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ORIGINAL ARTICLE

Molecular Characterization of Red Flour Beetle *Tribolium Castaneum* (Herbst) (Coleoptera: Tenebrionidae) from Various Warehouses In India

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ABSTRACT
Stored food products are prone to infestations by a huge number of pests such as the red flour beetle, Tribolium castaneum. Due to its small size, the identification process seems complicated and tedious. Hence molecular approaches for the identification and diversity analysis are preferred. Mitochondrial COI gene is used for this purpose owing to its fast evolution, high polymorphism, easy amplification and sequencing. In this study, 10 samples of T.castaneum (S1-S10) were collected from various warehouses in India. The DNA of each sample was extracted, amplified (COI gene) and sequencing were performed. The obtained sequences were analyzed using bioinformatic tools. The dendrogram thus obtained reveals close similarity between the different clades. The tree reveals that all the samples have diverged from a common ancestor with the samples S3, S7, S5,S8,S1 and S2 (Clade 1) having a recent common ancestor and S4, S6,S9 and S10 having another recent common ancestor (Clade 2). The phylogenetic studies among the nucleotide sequences results hence reveal close similarity among the sequences.
Keywords: *Tribolium castaneum*, molecular diversity, Cytochrome Oxidase I, Storage pests, MEGA, Phylogeny

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INTRODUCTION

Stored products pests, which enjoy a cosmopolitan distribution, are the prime concern to food security. *Tribolium castaneum* Herbst (Coleoptera::Tenebrionidae) one of the most widespread and destructive pest is a polyphagous pest which feeds on different stored-grain and grain products, nuts and dried fruits [1].Management of this pest has proved to be a major obstacle in the food storage and processing industry. Though, the use of synthetic pesticides is the oldest method for storage pest control [2], its continuous use has resulted in severe problems such as insecticide resistance [3]. Hence, plant products and their secondary metabolites are receiving increasing attention in the management strategy. The conventional method for the identification of *Tribolium* species are based on the morphological characteristics of the adult. However this process is tedious as it is difficult to rapidly identify adult fragments and non-adult stages based on external morphological characteristics. Molecular techniques may be used for the rapid and accurate identification of *Tribolium* species chiefly for pest monitoring and the quarantine of stored products pests. The molecular studies may also pave way for the identification of targets and for selective insect control. Few studies have reported the use of molecular markers such as cytochrome b, mtCOI for the identification and Phylogeny analysis of stored product pests[4,5,6,7].The genetic structure of *Tribolium castaneum* in various regions of a mill in Kerala has been studied using the species specific microsatellites [8], but the molecular diversity of the insect from different regions of the country has not been studied much. Thus the present study focuses on the molecular characterization of different samples of *Tribolium castaneum* collected from various parts of India.

MATERIAL AND METHODS

Collection of Pest

Samples of the pest was collected from various warehouses of the Food Corporation of India. The samples were either collected in person or were sent by the respective depots and these were labelled as:

Sample 1	Kerala
Sample 2	Karnataka
Sample 3	Haryana
Sample 4	Arunachal Pradesh
Sample 5	Gujarat
Sample 6	Orissa
Sample 7	Punjab
Sample 8	Rajasthan
Sample 9	Andhra Pradesh
Sample 10	Tamil Nadu

Molecular Characterization of the Collected Insect Samples

Total genomic DNA of the collected insect samples was extracted via the CTAB Method [9]. Agarose Gel Electrophoresis was performed (BioBee Agarose Gel Electrophoresis Unit, Model-TM Wide Sub Plus) in order to check the quality of the obtained DNA samples. The pure isolated DNA samples were then subjected to amplification. In this study, 642 bp region of mitochondrial cytochrome c oxidase subunit I (COI) genes of *T. castaneum* samples were amplified and sequenced. A pair of universal forward LCO1490 (5′-GGTCAACAAA TCATAAAGATATTGG-3′) and reverse HCO2198 (5′-TAAACTTCAGGGTGACCA AAAAATCA-3′) primers were used for COI amplification. PCR was performed based on methods by Wang *et al.,*[10] and was modified for half volume reactions containing 12.5 μL MasterMix with loading dye, 10 μL sterilized distilled water, 1.5 μL extracted DNA and 0.5 μL forward and reverse primers. The PCR amplification included an initial denaturing step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 10 min. The reactions were carried out on a Benchtop Gradient PCR (Genmate Series Thermal Cycler,Model-K960). The PCR products were analyzed electrophoretically in 1.2% agarose gels. The amplified DNA fragments subjected to purification and sequencing. DNA purification and Bidirectional sequencing using the same amplification primers was performed at The Regional Facility For DNA Fingerprinting, Rajiv Gandhi Centre For Biotechnology, Thiruvananthapuram, Kerala. The contigs generated were assembled, refined and submitted to the Genbank. Further, MEGA-X software was used for multiple sequences alignment and dendrogram construction [11].

