and also performed the ML analysis which was found to be the best fit model for nucleotide substitution which was evaluated based on the ALCc value. Ha *et al.,* 2018 produced the sequences from the processed fishes and also compared the sequences with the sequences already present in the NCBI. Singhal and workers, 2018 produced a phylogenetic tree for phylogenetic analysis of the 54 species of coleoptera. Phylogenetic analysis of the Japanese click beetles revealed that the majority of the genera contain multiple species belonging to monophyletic groups (Oba *et al.,* 2015). Maximum literature reviewed for this research work was found to have employed Neighbor-Joining phylogenetic tree along with the Kimura-2-Parameter genetic distance method and the Bootstrap support value for the phylogenetic analysis and hence study under reference here has followed suit.

## Summary and Conclusion

The present study was the first approach for carrying out molecular characterization of beetles of Jammu region of UT of J&K. The use of mitochondrial cytochrome C oxidase 1 gene (COI) as molecular marker was proved effective in delimitation of species under reference. The current study is the first step towards building an online reference DNA barcode library which will help even a non-taxonomist for identifying the species easily without learning any morphology-based taxonomic approach. The work will be helpful to future works concerning molecular identification of coleoptera species from the region.

### Limitation of the Study

Small number of the species was subjected to the molecular characterization. A comprehensive approach should be followed in future to include as many as possible number of species from the region.

### Conflict of Interest

The authors declared no conflict of interest among them.

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