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Research Article

DNA BARCODING AND EVOLUTIONARY LINEAGE OF GREY WEEVIL, *MYLLOCERUS VIRIDANUS* (FABRICIUS) (COLEOPTERA: CURCULIONIDAE)

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ABSTRACT

Grey weevil, Myllocerus viridanus (Fabricius) is a polyphagous pest with a wide range of host plants. The pest is reported to cause damage to plants of agriculture and horticulture importance. Taxonomic identification plays a crucial role in designing the accurate management procedures against insect pests. Inaccurate identification often may form a hurdle in implementation of proper management strategies. This work utilizes the advancement in the field of molecular systematics such as DNA barcoding for tracing the phylogeny of the weevil pest by the partial sequencing of mitochondrial cytochrome oxidase I (CO I). The species discrimination studies based on DNA barcoding involved steps like DNA isolation and PCR amplification. Evolutionary analysis was carried out in Mega 7 software. GenBank deposition indicated the novel and first time records of M.viridanus and was provided with accession numbers KU871376&MG021103 Further analysis showed that M.viridanus has 96% similarity with M.undecimpustulatus (KX778624) and 85%. The translational product of aligned data showed a difference in two amino acid sequences between M.viridanus and M.undecimpustulatus. The nucleotide sequences with 96-84% similarity were retrieved from NCBI and included in the study. The protein translation yielded a short chain of 219 amino acids. This approach was very informative and it helped in the better understanding of genetic variation among closely related species.

INTRODUCTION

Grey weevils, *Myllocerus* species (Curculionidae: Coloptera) are one of the important pests in India with a wide range of hosts. The adult weevil of this genus feed on leaves nibbling the margin and eating away small patches of leaf lamina [1]. In this genus a total of 336 species are recognized as valid from Asia especially from south East Asia, the Indian subcontinent, Africa, North America, the Palearctic and Australia [2]. These weevils are slow flyers and can fly only for a short distance. When disturbed the adult weevil shows a peculiar deceptive death and falls down [3]. Eggs are usually laid in soil and the larvae which come out of eggs after hatching feeds on the fibrous rootlets of the host plant and thereby causing damage to the host plants. The larva transforms into pupa and pupation happens inside the earthen cocoon. Adult emerge from the soil and the adult weevil is polyphagous. *Myllocerus viridanus* (Fabricius) is a well-known pest of crops with a wide host range including agriculture crops, cash crops, shrubby vegetation and forest trees [4]. Stebbing (1914) reported this species for the first time from teak in many localities in Kerala and Tamil Nadu [5].

Accurate taxonomic identification is a key step in biodiversity, community ecology, bio monitoring and systematic studies [6]. For years experts were in search of a silver bullet for accurate taxonomic identification that would be helpful even in the recognition of exotic pests of agriculture importance [7]. Morphological phylogenetics has confronted with so many practical problems including discovering the most befitting representation of constantly deviating phylogenetic dimensions determining the appropriate trait as a measure to catalogue organisms, requirement of expertised minds to assume hypotheses based on evolutionary relevant phenotypic traits and the herculean task of amassing data on morphological characters from field observation or literature[8,9,10].These practical difficulties of morphological phylogenetics are easily overcome with the advent of molecular phylogenetics which has thrown light on solving this complicated issue by the introduction a new versatile technique namely DNA barcoding is the utilization of short DNA sequences to discriminate

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among species [11]. Hebert *et al* (2003a &2003b) detailed that the Folmer region of mitochondrial COI gene could attorney for the genetic barcoding for all animal life. DNA barcoding is an accurate and swift method which used 658 bp fragment located at 5' end of mitochondrial COI gene [12, 13]. Even the 15 variable sites seen in COI gene could combine the four bases in one billion ways (4^{15}) which gave plenty of barcode patterns possible for approximately 10 million organisms in the world [14].

