

DNA Barcoding and Evolutionary Lineage of Some Economically Important Scarabaeid Beetles in South India

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Abstract

Proper identification of the scarabaeid beetle's species and knowledge of their bio ecology is essential for developing environmentally compatible integrated pest management strategies. Lack of taxonomic understanding has been a major impediment to the study and management of scarabaeid beetles. Identification of scarabaeid species is a challenging task due to variable morphological differences among species and delineation among the immature forms, the grubs and adults. DNA barcoding facilitates prompt identification of the pest utilizing fragmentary body parts. Barcodes were generated to identify the scarabaeid beetles from various geographical locations in South India, based on the mitochondrial Cytochrome c oxidase I (COI) gene (648-656 bp size). Genomic DNA of 23 scarabaeid beetles obtained from different geographical locations were characterized and a total of 19 barcodes were generated with Barcode Identity Number (BIN) in Bold Systems V3 database. Phylogeny for evolutionary relationship and divergence was assessed using MEGA (Molecular Evolutionary Genetic Analysis) program and Neighbor -Joining (NJ) methods, the overall mean distance was 0.215% and the pairwise genetic distance ranged from 0.000 to 0.384 The phylogeny revealed formation of distinct species-specific clusters. Nucleotide composition, genetic variations and sequences similarities were worked out. The composition of the mitochondrial sequence of the COI gene in the present study was expectedly AT biased. Molecular sequence information from NCBI revealed relatedness in all the collected scarabaeids, accurately as revealed by their morphological characters. The implications of the information generated for species delineation of scarabaeid beetles and increased accuracy in their management is presented

Keywords: Barcoding; COI; DNA; Phylogeny; Scarabaeid Beetles

Introduction

Scarabaeidae is the second largest family within the order *Coleoptera*, and is cosmopolitan in distribution [1]. Scarab beetles are the most diverse and widely distributed insects which belong to the largest order *Coleoptera* under superfamily *Scarabaeoidea*, the family *Scarabaeidae* is composed of about 91% of all scarabaeoids and represented by 30,000 species worldwide [2,3]. Maximum numbers occur in the tropical areas of the world, particularly in the African and Oriental regions.

About 2500 species are reported from India [4] and a majority of these are phytophagous (sub families *Melonthinae*, *Rutelinae*,

Dynastinae and *Cetoniinae*) [2,5]. The adult beetles and their grubs cause extensive damage to fruit crops, vegetables, ornamental plants, plantation crops pastures, turf and meadow grasses, lawns, golf courses and forest trees [6-8]. Adults of the sub-family *Melolonthinae* and *Rutelinae* are pre-dominantly leaf feeders [8,9] where as those of *Cetoniinae* feed on flowers and fruits, and are popularly referred to as flower beetles, prefer nectar, sap or juice of ripening fruits and vegetables. Members of *Dynastinae* usually attack stems or roots of plants. Grubs of *Melolonthinae*, *Rutelinae* and *Dynastinae* commonly referred to as white grubs are often soil dwelling and cause extensive damage to the roots of cereals, legumes, small fruit plants, shrubs and trees [10-12] In India, the white grubs are pests of national importance [13,14]. The scarab fauna of India is very rich and diverse and has not been explored to a greater extent.

An authentic classification of species is a pre-requisite for research in ecology and biodiversity. Lack of taxonomic understanding has been a major impediment to the study and management of scarabaeid beetles. Proper identification of the species and knowledge of their distribution, geographical variation, population dynamics, feeding and reproductive behavior are the first steps in developing environmentally compatible/sustainable integrated pest management strategies. Identification of scarabaeid species is a challenging task due to variable morphological differences among species and delineation among the immature forms, the grubs and adults. Morphological identification keys are typically time consuming and are often effective only for a particular life stage or gender [15,16]. It also demands a high level of expertise in order to avoid misidentifications, as error in identifying species could lead to ineffective control measures for the pest and damage to crops may potentially increase [17]. Therefore, molecular method for identification is employed as it is rapid and reliable [18-20].

The use of taxonomic keys often requires proficiency to avoid inaccuracy for those similarities which cannot be easily deciphered. The indistinctness due to high similarity in morphology is overcome by application of molecular techniques. In the identification of scarabaeid beetles, although identification keys are available, uncertainties are still common especially for many unexplored species from the tropics [21]. Molecular markers such as Internal Transcribed Spacers (ITS-2), 16S rDNA, NADH dehydrogenase subunit 1 (nadh 1), Cytochrome b (cyt b) and Cytochrome c oxidase I subunit (COI) for effective identification, linking the grubs and adults and species delineation have been reported [17,22,23].

