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Cytochrome Oxidase Subunit II: Potential Marker for the Identification of Forensically Significant Species of Coleoptera—A Preliminary Study

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Abstract: The foremost concern in forensic entomology is the explicit identification of the species recovered from the crime scene. From the different orders of insects, Diptera is the prime focus in this field, followed by Coleoptera, whose identification can be extremely helpful for corpses in later decomposition stages. In this study, cytochrome oxidase subunit II (COII) was used to check its adequacy as a genetic marker and to create a reference database for eleven species belonging to five families of Coleoptera, namely, Silphidae, Staphylinidae, Histeridae, Dermestidae and Scarabaeidae, from two different states in India to assist in the accurate identification of imperative beetle species in medico-legal entomology. To achieve this, standard protocols of DNA isolation, amplification and sequencing were followed. We concluded that the COII gene can be used as a molecular marker for the identification of forensically relevant species, as observed from the similarities between the phylogenetic relationship constructed by COII and morphological data.

Keywords: beetles; COII gene; forensic entomology; Coleoptera; species identification

1. Introduction

In forensic entomology, estimation of the minimum postmortem interval (mPMI) is an essential component in the investigation of unnatural death [1] and the accurate identification of an insect specimen is usually the most crucial step in forensic entomological analysis for mPMI estimation [2]. For this estimation, fast and accurate species identification is needed, which poses a problem for forensic investigators [3]. Although identification keys are available, only a few experts can identify the immature stages of forensically relevant insects up to the species level, whereas the time-consuming rearing of larvae to adults for identification may delay the criminal investigation. In recent years, the use of DNA has been popularized due to its specificity, especially mitochondrial DNA. Wells and Williams [4] validated molecular methods for the identification of blow flies of Calliphoridae Chrysomyinae. They performed a phylogenetic analysis of mitochondrial gene for cytochrome oxidase one (COI). COI sequences were obtained from 245 newly

sequenced specimens and 51 specimens from the literature. They validated this molecular marker as an accurate and vigorous technique for insect identification. However, to select a molecular marker, it is important that the chosen fragment is easy to isolate and amplify and has a mutation rate fast enough to allow for species discrimination, but sufficiently slow to minimize intraspecific variation [5,6]. Cytochrome oxidase II sequence analysis offers ample phylogenetically informative nucleotide substitutions that facilitate the identification of Coleoptera at the species level.

Out of the two most forensically important orders, most of the research has focused on flies, as they are early colonizers on a corpse, and beetles (Coleoptera) have been underemphasized [3], although they are the second-largest order of forensic interest [7,8] and can support the mPMI estimation along with Diptera data [9]. Coleoptera are very diverse, both taxonomically and ecologically; little molecular knowledge is available about this group [10]. Some molecular studies have been conducted on beetles in the forensic context using various markers such as COI, COII, 16s rRNA, ITS 2 and cytochrome b [7,8,11–19]. Most of these studies were based upon COI, given that it is highly conserved at the species level. A few coleopteran species, which are detritivorous in nature, contribute to the decay of a corpse in the case of mummification. Therefore, they provide the main entomological evidence when the skeletonized corpse is found [11].

In India, forensic entomology is a growing field, with most studies concentrating on insect succession, along with their composition, development, and toxicology, with few studies being related to the actual case studies. The molecular approach is limited to Diptera identification [20–24] to the best of our knowledge. Although the COI gene is the most widely used tool in the identification of sibling and cryptic species, phylogenetic relationships based on the COII gene were found to be more consistent with the morphological data, which made this gene a reliable molecular marker [25]. The present study was, therefore, concentrated on the DNA-based identification of forensically important beetles from India and the creation of a COII gene database to aid in future forensic investigations and research.

2. Materials and Methods

2.1. Specimen Collection

This study included 150 specimens of 10 species belonging to 8 genera and 5 families of the order Coleoptera (Table 1 and Figure 1. All the adult specimens were collected from five districts in the Northern Indian states of Himachal Pradesh and Punjab (Figure 2), either by placing bait traps or from dead animal farms. Dead animal farms are the open-space areas used to dispose animal carcasses, mostly cattle, where various tasks such as the skinning of carcasses and cleaning of bones are carried out. Prior to molecular study, specimens were identified with the help of various morphological keys to validate that molecular identification is accurate [26–32]. Specimens were stored in 95% alcohol. From 8 to 10 specimens per species were utilized for DNA extraction, except for *Necrophila* (*Calosilpha*) *ioptera*, *Necrophila* (*Calosilpha*) *cyvaniventris* and *Creophilus flavipennis,* for which only 2–3 specimens were available.

