International Journal of Environment, Agriculture and Biotechnology, 5(4)
Jul-Aug, 2020 | Available: https://ijeab.com/

Molecular Identification of Phytophagous Scarabaeid from different regions of India

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Abstract*—Identification of scarabaeid beetles is a challenging task due to variable morphological differences among species and delineation among the immature forms, the grubs and adults. A method for easy and accurate species-level identification at any life stage is required. In this study, a 658-base pair region of the mitochondrial cytochrome oxidase I (COI) gene was used to explore its utility in the identification of important beetles. Twenty seven specimens were collected from 25 locations in different states, were characterized using specific primers for their identification. Sequence analysis and divergence among the species was assessed. The composition of the mitochondrial sequence of the COI gene in the present study was expectedly AT biased. Genbank accession numbers were obtained for the species. Molecular sequence information from NCBI revealed relatedness in all the collected scarabaeids, accurately as revealed by their morphological characters. The studies indicate the relevance of DNA sequencing to match different forms of beetles and address ambiguities in morphological identification and information on species diversity would help plan strategies for pest management.*

***Keywords*— Beetle, Characterisation, COI gene, Scarabaeid, Sequence.**

I. INTRODUCTION

Scarab beetles are the most diverse and widely distributed insects which belong to the largest order *Coleoptera.* The family *Scarabaeidae* is composed of about 91% of all scarabaeoids and represented by 30,000 species worldwide (Pathania *et.al*., 2015). About 2500 species are reported from India (Chandra and Gupta, 2011) and a majority of these are phytophagous (sub families *Melolonthinae, Rutelinae,Dynastiae* and *Cetoniinae*) (Ali, 2000., Dadmal *et.al.*, 2013). 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 (Dashad *et.al*., 2008 .,Lawerence *et.al*., 2013). Adults of the sub-family *Melolonthinae* and *Rutelinae* are pre-dominantly leaf feeders 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 (Bhat *et.al.*, 2005). 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 (Thakre and Zade, 2012). In India, the white grubs are pests of national importance (Chandra and Ahirwar, 2007., Chandra and Singh, 2010).

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. Species identification of larval specimens requires a sophisticated technique and also vast knowledge of the cephalopharyngeal skeleton morphology (Greenberg, 2002).

Morphological identification keys are often effective only for a particular life stage or gender (Hebert et.al.,2003., Chandra and Gupta, 2012., Gupta et.al., 2014.,). The use of taxonomic keys often requires proficiency to avoid inaccuracy for those similarities which cannot be easily deciphered. Under these circumstances, DNA analysis appears promising to solve the species identification problem owing to the durability and stability of the DNA

ISSN: 2456-1878
https://dx.doi.org/10.22161/ijeab.54.16

(Wallman and Donnellan, 2001). It also can solve the problems of morphological identification with damaged specimens (Judith and Nicola, 2008). This technique is based on the mitochondrial DNA (mtDNA) encoded cytochrome oxidase I gene (COI) (Wells and Sperling ,2001., Sharma *et.al*., 2004, Theurkar *et.al.*, 2013). Partial sequences of this COI gene have been shown to have sufficient discrimination power (Stijn and Matthias, 2009., Zahoor et.al., 2013), which makes it suitable for a diagnostic taxonomy. The COI gene has been used for inferring phenogram analysis at various taxonomic levels of many animal groups (Avise,2000). Efficient Mitochondrial DNA (mtDNA) -COI based methods in the delineation and identification of scarabaeid species have been reported (Blaxter et.al., 2004., Dittrich et.al., 2009, Dirk et.al., 2007, Dirk et.al., 2011, Tauz et.al., 2003, Fang, 2009., Paul et.al, 2009).

II. 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 0.25-0.05 m depth 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 (arecanut, 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 Genomic Resources, ICAR-NBAIR, India for characterization and identification.

Identification of the Beetles:

The scarab adults and grubs 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 (Dirk, *et.al.*, 2011). 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 (Dashad *et.al*, 2008, Dirk *et.al*, 2011).

Extraction of Genomic DNA:

Total genomic DNA was isolated using the method described by (Gavarāne *et.al*, 2011) . Genomic DNA was isolated using modified Qiagen DNeasy blood tissue kit method. The insects were washed thoroughly in double distilled water. Total genomic DNA was isolated from the leg portion of the insect. The cleaned insect leg portion was homogenized in 1.5 ml appendorf tube in 500 µl of TE (Tris-EDTA-pH 8), with hand pestle and the homogenate was centrifuged at 10,000 rpm for 10 minutes in cooling centrifuge (-40C). The supernatant was discarded and the pellet was dissolved in 500 µl of lysis buffer (400 µl of TE and 100 µl of 5% SDS), followed by the addition of 6 µl of Proteinase K, and the solution was incubated at 650C for one and half hours. A mixture of 120 µl phenol

chloroform isomyl alcohol (25:24:1) was added and the tubes vortexed for 30 seconds and then centrifuged for 10 minutes at 10,000 rpm in cooling centrifuge. The upper aqueous layer was carefully transferred in to fresh tube, without disturbing the protein layer at the interphase. Isopraponal (500 µl) was added to this aqueous layer and stored at -40C overnight and then centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 70% alcohol and later the acohol was drained out, the pellet was dried and dissolved in 30 µl of TE-I was stored at -200C after checking on 0.8% agarose gel and visualized after staining with ethidum bromide.

