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Genetic Diversity and Phylogenetic Analysis of Lepidopteran Species by Molecular Barcoding Using Co I Gene Sequences

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Abstract: ***Lepidoptera is one of the largest orders of insects that include moths and butterflies. Most of the Lepidopterans are morphologically similar, therefore the identification of these insects is tedious using morphotaxonomy and that is detrimental in designing a better strategy to control and conserve them. These are extremely important group of 'model' organisms and are used to investigate many areas of biological research, including such diverse fields as navigation, pest control, embryology, mimicry, evolution, genetics, population dynamics and biodiversity conservation. The knowledge of Lepidopteran genomics will create new methods of insect pest management and will contribute to sustainable agriculture, protection of the environment and the maintenance of biodiversity. In this study we amplified cytochrome oxidase I gene of Junonia atlites for species identification and constructed phylogenetic tree for recognizing evolutionary relationship.***

Keywords: Molecular systematics, Lepidoptera, DNA barcoding, CO I gene sequences, Mitochondrial DNA, Junonia atlites.

1. Introduction

Identification and conservation of genetic diversity is important for Lepidoptera since it is one of the widespread and widely recognizable insect orders in the world. The strength of Lepidopteran genomics lies in the diversity of the group as a whole. Although there are clear insect models for genetic analysis (Drosophila) and disease vectors (Anopheles), the Lepidoptera are rich in diverse model systems for a variety of biological processes. Much of our knowledge of endocrinology, reproduction, behavior and immunity is derived from the studies in Lepidoptera. They are important test case for the use of mitochondrial DNA in species identification. This is the important order among class insecta that have appeared on endangered species list.

The genome of lepidoptera is characterized by larger size and higher chromosome number, typically about 30. Genetic crosses are routinely accomplished in Lepidoptera. The GC content of Lepidopteran DNA is about 35-40%. The large body size, accessible genetics, and extreme diversity of Lepidopteran species are important experimental advantages. Identification at the molecular level is important since phenotypic variability and convergent evolution causes misidentification of many cryptic species, in addition sexual dimorphism causes more confusion to species identification. To solve these problems in the taxonomy, recently a short nucleotide sequence of mitochondrial DNA (CO I) is widely accepted as a marker for the accurate and easy identification of species. DNA sequences of the mitochondrial cytochrome oxidase I (CO I) gene can serve as a DNA barcode for identifying all kinds of animals [1]. It is an important advancement in molecular biology for rapidly and cost-efficiently catalog fauna using a short reference sequence of DNA. The present work reveals the partial mitochondrial CO I gene sequences of Junonia atlites (Figure 1) their genetic divergence and phylogenetic status and also the effectiveness of DNA barcoding in identification of cryptic species.

2. Materials and Methods

2.1 Collection and Preservation

The samples were collected from Calicut University Botanical Garden (CUBG), Kerala using hand sweeping net. The sample was identified as Junonia atlites and placed in separate glassine envelope with 70% ethanol, assigned code number and stored at -20°C as voucher specimen until further use.

Figure 1: a) Junonia atlites

2.2 DNA Extraction, Amplification and Sequencing

DNA was extracted from the leg piece of the specimen using phenol chloroform method [7]. The obtained DNA was amplified for CO I gene using forward primer (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). PCR reaction was carried out in total volume of 50 µl containing 2 ng of genomic DNA (1 µl), 1 µl of each forward and reverse primer at a concentration of 10 µM, 1 µl of dNTPs (2 Mm), 5 µl of $MgCl_2$, 5 µl of 10X reaction

buffer, 0.5 µl Taq polymerase (5U/µl) and 36.5 µl of water. The PCR profile consisted of an initial denaturation step of 5 min at 95°C followed by 30 cycles of 10 sec at 95°C, 10 sec at 50°C and 45 sec at 72°C and ending with a final phase of 72°C for 30 min. The obtained PCR product was checked on 2% agarose gel and was column purified using Gene JET$_{TM}$ PCR Purification kit (Fermentas Life science). The purified PCR product was sequenced at Sci Genome Laboratories Ltd., Cochin, Kerala. The obtained sequence was checked for its quality by examining chromatograms and the forward and reverse sequence were assembled using Clustal W. Sequence analysis and sample identification were done by the trimmed sequence in NCBI's BLAST tool. Phylogenetic tree was then constructed using MEGA 6 software [8].

3. Result and Discussion

DNA Sequences of good quality and length of 543 bp were generated in the present study. The forward and reverse sequences obtained were trimmed for primer sequence and assembled using Clustal W (Figure 2). The nucleotide BLAST against the nucleotide redundant database revealed that the CO I gene sequence obtained is showing 100% similarity to Junonia atlites. The CO I hyper variable region of Junonia atlites DNA is 100% similar to that of Junonia atlites CO I gene reported from Western Ghats (GenBank accession number GU0126131.1). Here geographical barrier may act as evolutionary tool for the sequence divergence.

The evolutionary history of Junonia atlites is inferred using Neighobour joining method of MEGA 6 software which shows clearly the inter and intra species divergence (Figure 3). NJ clustering analysis showed that Junonia atlites from Calicut, Kerala and Western Ghats belong to same clade without any overlap while Junonia atlites from Africa (GenBank accession number EU053290.1) belongs to another clade with 100% similarity. The above phylogenetic tree shows that the Junonia atlites from Calicut, Kerala, Western Ghats and Africa have same ancestors. DNA barcoding provide rapid and automatable species identification by short standardized DNA fragment as species tag and its makes the Linnaean classification system more accessible [5].