2.2. DNA Extraction

Genomic DNA was extracted from adult beetles by 2% CTAB method [33] with some modifications, which mostly included a change in the quantity of reagents used during extraction. For large specimens, legs were used for DNA extraction, while in the case of small specimens, the whole specimen, excluding abdomen, was used.

Table 1. Details of species included in this study, their locality and accession number.

Species	Locality	Collected by	Accession No.	Date of Collection
Silphidae				
Thanatophilus minutus *	Kullu, Himachal Pradesh	N.Singh	MH023411	5/26/2014
Necrophila (Calosilpha) Ioptera *	Kullu, Himachal Pradesh	N.Singh	MH785185	5/26/2014
Necrophila (Calosilpha) cyvaniventris *	Kullu, Himachal Pradesh	N.Singh	MG958644	5/27/2014
Necrophila (Deutosilpha) Rufithorax *	Gagret, Himachal Pradesh	N.Singh	MG940969	5/27/2014
			MG772614	6/10/2014
			MH796140	6/10/2014
Histeridae				
Saprinus interruptus *	Gagret, Himachal Pradesh	N.Singh	MH023410	6/10/2014
			MH785186	6/10/2014
Staphylinidae			MG958643	6/30/2015
Aleochara nigra	Manali, Himachal Pradesh	N.Singh	MH777400	6/30/2015
Creophilus maxillosus	Kullu, Himachal Pradesh	N.Singh	MG958645	5/26/2014
Creophilus flavipennis *	Hamirpur, Himachal Pradesh	N.Singh	MH778535	5/26/2014
			MG940970	7/6/2015
Dermestidae			MG891837	4/10/2014
Dermestes maculatus	Patiala, Punjab	N.Singh	MH764581	4/10/2014
			MH814725	4/10/2014
Scarabaeidae			MH777399	5/26/2014
Onthophagus cervus *	Kullu, Himachal Pradesh	N.Singh	MH814724	5/26/2014
			MG920500	6/10/2015

* Reference data for these sequences were not available at the time of submission in GenBank.