DNA Quantification- PCR amplification and sequencing of COI gene fragment:

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.

The extracted DNA samples were subjected to PCR amplification of 658bp cytochromeoxidase I (COI) gene fragment. The amplification was carried out using the universal CO I primers: C@I 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 µl containing 2.5 µl of template DNA, 4 µl of GeNeiTM 10X Taq buffer containing 15mM of MgCl , 2.5 µl of GeNieTM 10mM dNTP mix, 1 µl of forward primer (10pmol/µl), 1 µl of reverse primer (10pmol/µl), 1 µl of GeNieTM Taq DNA polymerase and 13 µl of sterile water. Temperature conditions for amplification were as follows: initial denaturation at 95^0C for 2 minutes, denaturation at 95^0 C for 30 seconds, annealing at 50^0C for 1 minute, extension at 72^0C for 2 minutes followed by 34 cycles and final extension at 72^0C for 7 minutes were carried out in thermal- cycler (BioRad, USA). PCR-amplified products were purified using Bioneer's PCR purification Kit (www.Bioneer.com). 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.

Sequence analysis and data interpretation:

The most commonly used method of DNA sequencing is the dideoxy method or chain termination method. The amplified products of COI gene were got sequenced at M/s. Eurofin Pvt Ltd, Bangalore. 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 (Binneck *et.al*., 2004) to evaluate the reliability of the data, and good quality fragments were used to construct a consensus sequence for each sample. Chromatograms were edited to discard ambiguous bases, and edited sequences were aligned by using the Basic Local Alignment Search Tool (BLAST), with the sequences of same or related genera retrieved from the nucleotide database (PUBMED) of National Centre for Biotechnology Information (NCBI). 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) (Thompson *et.al*., 1994., Tauz *et.al*, 2003).

III. RESULTS AND DISCUSSION

Molecular identification:

The genomic DNA was isolated from the populations of scarabaeids collected from different states viz, Andhra pradesh, Himachal Pradesh, Karnataka, Kerala, Meghalaya, Tamilnadu and Uttar Pradesh. 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 (Cetoniinae, Dynastinae, Melolonthinae and Rutelinae) were collected

In the present studies, the isolated genomic DNA of scarab beetles from various locations was characterised through COI gene fragment (648-656 bp size) was successfully sequenced for all the specimens, and the alignment of all specimens considered in this study lacked any insertion or deletion. A total of 27 species were sequenced over COI regions and the Blast done with NCBI database to decipher the identity of the scarabaeids from

various locations is given in 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 (Dittrich et.al., 2006, Zahur et.al., 2013, Wardani and Sugiyarto, 2009).

The utility of DNA data in taxonomy and species diagnosis in the scarabaeid beetles was reported by (Dirk et.al., 2007 and 2011), based on the sequence variation in DNA based groups which highly structured. The population of scarabaeids from various locations were characterised using Cytochrome C oxidase subunit I (COI) gene, which has been recognised as an effective marker not only for species identification but also for phylogenetic relationship (Hebert et al., 2003, Dhoj et.al., 2009, Maryati and Sugiyato, 2009, Imura et al., 2014).

Nucleotide Analysis:

Nucleotide analysis of the sequences was carried out in order to find out the MCL (Maximum Composite Likelihood) 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) (Khannal *et.al.*, 2012). 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 transversionsal 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).

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% . 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 between46-48%withaverageof44%,andtheATpercentage at second and third codon position is nearly invariant 27% and 28% respectively.

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 (Williams *et.al.*,, 1990). In general, the frequency of transitional substitutions is known to be higher than transversion substitutions in the genome (Tauz *et.al*, 2003), 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.

Results indicated that the COI-based pest identification was extremely effective for the beetles based on the COI marker profile. DNA sequence data have been employed successfully to elucidate the relationships of many groups of insect species at generic level. 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 (Richards *et.al*, 1997., Zhu *et.al*., 2000, Blaxter *et.al*., 2004.,Monti *et.al*, 2005). Qiu , *et.al*, (2009), 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. Mgocheki *et.al.*,(2012), 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.

The species diversity is influenced by the cropping pattern, the climatological factors and the altitude. The abundance was reported to be negatively and significantly correlated with altitude. Low temperatures at higher altitudes inhibit the growth and development of beetles (Dhoj *et.al.*, 2009., Khanal *et.al.*, 2012) .

Our findings contribute to a better understanding of the identification of pests by COI genes and aid in formulating better management strategies.

IV. CONCLUSIONS

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, through surveys would be helpful in planning strategies for conservation of natural enemies, habitat management, design and develop pest management strategies.