Taxonomic data basing [24] and species richness of scarabaeid beetles needs to be ascertained due to the diminution of biodiversity. Additionally, phylogenetic analyses of evolutionary relationships may lead to increased accuracy of insecticide management on the basis that related species are likely to share similar physiology. In the case of white grubs, linking larvae with the adult based species delineation that require authenticated sequences is also possible through molecular approaches and integrative taxonomy.

The DNA analysis would also aid in generation of barcodes to construct on line libraries of barcode sequences that could be matched with all known species, leading to species discovery and divergent taxa available that may represent a new species. Such studies on soil arthropods in the Indian context are less well studied. The enormous number of species of these subterranean insect pests exemplifies the need for taxonomic system Complementary to morphometric diagnostics overcoming the limitations and ambiguities of the latter.

DNA barcoding is a cost effective and highly sensitive technique [16,25-27] in which a short standardized partial

mitochondrial DNA sequences, the Cytochrome c oxidase subunit (CO I) gene, is the key enzyme of aerobic metabolism with a 648-base pair fragment [15] and a highly conserved protein-coding gene is used as molecular marker for identification. They are smaller than nuclear genome and inherited maternally with rapid rates of mutation to easily differentiate the relatedness of the species and construct their phylogeny [28]. In the present study, DNA barcoding tool was used for identification of species. Mitochondrial COI region was examined for 23 species of scarabaeids collected from different locations in South India

Materials and Methods

Field survey and Collection of Scarabaeid Beetles

The diversity of scarab beetles depends on the availability of food for larvae and adult, weather conditions and soil type. Collection of scarab beetles was made randomly by hand picking and light trapping. Grubs were collected from a soil depth of 0.25-0.5 nm in cultivated fields. The beetles were collected during May-June which is the major activity period to assess the diversity. The populations were collected from different states and geographical locations of the country from various trees and crop plants (are canut, coconut, groundnut, mulberry, millets, neem, soybean, sugarcane and vegetables).

Collection of Adult Beetles Using Light Traps

Light traps were used for four months (May - September) to collect the beetle populations. The light traps were placed in the center of the fields at a height of about 3 meters above the ground and operated between 7:00 PM to 5:00 AM to attract the scarabaeid beetles which are positively heliotactic in nature. The light trap comprised of PVC plastic funnel of 25 cm in height, and 30 cm. diameter. The bottom diameter of the funnel was 5 cm. The rain shed cone for protecting the bulb was fixed at 17 cm above the funnel with the help of three white metal sheets. The diameter of the rain shed cone was 20 cm. The light source consisted of a 125-watt incandescent light bulb with copper wire choke. The light trap had three baffles (30 cm x 10 cm), placed at a uniform distance of 10 cm around the circumference of funnel. The baffles were fixed to emit light uniformly in all directions without any interference, when the beetles are attracted to light they collide with baffles and fall into the trap. A nylon bag was attached to the bottom of this funnel for collection of beetles. The collected beetles were preserved in a vial containing 70% alcohol and taken to the laboratory for morphological identification and a few samples stored at -80°C at the Division of Molecular Entomology, NBAIR-ICAR Bangalore, India for characterization, phylogeny studies and generation of barcodes.

Identification of the Beetles

The scarab adults and grubs collected from larvae collected

from different locations were identified up to the genus level at the Department of Entomology, University of Agricultural Sciences, Bangalore and the Division of Entomology, Indian Agricultural Research Institute, New Delhi, based on the keys and characters listed by [29]. Adult beetles were identified based on the morphological characters such as body size, coloration, surface sculpture and male genitalia, while the grubs were differentiated based on the color, size of the cephalic capsule, number and form of dorsal sensorial maculae of the last antennomere, distribution, stimulatory structures in the maxilla and mandible, raster pattern arrangement of bristles and hairs on the underside of the abdomen, shape of anal slit (crescent, Y shaped, strongly Y shaped), shape and size of the respiratory plates, proportions of each pair of legs and tarsungulus size [7,29].

