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MOLECULAR IDENTIFICATION OF THE SCUTELLERID BUG *SCUTELLERA NOBILIS* (FABRICIUS) (HEMIPTERA: PENTATOMOIDAE) FOR BIODIVERSITY STUDIES

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Abstract: The present study was to identify the pest of biodiesel plant (*Jatropha curcas*), *Scutellera nobilis* and substantiating it through molecular studies, phylogenetic relationship and the evolutionary sequence divergence among the closely related individuals belonging to the Super family Pentatomoidea. The computed evolutionary sequence divergence is <0.35 within the COI gene sequences and concludes that Cytochrome Oxidase I gene sequence can potentially serve as a barcode data in the species identification among the studied group.

Keywords: *Scutellera nobilis*, molecular studies, COI gene, sequence divergence

INTRODUCTION

The scutellerid bug *Scutellera nobilis Fabricius (Hemiptera : Pentatomidae)* lays its eggs on stem, petiole and leaves of *Jatropha curcas* in longitudinal manner. The laid egg hatches in five to nine days period. It takes about 33 days to develop as an adult from an egg stage. The *Scutellera nobilis* nymphs are pinkish in colour with black abdomen. Both the nymph and adults of *Scutellera nobilis* feed on developing fruits resulting in shrinking of fruits. Early symptoms include oozing out brown coloured secretion from the feeding area and later the fruit turns black. The infested fruits have less weight and shelling percentage thus directly affecting the seed yield and results in significant economic loss (Rao *et al*., 2010).

The identification of infested pest forms the initial step for pest management. Due to illiteracy among farmers and lack of expert field taxonomist, proper identification of insect pests has become problematic. Hence, the present work was aimed to identify the scutellerid bug species based on morphology and to submit the cytochrome oxidase subunit- I (COI gene) sequence of the mitochondrial DNA to data base library (gene bank) and also to barcode of life data base to serve as DNA barcode for the particular bug species. The DNA barcoding work carried out in scutellerid bug would of great help to the scientific community around world particularly in molecular identification by referring to the submitted DNA sequence as a reference sequence for future identification studies.

MATERIALS AND METHODS

The specimens were identified using conventional morphological base method (Rao *et al*., 2010). The tissue samples were obtained from the collected specimen by dissecting the animal and cutting a small amount of tissue around the anal tube, to recover muscle tissue, which is rich in mitochondria (Ball and Armstrong, 2007). The DNA was then isolated by using the DNA isolation kit and Insta Gene Matrix.

The extracted DNA sample was run on agrose gel 1% using TAE buffer (Tris base- 12.1g, 0.5M EDTA ($_p$H 8.0) - 50ml and Glacial acetic acid- 2.075ml). The gel incorporated with ethidium bromide was visualized by placing on a UV light source and photographed. PCR reaction was performed with 20 µl of genomic DNA as the template in a 30 µl reaction mixture by using a EF-Taq (SolGent, Korea) and the PCR was performed under the following conditions: 2 min at 95°C; 5 cycles of 40 sec at 94°C, 40 sec at 45°C, 1 min at 72°C; 35 cycles of 40 sec at 94°C, 40 sec at 51°C, 1 min at 72°C; 5 min at 72°C; held at 4°C. Universal primer LCO and HCO were used for polymerase chain reaction amplification of the mitochondrial gene cytochrome oxidase subunit I gene (Folmer *et al*., 1994). LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' HC02198: 5'-taaacttcagggtgaccaaaaaatca-3'

Sequencing reaction was performed using a PRISM big dye terminator v3.1 cycle sequencing kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

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Phylogenetic tree building and evolutionary distance analysis

The sequenced mitochondrial cytochrome oxidase subunit I gene sequence was analyzed for sequence similarity using the bioinformatics tool BLAST. For species identification studies the barcoded sequence data was analyzed in the BOLD database. The barcoded Scutellerid bug *Scutellera nobilis* belongs to the super family Pentatomoidea of the Suborder Heteroptera of Order Hemiptera. In order to study the interspecies sequence divergence relationship, four COI sequence were retrieved from the NCBI website containing the GenBank Accession numbers (AY253006.1- *Choerocoris variegates*, GU247489.1- *Calliphara nobilis*, GU247490.1- *Chrysocoris purpureus*, AY627331.1- *Tessaratoma papillosa*) all belonging to the super family Pentatomoidea as that of the studied Scutellerid bug *Scutellera nobilis*. The phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) Version 5 software.