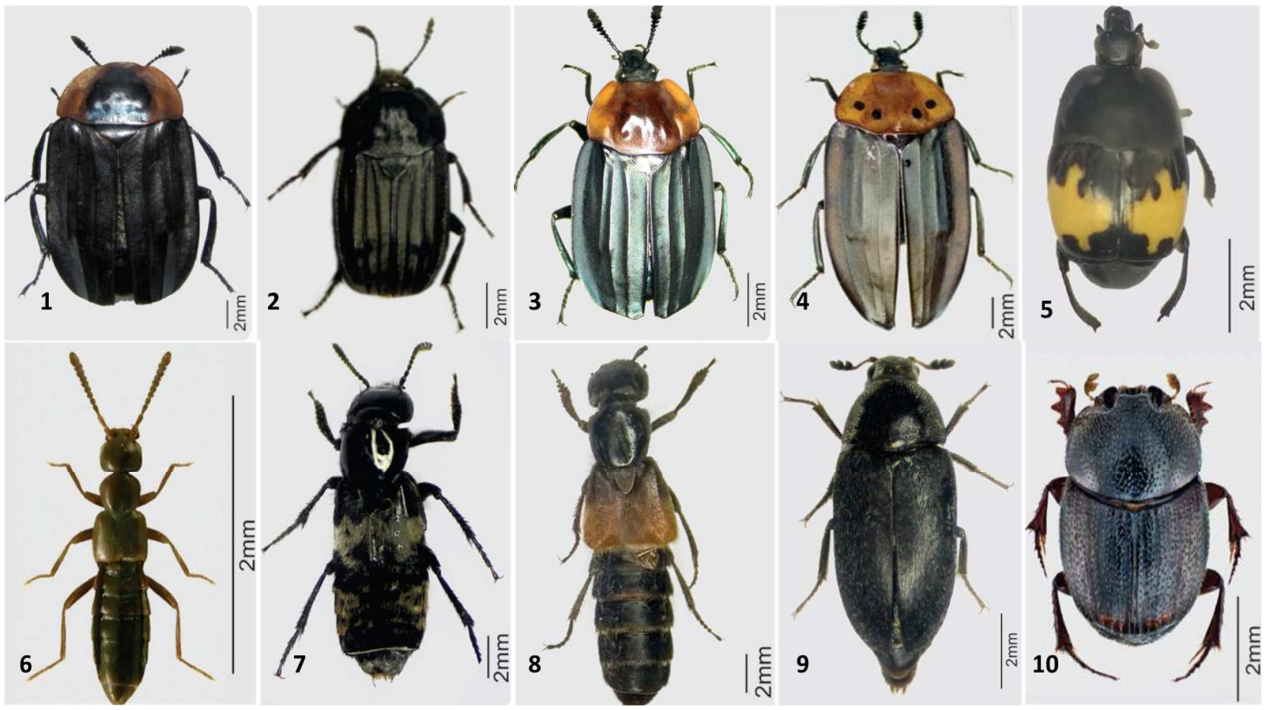

Figure 1. Coleoptera species collected and identified during the present study 1. *Thanatophilus minutes* (Kraatz) 2. *Necrophila* (*Calosilpha*) *cyvaniventris* (Motschulsky) 3. *Necrophila* (*Calosilpha*) *ioptera* (Kollar and Redtenbacher) 4. *Necrophila* (*Deutosilpha*) *rufithorax* (Wiedemann) 5. *Saprinus interruptus* (Paykull) 6. *Aleochara nigra* Kraatz 7. *Creophilus maxillosus* (Linnaeus) 8. *Creophilus flavipennis* (Hope) 9. *Dermestes maculatus* Brahm 10. *Onthophagus cervus* (Fabricius).

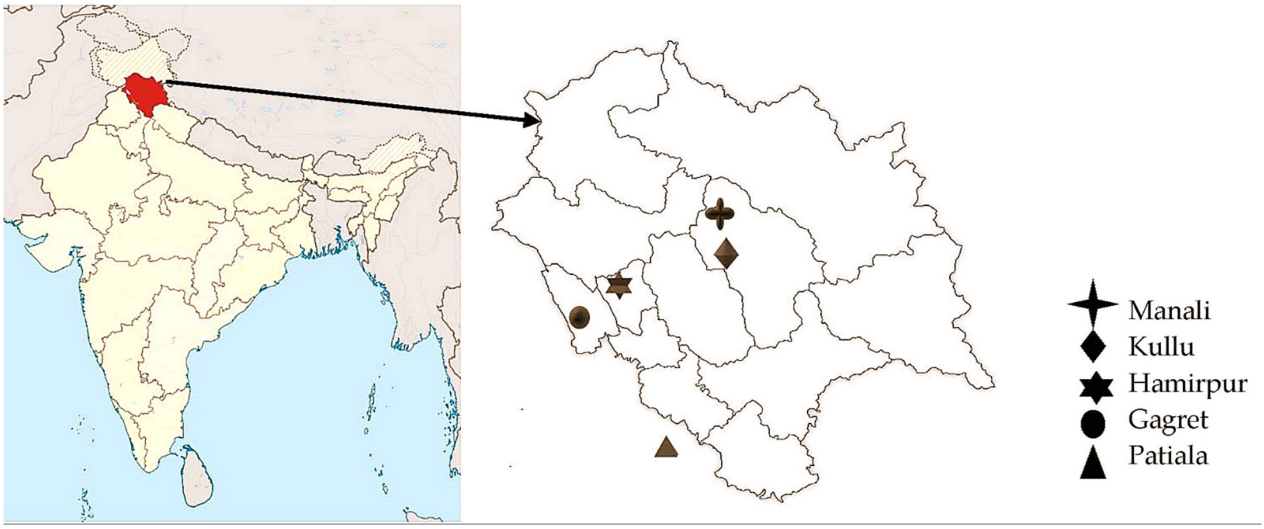

Figure 2. Map showing collection localities of beetle species.