DNA Extraction

Genomic DNA was isolated using modified Qiagen DNeasy blood tissue kit method. For extraction of DNA, the head was dissected from each of the specimens and collected in 1.5ml Eppendorf tube. It was then homogenized with 100 μ l of ATL buffer using autoclaved micro pestle. 10 μ l of proteinase K was added to it and kept for digestion at 56°C in water bath for 24 hours. Further 100 μ l AL buffer was added and incubated at 56°C for 10 minutes. After incubation the solution was then transferred to mini spin columns with subsequent addition of 100 μ l of 100% ethanol. These columns were then centrifuged at 8000 rpm for 5 minutes, the flow-through collected in the collection tube was discarded. Further the columns were subjected for wash centrifuged at 800 rpm for 5 minutes simultaneously with two different wash buffers present in kit i.e., AW1 and AW2. The DNA was then eluted by transferring the columns to fresh autoclaved 1.5ml Eppendorf tubes. The DNA was checked at 1% agarose gel and stored at -4°C.

DNA Quantification

The isolated DNA samples were quantified in order to find out the amount of DNA using Nanodrop Spectrophotometer. The absorption was measured at 260nm as the nitrogenous bases in DNA show strong absorption at this wavelength.

PCR amplification and sequencing of COI gene fragment. The extracted DNA samples were subjected to PCR amplification of 658bp cytochrome oxidase I (COI) gene fragment. The amplification was carried out using the universal CO I primers: COI forward (LCO1490) 5' GGTCAACAAATCATAAAGATATTGG 3' and CO I reverse (HCO2198) 5' TAACTTCAGGCTGACCAAAAAATCA 3' obtained from M/S Bioserve biotechnologies (India) Pvt Ltd. Amplification was performed in 0.2 ml PCR tubes with total volume of 25 μ l containing 2.5 μ l of template DNA, 4 μ l of GeNei™ 10X Taq buffer containing 15mM of MgCl₂, 2.5 μ l of GeNei™ 10mM dNTP mix, 1 μ l of forward primer (10pmol/ μ l), 1 μ l of reverse primer (10pmol/ μ l), 1 μ l of GeNei™ Taq DNA polymerase

(1U/ μ l) and 13 μ l of sterile water. Temperature conditions for amplification were as follows: initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes followed by 34 cycles and final extension at 72°C for 7 minutes were carried out in thermal-cycler (BioRad, USA). Amplification of DNA was then checked by running the samples on 1% agarose gel using 250bp DNA ladder and visualized in gel dock. The amplified products were then sent to commercial sequencing at M/S. Eurofins Pvt Ltd, Bangalore where the chain termination method was used for sequencing. The results were obtained in.ab1 file format and .txt format.

Sequence Analysis, Data Interpretation and Barcode Generations

The amplified products of COI gene were got sequenced at M/s. Eurofin Pvt Ltd, Bangalore. The sequence data's was submitted to NCBI and accession numbers were obtained. Consensus sequences of COI partial gene were multiple aligned using Clustal W (ver. 1.83) [27]. Neighbor joining phylogenetic tree was drawn by using 'Meg Align' program of 'Lasergene' software package (DNASTAR Inc., USA).

The COI gene sequence data was retrieved in the form of Chromatograms. Several individuals from each species were sequenced and chromatograms were subjected to VSQual to evaluate the reliability of the data, and good quality, fragments were used to construct a consensus sequence for each sample. Homology sequence alignment was carried out using Basic Local Alignment Search Tool (BLAST) from National Centre for Biological Information (NCBI) server and also, checked for insertions, deletions and frame shifts. Barcodes and the Barcode Identification Numbers (BIN'S) were obtained for species with Genbank accession numbers by submitting the sequences of identified species to BOLD systems V3 (<http://www.boldsystems.org/>).

Phylogenetic Analysis

Phylogenetic analysis was carried out in MEGA (Molecular Evolutionary Genetic Analysis) version 7.0.14 software and the constructed phylogenetic tree was visualized using tree viewer program [30]. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N. and Nei M. 1987) with the bootstrap of 1000 replicates (Felsenstein J) that represents the evolutionary history of the taxa analyzed [31]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Kimura 2-parameter methods were employed to compute the evolutionary distances [32] and are in the units of the number of base substitutions per site. Analysis involved 22 nucleotide sequences and codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated and there was a total of 133 positions in the final dataset.

Results and Discussion

A good quality of genomic DNA for all the 23 samples was isolated and these samples were further characterized by amplifying the genomic DNA using the universal COI primers. The amplified products were sequenced at M/S. Eurofins Pvt Ltd, Bangalore and the results obtained were edited to remove any errors and submitted to NCBI- Genbank Bank It in order to obtain the accession numbers (Table 1). A total of 19 Barcode sequences were generated out of 23 species to which BIN (Barcode Identification Number) ID's were assigned in the BOLD systems V3 Database.