> *Junonia atlites* mitochondrial cytochrome oxidase subunit I (CO I) partial nucleotide sequences, 543 bp

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>GTGATGATCAAATTTATAATACTATTGTTACAGCTCATGCTTTTATTATAATTTTTTTTA
TAGTTATACCAATTATAATAGGAGGATTTGGTAATTGATTAGTACCCCTTATATTAGGTG
CTCCTGATATAGCTTTCCCACGAATAAACAATATAAGATTTTGATTATTACCCCCATCAT
TAATTTTATTAATTTCTAGAAGAATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTGT
ACCCCCCACTTTCATCTAATATCGCCCATGGAGGATCTTCAGTTGATTTAGCAATTTTTT
CCTTACATTTAGCTGGAATCTCATCTATTTTAGGAGCTATTAATTTTATTACAACAATTA
TTAATATACGAATTAACGGTATATCATTTGATCAAATACCTCTATTTGTTTGAGCAGTAG
GAATTACCGCTTTATTATTAGTTCTTTCTCTCCCAGTTTTAGCCGGAGCTATTACTATAT
TACTTACAGATCGAAATATTAATACTTCATTCTTTGATCCAGCTGGTGGAGGAGACCCAA
TTT
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Figure 2: The DNA sequence of the mitochondrial cytochrome oxidase I (CO I) gene of Junonia atlites

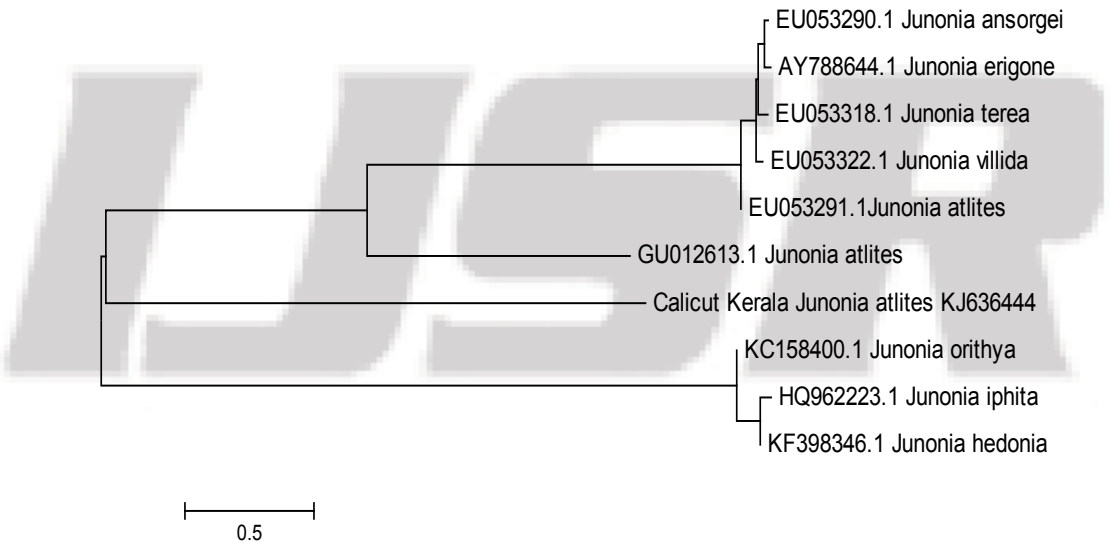

Figure 3: Phylogenetic Status of Junonia Atlites Using Neighbor Joining Method

There is no intra species nucleotide divergence between Junonia atlites since the nucleotide BLAST against the nucleotide redundant database is showing 100% similarity to Junonia atlites. The intra species divergence is found to be between 2-9%. The average nucleotide composition proportions for these twelve sequences were G, 15.3%; A,

31.7%; T, 37.9%; and C, 15.1%. The present results indicate that an identification system for insect life based on the COI gene will be highly effective. Transition/Transversion bias (R) calculated for selected species of Junonia atlites is 0.80. Maximum transitional substitutions are C to T and transversional substitutions are A to T. Variation in the nucleotide sequence is a fundamental property of all living organisms which can be used for their identification and phylogenetic status.

The conceptual peptide translation yielded 180 aminoacids. The peptide blast of nucleotide sequence showed 100% similarity to Junonia atlites which again confirmes the sample as Junonia atlites. The molecular barcode generated for Junonia atlites in the present study can be used for its accurate taxonomic identification. Sequences generated in this study were submitted to GenBank with accession number KJ636444 (Junonia atlites) which can be used as molecular barcode of this species and can be used for its accurate taxonomic identification.

The present study on molecular evolutionary analysis using partial mitochondrial cytochrome oxidase subunit I (CO I) gene sequence explicates phylogenetic relationships of Junonia atlites. The CO I gene is generally effective as a barcode sequence, delivering more than 95% species level resolution [1]. Thus present study reveals the importance of DNA barcoding in the identification of species. The molecular database will act as molecular field guide facilitating the identification of cryptic species [4].

4. Conclusion

The study concludes the efficacy of using COI gene for identification of species at molecular level and applying this molecular taxonomy for identification of cryptic biodiversity. The study also concludes that DNA barcoding is most effective tool for identification of species at molecular level than the morphotaxonomy that are ordinarily employed for identification. Morphotaxonomy often generates errors due to intraspecific variability between the species. The study developed a comprehensive DNA barcode database for Lepidoptera Junonia atlites (GenBank accession number KJ636444).

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