2.3. DNA Amplification and Sequencing

Mitochondrial COII gene sequences were amplified with the aid of primers from DNA isolated from different beetle species. The details of the forward and reverse primers used are given in Table 2. Each PCR cocktail consisted of 4 µL of 10X of Taq Buffer, 1.6 µL of $MgCl_2$ (50 mM), 3.2 µL of dNTPs (10 mM), 0.6 µL of each primer (10 pmol/µL), 2 µL of DNA template (10–20 ng), and the rest was double-distilled water, to obtain a final volume of 40 µL. All DNA amplifications were carried out in a thermal cycler (Biometra, Göttingen, Germany) using the PCR conditions, including initial denaturation at 94 °C (5 min), followed by 35 cycles of denaturation at 94 °C (60 s), annealing at 45 °C (45 s) and extension at 72 °C (60 s). Final extension of amplified products was carried out at 72 °C (7 min) to complete the reaction. The amplified products were first cleaned with Gel/PCR DNA fragments extraction kit (Cat.No. IB47020) following manufacturer's protocol in order to preserve the quality and quantity of DNA, and were sent to Chromous Biotech. Pvt. Ltd. (Bengaluru, India) for sequencing. The crude sequences were aligned in BioEdit Sequence Alignment Editor; after eliminating noise, sequences were submitted to GenBank using the BankIt submission tool.

Table 2. Primer sequences used to amplify COII gene.

Primer Name	Sequence (5′—3′)	Length	Reference
Forward TL2J 3037	ATGGCAGATTAGTGCAATGG	20	O'Grady (1999) [34]
Reverse TKN 3785	GTTTAAGAGACCAGTACTTG	20	Liu and Beckenbach (1992) [35]

2.4. Phylogenetic Analysis

All sequences were aligned in Clustal Omega software(Version:1.2.2., European Bioinformatics Institute, Cambridge, United Kingdom [36] and compared with the available reference sequences in GenBank maintained by NCBI by using BLAST to confirm the identification carried out with the help of traditional methods. Non-overlapping regions of the sequences were trimmed, and aligned sequences were used for pairwise divergence and phylogenetic analysis in Mega6 [37]. Neighbor-joining (NJ), a distance-based method, and maximum likelihood (ML), a character-based method, were used to study the lineage history using the Kimura-2-parameter model [38]. For all the methods, bootstrap values were calculated with 1000 replicates [34] to test the reliability of the derived trees. Two species from suborder Adephaga, viz. *Anisodactylus poeciloides* (EU839552.1) and *Dyschirius salinus* (EU839539.1), were taken as outgroups.

3. Results

3.1. Amplification of Mitochondrial COII Gene

The amplification of mitochondrial COII gene obtained a product of ~780 bp (Figure 2). The COII gene sequences from different beetles were aligned with NCBI, which suggested that the beetles belong to 11 different species with five distinct families. The respective accession numbers for the COII gene sequence of each beetle was obtained from NCBI (Table 1).

3.2. Divergence and Nucleotide Composition

Conspecifics showed <3% divergence (range: 0–2.8%). For some species, such as *Necrophila* (*Calosilpha*) *ioptera*, *Necrophila* (*Calosilpha*) *cyvaniventris* and *Creophilus flavipennis*, single sequences were present; hence, it was impossible to determine the intraspecific variation (Table 3).

Table 3. Intraspecific divergences between beetle specimens using the COII gene.

Species (Accession No.)	Species (Accession No.)	Intraspecific Divergence
Creophilus maxillosus MG958645	*Creophilus maxillosus* # GQ118443	10.6%
Dermestes maculatus MG891837	*Dermestes maculatus* # NC_037200	11.5%
Aleochara nigra MG958643	*Aleochara nigra* # AJ293051	12.8

Sequences retrieved from GenBank.

The lowest divergence of 0.6% was observed between *Creophilus maxillosus* (MG958645) and *C. maxillosus* (GQ118443.1) submitted from China. *Dermestes maculatus* (MG891837) sequences from the present study showed a variation of 1.5% with *D. maculatus* (NC_037200.1) submitted from South Korea. The highest intraspecific divergence (2.8%) was found between *Aleochara nigra* (MG958643) of the present study and *A. nigra* (AJ293051.1) submitted from Taiwan.

The interspecific divergence among the beetle species varied from 4.7 to 9.7% Due to the lack of GenBank data for many studied species, interspecific divergence was only studied for six species. *Necrophila (Deutosilpha) rufithorax* was compared with *Necrophila (Calosilpha) ioptera* and *Necrophila (Calosilpha) cyvaniventris* and a divergence of 7.3% and 8.3% was found; *Necrophila (Calosilpha) ioptera* and *Necrophila (Calosilpha) cyvaniventris* showed a divergence of 6.5% (Table 4).