Sl.No	Place	State	Code	Latitude	Longitude	Molecular identity	Sub-family	Genbank Accession no.	BIN
1	Dasarahalli	Karnataka	DAST SC-16	13.096°	77.835°	<i>Protaetia cuprea</i>	<i>Cetoniinae</i>	KT203778	ADA4139
2	Dasarahalli	Karnataka	DAST SC-14	13.096°	77.835°	<i>Holotrichia serrata</i>	<i>Melolonthinae</i>	KT254245	ADA3548
3	Dasarahalli	Karnataka	DAST SC-19	13.096°	77.835°	<i>Anomala ruficapilla</i>	<i>Rutelinae</i>	KT254246	AAP8590
4	Chikkabalapur	Karnataka	Chik SC-1	13.432°	77.728°	<i>Onthophagus nuchicornis</i>	<i>Scarabaeinae</i>	KT254248	AAZ7167
5	Yehalanka	Karnataka	Yella SC-1	13.135°	77.589°	<i>Exomala pallidipennis</i>	<i>Rutelinae</i>	KU317746	Not Available
6	Nandi Hills	Karnataka	Nand-SC-1	13.136°	75.64°	<i>Onthophagus coenobita</i>	<i>Scarabaeinae</i>	KU665394	ADA5611
7	Nandi Hills	Karnataka	Nand-SC-2	13.136°	75.64°	<i>Onthophagus coenobita</i>	<i>Scarabaeinae</i>	KU665395	ADA5611
8	Nandi Hills	Karnataka	Nand-SC-3	13.136°	75.64°	<i>Copris tripartitus</i>	<i>Scarabaeinae</i>	KU665396	ADA6490
9	Nandi Hills	Karnataka	Nand-SC-5	13.136°	75.64°	<i>Onthophagus coenobita</i>	<i>Scarabaeinae</i>	KU665397	ADA5611
10	chikkabalapur	Karnataka	Chikka SC-7	13.432°	77.728°	<i>Onthophagus nuchicornis</i>	<i>Scarabaeinae</i>	KU355552	AAZ7167
11	Chintamani	Karnataka	Chin-Sc-1	13.402°	78.055°	<i>Onthophagus nuchicornis</i>	<i>Scarabaeinae</i>	KU517667	AAZ7167
12	Rajankunte	Karnataka	Rajan-SC-1	12.971°	77.594°	<i>Onthophagus nuchicornis</i>	<i>Scarabaeinae</i>	KU517666	AAZ7167
13	Mudigere	Karnataka	Mudhi SC-2	13.136°	75.64°	<i>Onthophagus auritus</i>	<i>Scarabaeinae</i>	KU665398	ADA5841
14	Anekal	Tamil Nadu	Anekal SC-4	12.711°	77.691°	<i>Phyllopertha horticola</i>	<i>Rutelinae</i>	KT203779	ADA4139
15	Valpari	Tamil Nadu	Valam SC-1	10.323°	76.951°	<i>Exomala pallidipennis</i>	<i>Rutelinae</i>	KT203780	ADA4359
16	Krishnagiri	Tamil Nadu	Doddashiv SC-1	12.518°	78.213°	<i>Phyllopertha horticola</i>	<i>Rutelinae</i>	KU317744	ADA8332
17	Krishnagiri	Tamil Nadu	Doddashiv SC-2	12.518°	78.213°	<i>Phyllopertha horticola</i>	<i>Rutelinae</i>	KU317745	ADA8332

18	Theni	Tamil Nadu	ThenGB-1 (a)	10.010°	77.467°	<i>Anomala ruficapilla</i>	<i>Rutelinae</i>	KU517668	ADA6471
19	Theni	Tamil Nadu	Then GB-1(b)	10.010°	77.467°	<i>Anomala dimidiata</i>	<i>Rutelinae</i>	KU517664	Not Available
20	Theni	Tamil Nadu	Then GB-2(a)	10.010°	77.467°	<i>Calicnemis obesa</i>	<i>Dynastidae</i>	KU517665	ADA5610
21	Theni	Tamil Nadu	Then GB-2(b)	10.010°	77.467°	<i>Oryctes rhinoceros</i>	<i>Scarabaeinae</i>	KU517663	Not Available
22	Theni	Tamil Nadu	Then GB-3 (b)	10.010°	77.467°	<i>Hybosorus illigeri</i>	<i>Hybosoridae</i>	KU317747	Not Available
23	Gujarat	Gujarat	Guj Sc-4	22.258°	71.192°	<i>Adoretus flavus</i>	<i>Rutelinae</i>	KT254250	ADB2802

Table 1: Genbank accessions and barcode identity numbers of scarabaeid beetles collected from various locations.