RESULTS

With the obtained COI gene sequence, nucleotide BLAST was performed and the results exhibited less sequence similarity with the top match relating to human isolate DolniVestonice 13 mitochondrion, complete genome with the maximum score 40.1, query cover 3%, maximum identity 96% and E-value 1. COI gene sequence of the bug *Scutellera nobilis* was presented as a query sequence in the barcode of life database to find the reference sequence and no significant match was found. Results of present study conclude that the COI gene sequence of the *Scutellera nobilis* was not found to be present on the DNA Database libraries. Based on the evolutionary sequence divergence analysis of four closely related bug species along with the studied bug, *Scutellera nobilis* showed a mean sequence divergence <0.35 within their COI gene sequences. Thus the sequence divergence results conclude that, DNA barcodes can be successfully employed to discriminate a species within and between their closely related individuals of the studied group (Superfamily- Pentatomoidea).

Fasta format of sequence

>seq1 (organism=Scutellera nobilis) (cytochrome oxidase subunit I) (COI) gene, partial sequence; mitochondrial

GCGAATGGATATCGGTATGTATGGTAGTCTACTCCTCCG
GCGGGGTCGAAGAAGGTGGTGTTGAGGTTGCGGTCTGT
TAATAGTATAGTGATGCCAGCAGCTAGGACTGGGAGAG
ATAGGAGAAGTAGGACTGCTGTGATTAGGACGGATCAG
ACGAAGAGGGGCGTTTGGTATTGGGTTATGGCAGGGGG
TTTTATATTGATAATTGTTGTGATGAAATTGATGGCCCCT
AAGATAGAGGAGACACCTGCTAGGTGTAAGGAGAAGAT
GGTTAGGTCTACGGAGGCTCCAGGGTGGGATAGTTCCCT
GCTAAGGGAGGGTAGACTGTTCAACCTGTTCCTGCTCCG
GCCTCCACTATAGCAGATGCGAGCAGGAGTAGGAGAGA
GGGAGGTAAGAGTCAGAAGCTTATGTTGTTTATGCGGG
GAAACGCCATATCGGGGGCACCGATTATTAGGGGAACT
AGTCAGTTGCCAAAGCCTCCGATTATGATGGGTATTACT
ATGAAGAAGATTATTACAAATGCATGGGCTGTGACGAT
AACGTTGTAGATGTGGTCGTTACCTAGAAGGTTGCCTGG
CTGGCCCAGCTCGGCTCGAATAAGGAGGCTTAGAGCTGT
GCCTAGGACTCCAGCTCATGCGCCGAATAATAGGTATAG
TGTTCCAATATCTTTAATTGTGTTTTGTCACACAAANN

Table 1: Estimates of Evolutionary Divergence between COI Sequences

S.no	Species -1	Species-2	Evolutionary divergence between COI gene sequences
1.	*Choerocoris variegatus*	*Scutellera nobilis*	0.46
2.	*Calliphara nobilis*	*Scutellera nobilis*	0.45
3.	*Chrysocoris purpureus*	*Scutellera nobilis*	0.11
4.	*Tessaratoma papillosa*	*Scutellera nobilis*	0.12
5.	*Choerocoris variegatus*	*Calliphara nobilis*	0.44
6.	*Choerocoris variegatus*	*Chrysocoris purpureus*	0.44
7.	*Calliphara nobilis*	*Chrysocori spurpureus*	0.52
8.	*Choerocoris variegatus*	*Tessaratoma papillosa*	0.28
9.	*Calliphara nobilis*	*Tessaratoma papillosa*	0.28
10.	*Chrysocoris purpureus*	*Tessaratoma papillosa*	0.52
	Mean Divergence		**0.36**

The number of base differences per site from between sequences is shown. The analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 272 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5 software.

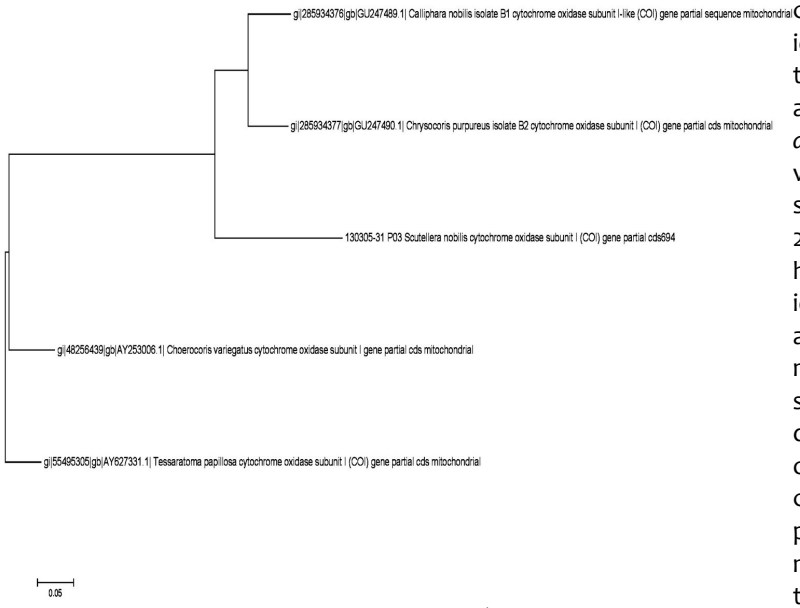

Figure 1: Phylogenetic tree (Neighbor joining method, constructed using p-distant method).