Worldwide DNA barcodes of 16% of the described species are created whereas in India molecular systematic based on DNA barcoding is still in its infancy with 4.6%barcodes are created out of 59,000 described insect pests [15].The struggle of keeping in pace with the insect which has an undesirable effect on environment and design management strategies to control them entirely depend on our potential for precise and swift identification. Factors like the field of action and trustworthiness etc of the accessible barcode libraries often influence the precision of species identification [16]. Unavailability of a reference data is one of the major causes that arise in identification based on DNA barcode [17]. Identification and description of species would be improved if the different types of information including ecological morphological, physiological and molecular data of a species have been integrated [18, 19]. In such cases an identification system which is rapid and accessible at a global level is vital for putting the adequate management strategies into practice. In the current study we analyzed the identity of *Myllocerus viridanus* based on DNA barcoding and phylogeny analysis.

MATERIALS AND METHODS

Specimen Collection

Adult weevils were collected from mango trees available in mango orchards and homesteads in Bekal (12°23'15.36"N; 75°02'31.2"E), Badiyadukka (12°35'0"N; 75°4'0"E), Padannakkad (12°15.095'N; 75°.07.077'E), Pilicode (12°11'59.5"N; 75°09'49.2"E) of Kasaragod district. Collections were made by handpicking method and specimens were kept in neat and clean bottles and brought to lab and were stored at -20^{0}C for extraction of DNA and as voucher specimen for future reference.

DNA Isolation and Extraction

The specimens were kept in the laboratory at -20ºC, till genomic DNA isolation. The genomic DNA of the weevil was isolated from whole body as the insect was small. Isolated DNA was extracted using 'Macherey-Nagel Nucleospin Tissue' kit (Machery-Nagel, Duren, Germany) according to the protocol of manufacturers.DNA amplification was carried out in a thermal cycler Sure Cycler 8800(Agilent Technologies). Amplification was performed following standard protocols for PCR and sequencing and aligning of Mitochondrial CO I gene region[20].PCR profile of temperature comprised of an initial denaturation of 95^{o}C for 3 minutes followed by 45 cycles at 95^{o}C /10 sec for denaturation ,an annealing temperature of 45^{o}C for 4 minutes and elongation step at 72^{0}C for 45 seconds. The final extension step was at 72^{0}C for 3 minutes. The primers used were universal primers LCO-1490 and HCO-2198.the reaction mixture of PCR consisted of 1 µl each of left and right primers at a concentration of 10 µM, 2 ng of genomic DNA in 1 µl , 2.5 µl 10X reaction buffer 2.5 µl of dNTPs with 2 mM concentration, 0.20 µl Taq polymerase with 5 U/µl and sterile water in a volume of 16.8 µl. Using Mo Bio Ultra Clean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California) the PCR amplified product was column purified following the Manufacturer's instructions. The amplified and purified PCR product was then resolved in 2% TAE Agarose Gel stained with Ethidium bromide and visualized on gel documentation system with trans illuminator.

Sequencing and Evolutionary Analysis

The amplified and purified PCR product were sequenced from both ends with left and right primers using the Sanger's dideoxy sequencing method at SciGenom Labs, Cochin. After sequencing both sequences were trimmed and assembled in Sequencher (Gene Codes Corporation,Arbor,MI,USA) to get a consesne sequences. These sequences were then deposited in NCBI GenBank (KU871376&MG021103) aligned and the consensus sequence was used for analysis. The evolutionary analysis was done using the NCBI nucleotide BLAST [21]. Intra and interspecific distances were measured in MEGA 7 software [22].

RESULT AND DISCUSSION

Phylogeny analysis of *M.viridanus* when carried out by the partial sequencing of the mitochondrial COI of *M.viridanus* yielded a 630 bp product by PCR amplification. GenBank deposition indicated the novel and first time records of *M.viridanus* and was provided with accession numbers KU871376&MG021103. The protein translation yielded a short chain of 209 amino acids Similarity of the nucleotide sequences were analyzed with the help of BLAST [23]. Genbank analysis showed the sequence *M.viridanus* has 96% similarity with that of *M.undecimpustulatus* (GenBank accession number: KX778624) reported from Calicut, Kerala and is its nearest neighbor. Further analysis revealed that sequences of both *M.viridanus* and *M.undecimpustulatus* have approximately 567 sequences out of 589 bp in common. The sequences of both *M.viridanus* and *M.undecimpustulatus* when analysed in Mega 7 showed a difference of two amino acids in their translation product. The translational product of aligned data showed that the first change in amino acid sequences were that of isoleucine(I) for *M.viridanus* while that was leucine (L) for *M.undecimpustulatus*. In the second change *M.viridanus* has Glycine (G) and *M.undecimpustulatus* has Tryptophan (W). *Polydrusus formosus* (GenBank accession number: KM439386) reported from Germany which has 85% similarity with that of *M.viridanus* with 546 out of 632 in common. The aligned data showed a difference about nine amino acids.