Persusal of the data on the collection of scarabaeid beetles from the different geographical locations and crops and their identification had revealed the diversity of beetles in the country. An array of phytophagous beetles (Table 1) belonging to the subfamilies (*dynastinae*, *melolonthinae* and *rutelinae*) were recorded. Such a diversity was earlier reported from various locations of the country. [33], reported the diversity and relative abundance of pleurostrict *scarabaeidae* in the Achanakmar-Amkarkantak biosphere reserve in Chattisgarh state. About 22 species belonging to 11 genera and 6 subfamilies were reported from the region, while in Madhya Pradesh, 47 species were reported [6,34,35]. The genus *Anomala* predominated over among all the scarabeids in both the states. In Maharashtra, the occurrence of different species of *Holotrichia* was widespread on sugarcane, sorghum, groundnut and soybean crops in South konkan and Vidharba regions [34] and in Pune [36,37]. *Holotrichia serrata* was predominant among all the species recorded. In Himachal Pradesh, *Anomala* sp. followed by *Brahmina* sp. were dominant in Chamba, Kanra, Kullu and Shimla areas [38,39]. Congenial habitat, natural vegetation, food availability and appropriate soil type contribute to the diversity of *scarabaeids* and the species richness [40,41] In addition, climatological factors rainfall, humidity, temperature and wind velocity play a decisive role on the emergence, movement, distribution and bioecology of *scarabaeids* [3]. Knowledge on species diversity, abundance, richness and dominance through surveys would be helpful in planning strategies for conservation of natural enemies, habitat management, design and develop pest management strategies

Nucleotide Analysis

Nucleotide analysis of the sequences was carried out in order to find out the MCL (Maximum Composite Liklyhood) estimate of the pattern of nucleotide substitution, AT%, GC% and the AT

content at first, second and third codon position. The MCL pattern showed the probability of substitution (r) from one base (row) to another base (column) [42]. The sum of r values was made equal to 100. Rates of different transitional are shown in substitutions which were 18.79, 21.13, 9.83 and 8.97 and the transversional substitutions are given in (Table 2). The nucleotide frequencies are 27.99% (A), 40.02% (T/U), 18.63% (C), and 13.36% (G). The transition/ transversion rate ratios are $k_1 = 3.253$ (purines) and $k_2 = 2.558$ (pyrimidines). The overall transition/ transversion bias is $R = 1.288$, where $R = [A^*G^*k_1 + T^*C^*k_2]/[(A+G)^*(T+C)]$.

	A	T	C	G
A	-	8.26	3.84	8.97
T	5.78	-	9.83	2.76
C	5.78	21.13	-	2.76
G	18.79	8.26	3.84	-

Table 2: MCL estimate of the pattern of nucleotide substitution of COI sequences of scarabaeid beetles

The analysis also revealed that the percentage of AT was comparatively more i.e., 33.25% ranging between 29.8-34.9% than that of GC which is 16.8% with a minimum of 15.1% and maximum of 19.4% (Table 3), indicating that the sequences were AT biased. This difference was attributed to the AT percentage at different codon position. The AT content at first codon position ranged between 46-48% with average of 44%, and the AT percentage at second and third codon position is nearly invariant 27% and 28% respectively (Table 3). Therefore, higher genetic distance was observed at first codon position comparatively to the second and third codon position. The higher values at first codon position indicated that detailed studies on first codon position may reveal possible evolutionary information among the closely related species.