Table 4. Interspecific divergences between beetle species using COII gene.

Species (Accession No.)	Species (Accession No.)	Intraspecific Divergence
Onthophagus cervus MH777399	*Onthophagus* cf. *taurinus* # KU739462	14.7%
Onthophagus cervus MH777399	*Onthophagus obscurior* # KU739477	19.6%
Creophilus flavipennis MG940970	*Creophilus maxillosus* # GQ118443	18.9%
Creophilus flavipennis MG940970	*Creophilus maxillosus* MG958645	9.7%
Necrophila (Deutosilpha) rufithorax MG772614	*Necrophila (Calosilpha) ioptera* MG958644	7.3%
Necrophila (Deutosilpha) rufithorax MG772614	*Necrophila (Calosilpha) cyvaniventris* MG940969	8.3%
Necrophila (Calosilpha) ioptera MG958644	*Necrophila (Calosilpha) cyvaniventris* MG940969	6.5%

Sequences retrieved from GenBank.

Creophilus flavipennis (MG940970) of the present study and *Creophilus maxillosus* (GQ 118443.1) submitted from China showed 8.9% divergence, while *C. maxillosus* (MG958645) and *C. flavipennis* (MG940970) showed a variation of 9.7%.

Three sequences of *Onthophagus cervus* were compared with *Onthophagus* cf. *taurinus* (KU739462.1) submitted from Cambodia, and *Onthophagus obscurior* (KU739477.1) from Indonesia. Interspecific divergence ranged from a minimum of 4.7% (*O.cervus* and *O. taurinus*) to a maximum of 9.6% (*O.cervus* and *O. obscurior*). As for the nucleotide composition, the obtained sequences were AT-rich, with an average AT of 75.10% and GC of 24.90%, in accordance with previous studies conducted on the COII gene of beetles [13,35]. AT bias was observed in all the positions, but was most extreme in the third position (A = 38.6% and T = 44%).

3.3. Phylogenetic Analysis

The evolutionary history of the studied taxa was inferred using neighbour joining and maximum likelihood methods, which resulted in congruent trees. Phylogenetic trees (Figures 3 and 4 showed the formation of four major monophyletic clades. The grouping of all species of family Staphylinidae under study in a single clade was observed. As for *Creophilus flavipennis*, phylogeny allows for confirmation at the genus level. Conspecifics belonging to the genus *Onthophagus* formed a separate cluster, showing a close relationship with other congeneric species. The species of the Silphidae family were assembled in a single clade, while *Saprinus interruptus* formed a separate clade. No reference sequence of *S. interruptus* was found for comparison, and this is the first report of the COII gene of *S. interruptus*, so their morphological identification was considered accurate.