Protien BLAST analysis in NCBI revealed that the protein sequence of *M.viridanus* has 99% similarity with that of *Myllocerus blandus* (Sequence ID : AMS34677) apart from six other sequences including that of *Apodrosus quisqueyanus* (Sequence ID: AKP94966.1), Myllocerus undecimpustulatus (Sequence ID: AKP94971.1, AKP94972.1) and *Myllocerus undatus* (Sequence ID: AMO26050.1, ATU31563.1, ATU31564.1, AUB31029).

Variation among nucleotides is one of most characteristic features of life on earth and it is the same feature that researchers make use of to trace the root of their phylogeny analysis. Analysis of mitochondrial cytochrome oxidase I

(COI) can provide a lot of information about the species. So the mitochondrial cytochrome oxidase I (COI) of sequences that has similarity with that of our species of interest was considered for phylogeny analysis. For that apart from sequences of *M. viridanus* 25 other sequences were involved in the study and were retrieved from NCBI that showed 90-80% similarity in BLAST analysis. The sequences are enlisted in table 1.

Generally the model which has low Bayesian Information Criterion, BIC value was seleced as the best model to describe the substitution pattern. The best fit test model suggested GTR+G+I as the optimum model to describe the substitution pattern. based on Bayesian Information Criterion, BIC (11767.094) and Akaike Infromation Criterion,corrected AICc(11301.782) values. The estimation of transition/transversion bias study revealed that the estimated transition/transversion bias (R) was 1.65 under General Time Reversible (+G+I) model[24].The nucleotide frequencies were A (30.60%), T/U (35.50%), C (18.60%) and G (15.30%). When the number of base substitution per site from averaging overall sequence pairs are computed using Kimura 2-Parameter model [25] the average evolutionary divergence was found to be 0.202 with a standard error of 0.010 (0.202± 0.010).There were a total of 567 positions in the final data set form 27 sequences. In phylogeny analysis by constructing phylogenetic tree of the selected 27 sequences 1000 replications of boot strapping was implemented and Neighbour-joining method was adopted using Kimura 2-Parameter model [26,25].

The optimal tree was with the sum of branch length is 2.1538. The phylogenetic tree revealed the monophyly between *M.viridanus* and *M.undecimpustulatus* (Fig 1). The upper clad they shared with *Strophosoma capitatum* (KU908974) and *Scobius* sp (JX412782).

Table 1 Specimens with similarity retrieved from NCBI with associated geographic localities and Genbank accession number

Sl no	Insect group	Accession number	Country
1	*Myllocerus undecimpustulatus*	KX778624	India
2	Curculionidae sp.	KY841554	Pakistan
3	*Polydrusus formosus*	KM439386	Germany
4	Curculionidae sp.	KR489496	Canada
5	*Phyllobius pomaceus*	KM446832	Germany
6	Coleoptera sp	JN290423	Canada
7	*Strophosoma capitatum*	KU908974	Germany
8	*Celebia suturalis*	LT799107	USA
9	Naupactus aff. chloraspis	MH734215	Argentina
10	*Artipus sp*	HQ891429	USA
11	*Myllocerus blandus*	KR868760	India
12	*Monochamus scutellatus*	KC593331	New Zealand
13	*Heteroglymma nr. biramosa*	LN866384	Germany
14	*Airosimus Jacobi*	MH734216.	Argentina
15	*Phyllobius calcaratus*	KU910243	Germany
16	*Polydrusus picus*	KU910227	Germany
17	*Myosides seriehispidus*	MG057929	Canada
18	*Phyllobius glaucus*	KJ962147	Finland
19	*Celeuthetini gen. 3 sp. 1*	LN866338	Germany
20	*Sciobius sp*	JX412782	United Kingdom
21	*Phyllobius virideaeris*	KM449062	Germany
22	*Blosyrus sp*	MG462881	Canada
23	*Apodrosus quisqueyanus*	KP851140	India
24	*Molytinae sp*	KY034264	Canada
25	*Stibara nigricornis*	KP233786	India