Identified species	AT%	GC%	First	Second	Third
<i>Protaetia cuprea</i> (DAST-SC-16)	29.8	20.2	36	24	29
<i>Phyllopertha horticola</i> (Anekal-SC-4)	34.9	15.1	47	29	29
<i>Exomala pallidipennis</i> (Valam-SC-1)	32.3	17.7	43	26	28
<i>Holotrichia serrata</i> (DAST-SC-14)	29.9	20.1	38	24	28
<i>Anomala ruficapilla</i> (DAST-SC-19)	33.8	16.2	45	28	28
<i>Onthophagus nuchicornis</i> (Chick-SC-1)	34.5	15.5	47	29	28
<i>Adoretus flavus</i> (Guj-SC-4)	34	16	47	27	29
<i>Phyllopertha horticola</i> (Doddashiv-SC-1)	34.9	15.1	47	29	29
<i>Phyllopertha horticola</i> (Doddashiv-SC-2)	34.5	15.5	45	28	29
<i>Exomala pallidipennis</i> (Yella-SC-1)	34.7	15.3	48	29	28
<i>Onthophagus auritus</i> (Mudhi-Sc-2)	33.8	16.2	46	27	28
<i>Onthophagus coenobita</i> (Nand-SC-1)	33.1	16.9	43	28	28
<i>Onthophagus coenobita</i> (Nand-SC-2)	32.9	17.1	43	28	28
<i>Copris tripartitus</i> (Nand-SC-3)	34.3	15.7	47	27	29
<i>Onthophagus coenobita</i> (Nand-SC-5)	32.9	17.1	43	27	28
<i>Onthophagus nuchicornis</i> (Chikka-SC-7)	34.3	15.7	47	28	28
<i>Onthophagus nuchicornis</i> (Chin-SC-1)	34.8	15.2	48	29	28
<i>Onthophagus nuchicornis</i> (Rajan-SC-1)	34.5	15.5	47	29	28
<i>Anomala ruficapilla</i> (Then-GB-1[a])	33.9	16.1	46	27	29
<i>Calicnemis obesa</i> (Then-GB-2[a])	30.7	19.3	38	26	28
<i>Oryctes rhinoceros</i> (Then-GB-2[b])	31.4	18.6	39	26	29
<i>Hybosorus illigeri</i> (Theni GB-3b)	30.6	19.4	38	26	28
MEAN	33.2	16.8	44	27	28

Table 3: AT%, GC% and AT% at first, second and third codon position of scarabaeid beetles

The utility of DNA data in taxonomy and species diagnosis in the scarabaeid beetles was reported by [43,44] based on the sequence variation in DNA based groups which was highly structured. The population of scarabaeids from various locations were characterized using Cytochrome C oxidase subunit I (COI) gene, which has been recognized as an effective marker not only for species identification and also for phylogenetic relationship [15,41,45]. In the present studies, the isolated genomic DNA of scarab beetles from various locations was characterized through COI gene fragment (648-656 bp size). The amplified gene was sequenced and the Blast done with NCBI database to decipher the identity of the scarabaeids from various locations and crops (Table-1). Molecular sequence information from NCBI revealed relatedness in all the collected scarabaeids, accurately as revealed by their morphological characters. Our observations, corroborate with the reports of [46-49,45,25]. [47], suggested that where sequence information is available in Genbank for morphologically defined species, which can be matched with some DNA based clusters, close relationship can be identified readily in sequence variation in field collected field samples and these clusters are likely to correspond to previously described unknown species [21,50]. reported that the sequence information based on mitochondrial markers can be utilized for species delineation of adults and grubs of scarabaeids inferring larval taxonomy. Our studies indicate the relevance of DNA sequencing to match different forms of scarabs and address the issues of having to depend exclusively on morphological features and avoid misdiagnosis.

Phylogenetic Analysis

Analysis involved 22 nucleotide sequences which involved the 1st+2nd+3rd+Noncoding codon positions. A total of 133 positions for comparison were obtained in the final dataset using Kimura 2-parameter method, where all the positions containing gaps and missing data were eliminated. The overall mean distance was 0.215% and the pairwise genetic distance ranged from 0.000 to 0.384 (Table 4). The lowest genetic variation was observed between *Hybosorus illigeri* (Theni-GB-3b) and *Oryctes rhinoceros* (Theni-GB-2[b]) i.e.,

0.008, whereas the highest genetic variation was 0.384 observed between *Onthophagus auritus* (Mudhi-Sc-2) with *Calcinemis obesa* (Theni-GB-2[a]) and *Oryctes rhinoceros* (Theni-GB-2[b]) (Table 4).