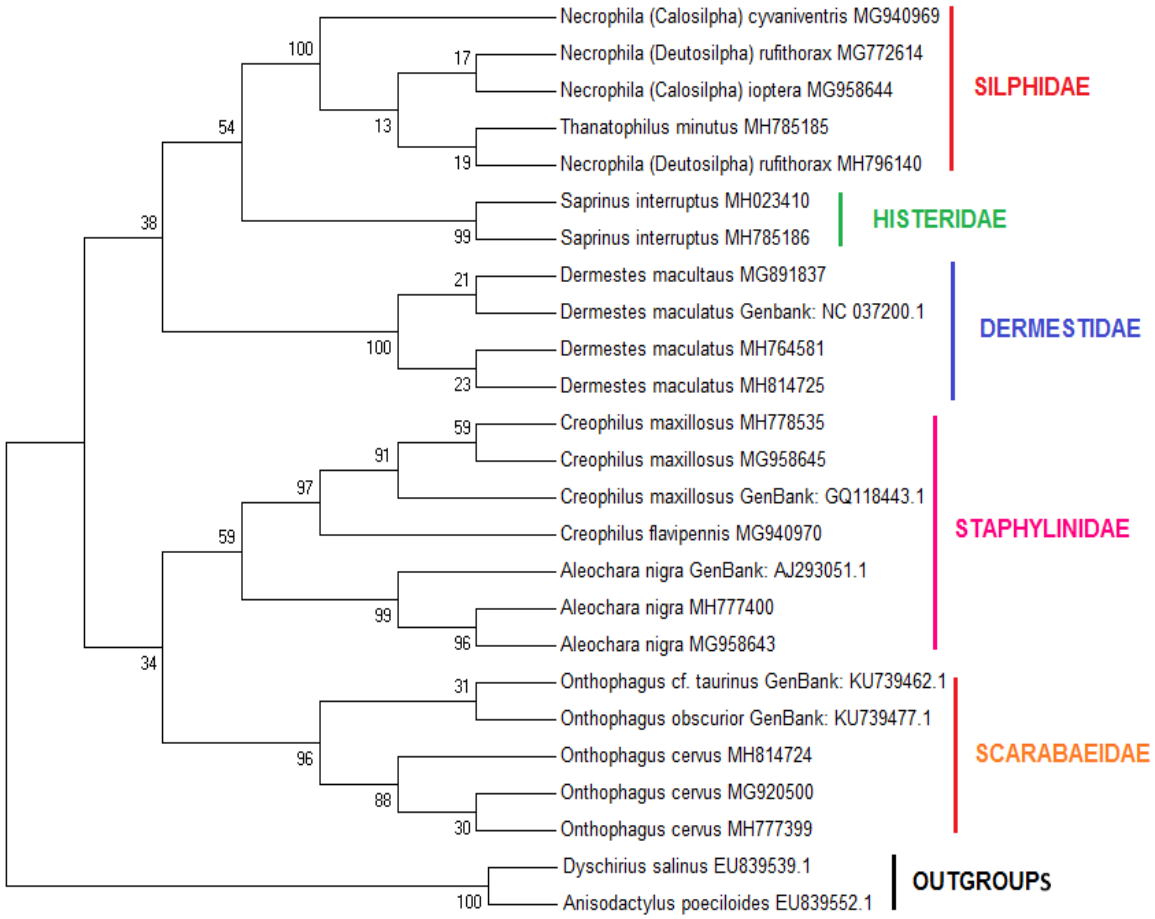

Figure 3. Neighbor joining (NJ) for beetle species of forensic interest in India inferred from COII gene sequence.