Table 1. Scarabaeid beetles collected from various locations and their accession numbers

Sl.No	Species	Subfamily	Location	Latitude/Longitude	Acc.No.
1	***Holotrichia consanguinea***	**Melolonthinae**	Anakapalle	17 .38. ^{0}N, 83.2 ^{0}E	KU35553
			Samarlakota	17 .5 ^{0}N, 82.2 ^{0}E	KU35552
			Tirupathi	13.65 ^{0}N, 79.42 ^{0}E	
2	***Holotrichia serrata***		Bangalore	12.97 ^{0}N., 77.57 ^{0}E	KT254245
3	***Schizonycha* sp.**		Shimoga	13.92° N, 75.56° E	1762749
4	***Maladera insanabilis***		Phasighat	25.34.11°N, 91°59.2E	KU35551
					KU35551
5	***Apogonia* sp.**		Aligarh (UP)	27.89° N, 78.08° E	1762764
6	***Leucopholis lepidophora***		Sringeri	12.57 -13.52 ^{0}N., 75.72 -75.22 ^{0}E	KU665428
			Shivamoga		KU665428
			Thirtahalli		KU665428
			Sirsi	14.6196° N, 74.84° E	KU665428
			Sultan Betheri (Kerala)	11.67 ^{0}N, 76.28 ^{0}E	KU665428
7	***Leucopholis burmeisteri***		Chikmagalur	13.40 ^{0}N., 78.05 ^{0}E	KU665432
			Belgaum	15.51 ^{0}N., 74.29 ^{0}E	KU665432
			Kannur (Kerala)	11.8 ^{0}N, 75.32 ^{0}E	KU665432
8	***Leucopholis coneophora***		Thrissur	10.52 ^{0}N, 76. 2 ^{0}E	KU665428
9	***Heterorrhina sp.***	**Cetoninae**	Ooty(TN)	11.41 ^{0}N., 70.58 ^{0}E	KM657485
10	***Protaetia* sp.**		Tirupathi (AP)		KM657490

			Phasighat	28.069° N, 95.32° E	KT1762766
			Shillong	25.34.11°N, 91°59.2E	KM657489
			Ooty (TN)	11.41 ^{0} N., 70.58 ^{0} E	KM657486
			Gudalur	11.59 ^{0} N., 76.50 ^{0} E	KT1762776
					1762777
11	***Protaetia cuprea ignicollis***		Bangalore	12.97 ^{0} N., 77.57 ^{0} E	KT203778
12	***Adoretus cupreus***	**Rutelinae**	Anand (Gujarat)	22.56° N, 72.92° E	KT254249
13	***Adoretus falvus***				KT254250
14	***Anomala* sp.**		Shimla (HP)	31.18° N, 77.17° E	1762765
			Bangalore (Dasarahalli)	12.97 ^{0} N., 77.57 ^{0} E	KM657492
				25.34.11°N, 91°59.2E	KM657491
15	***Anomala ruficapilla***		Bangalore (Dasarahalli),	12.97 ^{0} N., 77.57 ^{0} E	KT254246
16	***Anomala dimidata***		Theni		KU517668
	Anomala dimidata		Theni (Tamilnadu)	15.51 ^{0} N., 77. 79 ^{0} E	KU517664
17	***Phyllopertha horticola***		Anekal (Tamilnadu)	12.70° N, 77.69° E	KT203779
18	***Exomala pallidipennis***		Bangalore		KU317746
			Valampari (T.N)	15.51 ^{0} N.,74.29 ^{0} E	KT203780
19	***Onthophagus nuchicornis***	**Scarabaeinae**	Chintamani	13°24 ^{0} N 78°04'12. E	KU517667
			Bangalore		KU517666
20	***Onthophagus auritus***		Mudhigeri	13.1365° N, 75.64° E	KU665401
			Mudhigeri		KU665398
21	***Onthophagus coenobita***		Bangalore (Nandi hills)	13.3702° N, 77.68° E	KU665397
22	***Copris tripartitus***		Bangalore (Nandi Hills)	13.3702° N, 77.68° E	KU665396
23	***Basilepta sp***		Mudhigeri	13.1365° N, 75.64° E	KU665400

24	***Calicnemis obesa***	**Dynastinae**	Theni	15.51 ^{0} N., 77. 79 ^{0} E	KU517665
25	***Oryctes rhinoceros***		Theni (TN)	15.51 ^{0} N., 77. 79 ^{0} E	KU517993
26	***Alissonotum sp***		Kapatganj (UP)	26.920° N, 83.77° E	1762754
27	***Aethina concolor***	**Nitidulinae**	Mudhigeri	13.1365° N, 75.64° E	KU665399

Table 2. Maximum Composite Likelihood estimate of the pattern of nucleotide substitution of COI

	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	----

ACKNOWLEDGEMENTS

The authors are thankful to the Director, ICAR-NBAIR, for providing the facilities. Identification of the scarabaeids by Dr. K. Sreedevi, ICAR – NBAIR and Dr. ARV Kumar Department of Entomology, GKVK , Bangalore is duly acknowledged. We also thank Dr. Veena kumari, Dr. A.N.Shylesha , Dr. Mahendirn and Dr. Jagdish Patil of ICAR –NBAIR for the collections.

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