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
2	0.250																				
3	0.258	0.158																			
4	0.149	0.218	0.247																		
5	0.207	0.197	0.159	0.217																	
6	0.280	0.168	0.178	0.291	0.158																
7	0.216	0.197	0.187	0.217	0.131	0.159															
8	0.250	0.015	0.177	0.208	0.206	0.178	0.207														
9	0.250	0.015	0.177	0.208	0.206	0.178	0.207	0.000													
10	0.258	0.206	0.178	0.247	0.150	0.158	0.150	0.206	0.206												
11	0.313	0.197	0.208	0.296	0.237	0.159	0.197	0.206	0.206	0.227											
12	0.315	0.291	0.281	0.318	0.216	0.168	0.237	0.302	0.302	0.269	0.178										
13	0.315	0.291	0.281	0.318	0.216	0.168	0.237	0.302	0.302	0.269	0.178	0.000									
14	0.247	0.197	0.258	0.228	0.206	0.177	0.123	0.206	0.206	0.207	0.227	0.268	0.268								
15	0.315	0.291	0.281	0.318	0.216	0.168	0.237	0.302	0.302	0.269	0.178	0.000	0.000	0.268							
16	0.280	0.168	0.178	0.291	0.158	0.000	0.159	0.178	0.178	0.158	0.159	0.168	0.168	0.177	0.168						
17	0.280	0.168	0.178	0.291	0.158	0.000	0.159	0.178	0.178	0.158	0.159	0.168	0.168	0.177	0.168	0.000					
18	0.280	0.168	0.178	0.291	0.158	0.000	0.159	0.178	0.178	0.158	0.159	0.168	0.168	0.177	0.168	0.000	0.000				
19	0.247	0.168	0.237	0.259	0.187	0.227	0.159	0.187	0.187	0.197	0.177	0.248	0.248	0.217	0.248	0.227	0.227	0.227			
20	0.281	0.269	0.259	0.269	0.237	0.292	0.250	0.258	0.258	0.384	0.315	0.259	0.259	0.269	0.259	0.292	0.292	0.292	0.292	0.279	
21	0.281	0.269	0.259	0.269	0.237	0.292	0.250	0.258	0.258	0.384	0.315	0.259	0.259	0.269	0.259	0.292	0.292	0.292	0.292	0.279	0.000
22	0.270	0.258	0.248	0.258	0.227	0.280	0.239	0.247	0.247	0.372	0.303	0.270	0.270	0.258	0.270	0.280	0.280	0.280	0.268	0.008	0.008

Table 4: Pairwise genetic distance for partial COI sequences of scarabaeid beetles.

A phylogenetic tree of the species using Neighbour-Joining method [31] was drawn on the basis of multiple sequence alignment of COI gene. e tree elucidates of 22 concatenated samples of the COI genes from 12 different areas in Karnataka, Tamilnadu and Gujarat states. The phylogeny clearly indicated the phylogenetic generalized least squares (PGLS) and revealed formation of distinct species-specific clusters based on the geographical location of the scarabaeid population obtained. All the *Onthophagus nuchicornis* collected from parts of Karnataka were placed in the top cluster with the bootstrap value and branch length range of 19-100 and 0.00-0.10 respectively (Table 5).

Sl. No.	Organism name
1	<i>Protaetia cuprea</i> (DAST-SC-16)
2	<i>Phyllopertha horticola</i> (Aneka-SC-4)
3	<i>Exomala pallidipennis</i> (Valam-SC-1)
4	<i>Holotrichia serrata</i> (DAST-SC-14)
5	<i>Anomala ruficapilla</i> (DAST-SC-19)
6	<i>Onthophagus nuchicornis</i> (Chick-SC-1)
7	<i>Adoretus flavus</i> (Guj-SC-4)
8	<i>Phyllopertha horticola</i> (Doddashiv-SC-1)

9	<i>Phyllopertha horticola</i> (Doddashiv-SC-2)
10	<i>Exomala pallidipennis</i> (Yella-SC-1)
11	<i>Onthophagus auritus</i> (Mudhi-Sc-2)
12	<i>Onthophagus coenobita</i> (Nand-SC-1)
13	<i>Onthophagus coenobita</i> (Nand-SC-2)
14	<i>Copris tripartitus</i> (Nand-SC-3)
15	<i>Onthophagus coenobita</i> (Nand-SC-5)
16	<i>Onthophagus nuchicornis</i> (Chikka-SC-7)
17	<i>Onthophagus nuchicornis</i> (Chin-SC-1)
18	<i>Onthophagus nuchicornis</i> (Rajan-SC-1)
19	<i>Anomala ruficapilla</i> (Then-GB-1[a])
20	<i>Calcinemis obesa</i> (Then-GB-2[a])
21	<i>Oryctes rhinoceros</i> (Then-GB-2[b])
22	<i>Hybosorus illigeri</i> (Theni-GB-3b)

Table 5: Chronological order of the scarabaeid beetles as indicated in the similarity matrix.