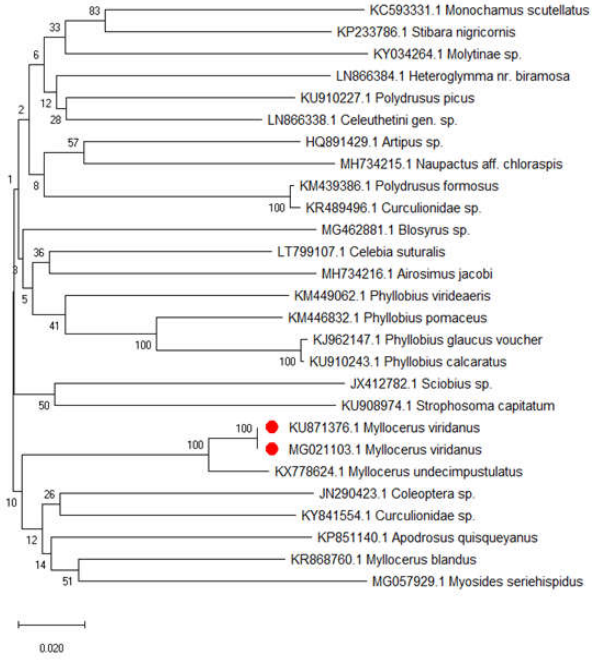

Fig 1 Neighbour joining tree for *M.viridanus* formed using Kimura 2-Parameter model

CONCLUSIONS

When the protein product of *M.viridanus* and its nearest neighbor *M.undecimpustulatus* (KX778624) was compared it was found that out of two aminoacid difference that exist between them one was conservative (Isoleucine (I) in *M.viridanus* and Leucine(L) in *M.undecimpustulatus*). Here Isoleucine (I) in *M.viridanus* and Leucine(L) in *M.undecimpustulatus* are both aliphatic aminoacids and hydrophobes. The other difference in aminoacid was glycine in *M.viridanus* and tryptophan (W) in *M.undecimpustulatus* and this difference might have created a different protein as these differences are radical. Glycine is coded by GGA, GGC, GGG and GGU while UGG codes for Tryptophan. So the change may due to a non synonymous mutation.

From the current investigation it is very clear that mitochondrial cytochrome oxidase I has the potential to become the molecular marker in species delineation studies based on barcoding. Unambiguous identification of pest of agriculture importance can confront with hurdles like cryptic species, errors regarding misidentified specimens in literature, specimens arranged wrong taxa etc which can be currently meet with DNA barcoding [27,28]. Even though DNA barcoding has proved to be effective tool in species discrimination studies for taxonomy it's not a substitute for conventional taxonomy it is only an easy door to attain taxonomic features that can help in species discrimination studies [18]. For the better understanding of a species at molecular level there must be an integration of information from the nuclear genes and mitochondrial genes and this can throw light on the constraints of molecular systematics.

It could be a better way to explore the diversity of nature by efficient utilisation of the technique of barcoding. The study gave a clear visualization of their molecular evolution. In future it can be used to imply management strategies at molecular level. It would have been more helpful if more species in the

genus *Myllocerus* have their sequence barcoded and deposited. Overall the present study indicates how the application of single gene sequence (COI) can be helpful in identifying and discriminating species. Therefore in near future if all the species in the genus *Myllocerus* have at least their single gene segment barcoded, identification and thereby implication of proper management strategies could be done even by farmers.

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