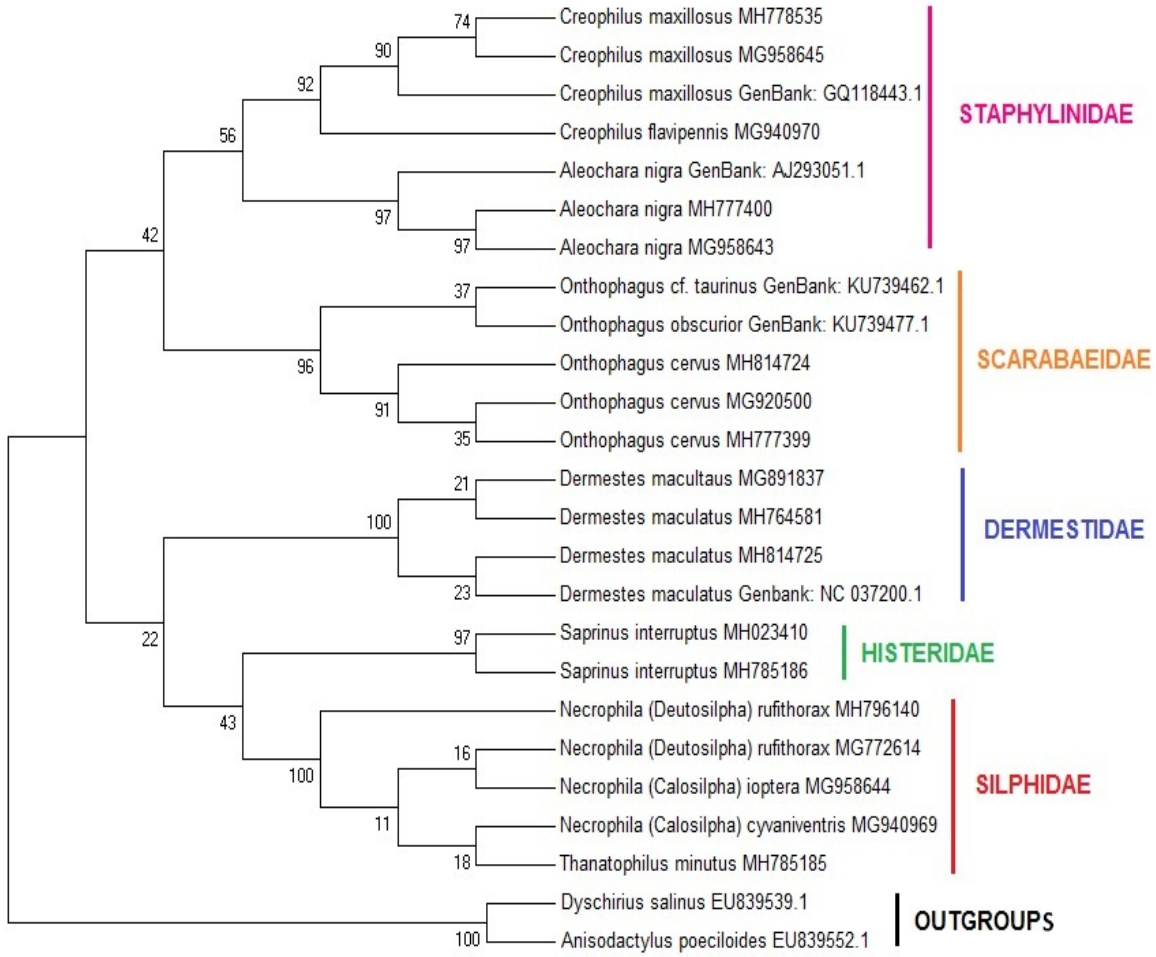

Figure 4. Maximum likelihood (ML) for beetle species of forensic interest in India inferred from COII gene sequence.

4. Discussion

Our study is the first attempt at the molecular identification of forensically relevant beetle species from northern India. The present study aimed to generate a mitochondrial COII gene molecular database of forensically important beetles, which could be of great value to the field of forensic entomology, as they all share a common habitat of decaying organic matter. The similarity between the classical morphological taxonomy and molecular phylogeny in this study validates the COII gene as a potential marker to distinguish between species, as the phylogenetic relationships reconstructed with the help of COII were found to be similar to the morphological data.

According to Hebert et al. [39], intraspecific variation should not exceed 3%, while interspecific variation needs to exceed that threshold value to allow for species differentiation. We found low intraspecific and high interspecific divergences for all the species and no overlap between intraspecific and interspecific divergences. Hence, it is safe to assume that the COII gene sequences have sufficient discriminatory power for accurate species identification. As far as phylogeny is concerned, data were analyzed using the two most frequent methods and a similar topology was observed among the trees, with slight differences in bootstrap values. The small size of our data, and the lack of enough data from GenBank, made it impossible to infer any conclusions related to barcoding gaps.

Most of the clades in our analysis form distinguished branches with a bootstrap value of >90% at family level. At the genus or species level, some of the bootstrap values were below 50, but species were correctly assigned to their genus, with only one exception, Silphidae, for which further research is needed. An important caveat in insect molecular

identification is that entire insect biomes can be affected by disruptive *Wolbachia* genetic expression and horizontal gene transfer (HGT), leading to instability. In a study by Whiteworth et al. [40], they noticed 12 species of the blow-fly of genus *Protocalliphora*, known to be infected with the endosymbiotic bacteria *Wolbachia*. They found that the barcoding approach showed very limited success in accurate species identification for 60% of the species. This very low success rate of the barcoding approach is due to the non-monophyly of many of the species at the mitochondrial level. *Wolbachia* is known to infect between 15 and 75% of insect species; therefore, identification at the species level based on mitochondrial sequence might not be possible for many insect species.

5. Conclusions

COII gene sequence analysis has the power to accurately delimit species; however, a lot of work is needed to create a database for distinct families of forensically important insects. To achieve this, multiple specimens of a species should be studied, using efficient methods for DNA extraction and amplification, as well as the sequencing of different gene/molecular markers. Developing suitable amplification methods and integrated identification protocols is in the best interest of forensic entomology. More extensive studies, with a larger COII gene sample size, are needed to explore this marker's usefulness in insect identification in forensic entomology. Having numerous sequences in the Gen bank is a powerful tool in forensic entomology.

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Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

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Conflicts of Interest: The authors declare no conflict of interest.

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