The *Anomala ruficapilla* (sub family - *Rutelinae*) was clustered with *Exomala pallidipennis* (*Rutelinae*) and diversified

as a polyphyletic clade in the tree, the interesting observation among the polyphyletics was that one cluster was from Karnataka (cluster-2) whereas the other one was from Tamilnadu (cluster-4). The geo-specie specific cluster 2 and 4 was mediated by *Adoretus flavus* obtained from Gujarat which clustered with that of *Copris tripartitus* (*Scarabaeidae*) from Karnataka. Apart from the above said clusters (1-4), three samples of garden chafer *Phyllopertha horticola* were placed Cluster-5 which were all from Karnataka region.

Our findings, concur with the observations made by [16,51], on the significance of phylogeny based on DNA data. Weak phylogenetic foundation is related to issues like rare information about scarabaeid taxa at the family level. The classification of the world *dynastinae* is fairly well established, while in *melolonthinae*, *rutelinae* and *cetoniinae* that are poorly known taxonomically, new genera cannot be reliably identified. In the present study the species specificity might be due variations in the geographical location, the cropping pattern and the prevailing environmental conditions

Scarabaeidae species are often soil dwelling, their population densities and the associated damage risk probably depend on the site characteristics that influence soil temperature, soil moisture and humus content or the impact of management strategies imposed on the crop. The environmental conditions that are determining grub populations and the damage risk are fairly understood.

Nevertheless, a larger study of many populations and different genes may help reconstruction of the phylogeny and understand the evolutionary relationship. Further, phylogenetically, closely related species are likely to have a comparable physiology [15] which would facilitate precision management of the pest with insecticides. The present studies do not lead to exclusive inference, since a large number of scarabaeids are yet to be analyzed

COI barcoding has the added advantage of not being limited by polymorphism, sexual form (asexual/sexual) and life stages of the target species [42]. Overall rate of nucleotide substitution, ratio of two specific instantaneous rates of substitution rate at which transitions and transversions occur and the rate variation among sites play a significant role and are necessary for accurate reconstruction of phylogeny [12,36]. The present study was based on the molecular identification of scarabaeid beetles attacking crops and comprised of 23 nucleotide sequences were identified by COI.

Results indicated that the COI-based pest identification was extremely effective for the beetles based on the COI marker profile. Most of the phylogenetic information has been derived from mitochondrial DNA variations and recently DNA sequence data have been employed successfully to elucidate the relationships of many groups of insect species at generic level. The composition of the mitochondrial sequence of the COI gene in the present study was expectedly AT biased and this was generally observed in several previous studies [37]. In general, the frequency of transitional

substitutions is known to be higher than transversion substitutions in the genome [27], According to 10X rule the percentage of nucleotide divergence between the intraspecies should be less than 3% and that of interspecies should be more than 3%. Hence the sequences analyzed in the present study exhibited high inter species variability on the basis of nucleotide sequences. Therefore, the intra specific divergence was higher enough to discriminate between the individuals.

Molecular identification was done for several pests worldwide, in *Orius* (*Hemiptera: Anthocoridae*) [19] and potato flea beetles (*Coleoptera: Chrysomelidae*) [18], Japanese beetles [52] and other scarabaeids [53]. Moreover, discrimination of aphids of 32 species collected in various host plants in South India was also reported [49]. This investigation of COI barcoding could potentially be applied in agricultural and horticultural researches to rapid identification of pests. The phylogenetic signal is a direct function of the length of the branch (in units of the expected number of substitutions per site), which sheds light the evolutionary relationship [45].

DNA barcoding using COI genes could be an effective method for screening insect pests and to shed light on their genetic variations in addition the integration of traditional taxonomy especially for the scarabaeids where the species delineation and larval taxonomy is a challenging task Our findings contribute to a better understanding of the identification of pests by COI genes and aid in formulating better management strategies.

Conclusion

The diversity of phytophagous scarabaeid beetles from various geographical locations of India occurring in crops were morphologically identified and characterised using molecular tools, Molecular sequence information from NCBI revealed relatedness in all the collected scarabaeids, accurately as revealed by their morphological characters. Phylogenetic tree revealed the genetic relatedness among the beetles and understand the evolutionary relationship. The relevance of DNA sequencing to match different forms of beetles and address limitations in morphological identification is indicated. Knowledge on species diversity, abundance, richness and dominance through surveys would be helpful in planning strategies for conservation of natural enemies, habitat management, design and develop pest management strategies.

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