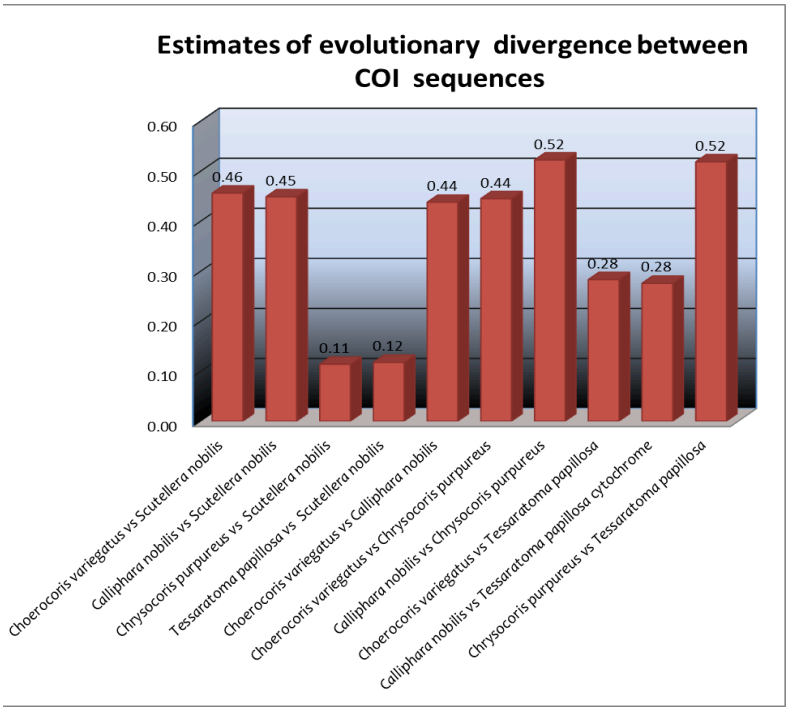

Fig.2: Estimates of evolutionary divergence between COI sequences.

DISCUSSION

DNA Barcoding is a molecular, bio - informatical and identification tool for taxonomical studies. DNA bar coding is new aspect, which involves the short gene markers in the mitochondrial DNA to identify an organism. Since mitochondrial DNA shows high rate of mutation than the nuclear DNA, it can be employed in the field of evolutionary analysis (Brown et al. 1979). Thus mitochondrial DNA serves the need of a highly variable sequence that can be standardized as a barcode for species identification. A short sequence of 648 bp region of the cytochrome *c* oxidase I (coI) gene is preferred as a primary sequence for animal barcoding (Hebert *et al*., 2003). Many experimental results derived from varied species of animals revealed that 95% of the species posses' diverse COI sequence (Hebert et al. 2003; Steinke et al. 2009; Hajibabaei *et al*. 2005). It has proven to be very useful technique for identification of lower forms of organisms like fungi and yeast in which classical identification based on morphology restricted the identification of many species for years (Groenewald, 2011). DNA barcode data was found reliable in the identification of lower organisms, whose identification by morphological characteristics is problematic or impossible due to phenotypic plasticity, lack of diagnostic morphological characters and immature life stage of the specimen (Ball and Armstrong, 2007).

DNA Barcoding technique not only assists the field scientist in species identification but also expedite the process of finding the new species (Kerr *et al.*, 2006). This Molecular technique was based on the premise that genetic variation between the species will be compared higher to variation within species and thus this new molecular technique will aid in identification of cryptic and sibling species, accurately supplementing the need of morphological based identification method (Hebert *et al*., 2003). "According to IUCN (2008), current estimated species number varies from 5 to 30 million out of which about 1.8 million so far has been described. IUCN updates include 44,838 species of which 869 (2%) are extinct or extinct in wild; 16,928 (38%) are threatened with extinction (with 3,246 critically endangered, 4,770 endangered and 8,912 vulnerable); 3,513 (8%) are near threatened; while 5,570 (12%) have insufficient information to determine their threat status (data deficient). The number of extinctions might well exceed 1,100 if the 257 critically endangered species tagged as 'possibly extinct' are considered". Scientists also predict that many unidentified species are facing extinction and threatened without even being identified. Thus the mitochondrial DNA sequence of the COI gene deposited in the DNA libraries like Gene bank and BOLD (Barcode of Life Database) will help non-taxonomist in identifying an unambiguous species and potentially speeds up the process of identifying new species, and many unidentified species to the biodiversity inventory list.

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