RESULTS AND DISCUSSION

In the recent times, due to the rapid progress of molecular biology techniques such as nucleic acid sequencing and analysis of large data, the mtDNA study is becoming more prevalent. When compared to the common nuclear markers, mitochondrial markers are more susceptible to the effects of genetic drift [12]. As a potent and widely used molecular marker, mtDNA has been applied in many organisms to determine the population structure and rates of genetic variation [13]. Since Mitochondrial DNA is maternally inherited with least intermolecular genetic recombination and rapid rate of evolution, it has become a key tool for the study of genetic structure of population and molecular variations [13,14]. COI is a protein-coding gene in mtDNA. Due to fast evolution, high polymorphism, easy amplification and sequencing, COI is now a widely used genetic mitochondrial marker for intra-specific analysis of genetic populations [13,14,15].

In the present study, the mtCOI region of the collected insect samples was amplified and sequenced using the standard protocols. The mtCOI sequences generated from different red flour beetles consisted of approximately 650bp. The nucleotide sequences obtained were submitted to the GenBank database via the GenBank submission portal. The obtained accession numbers for the sequences are mentioned below:

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Sequence ID	Accession Number
S1-Kerala	MT499223.1
S2-Karnataka	MT499224.1
S3-Haryana	MT499225.1
S4-Arunachal Pradesh	MT506943.1
S5-Gujarat	MT499226.1
S6-Orissa	MT506942.1
S7-Punjab	MT506940.1
S8-Rajasthan	MT506941.1
S9-Andhra Pradesh	MT499227.1
S10- Tamil Nadu	MT499228.1

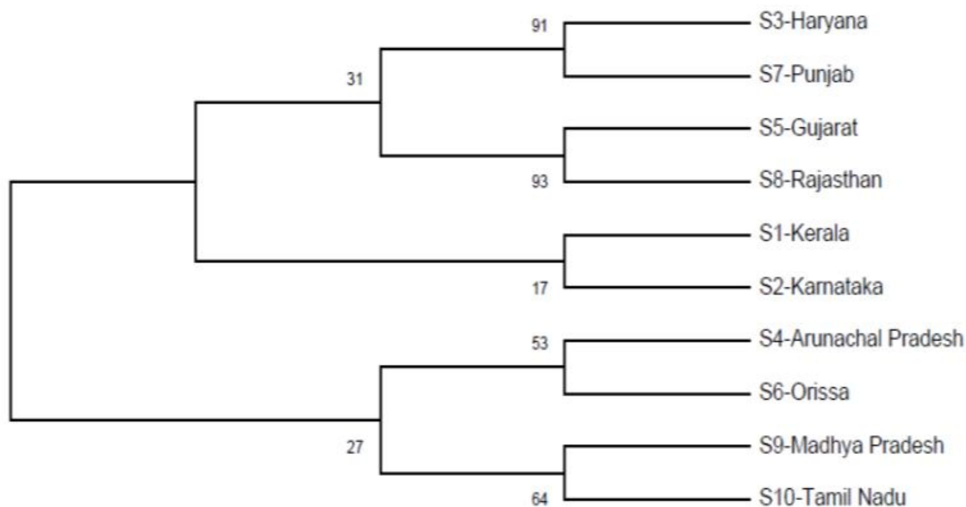

Fig1. The Dendrogram showing the Evolutionary Relationship among the 10 sequences

MEGA-X software was use to perform the phylogenetic analysis of the obtained nucleotide sequences after performing the sequence alignment of the obtained nucleotide sequences. The phylogenetic tree construction was done using this software using the Neighbor-Joining method. The evolutionary history was inferred using the distance based method i.e., Neighbor-Joining method [16] which takes into account the matrix of pairwise evolutionary distances between the sequences Further bootstrapping, a resampling technique used to estimate statistics on a population by sampling a dataset with replacement., was performed. Bootstrapping allots measures of accuracy to the sample estimates. The bootstrap consensus tree inferred from 500 replicates [17] was taken to represent the evolutionary history of the taxa analyzed [17]. Since all pairwise distances in a distance matrix are correlated due to the phylogenetic relationships among the sequences, the sum of their log-likelihoods is used for tree construction. This method is defined as Maximum Composite Likelihood method [18]. The bootstrap consensus tree of these sequences after 500 replicates of bootstrapping results was obtained finally (Fig.1).From the dendrogram it can be inferred that all the samples have a primary common ancestor with two major clusters i.e. Cluster I and Cluster II diverging from the common ancestor and that the members of each cluster are similar to each other. The tree reveals that all the samples have diverged from a common ancestor with the samples S3, S7, S5,S8,S1 and S2 (Clade 1) having a recent common ancestor and S4, S6,S9 and S10 having another recent common ancestor (Clade 2). This reveals that their nucleotide sequences would also be highly similar. Since, the bootstrapping values of most of the clades is >70, it is ascertained that these clade similarity is real [19]. Similar study conducted by Zhang *et al.,* [6]and Aslam*et al.,* [20] reveals close similarity between the *Tribolium castaneum* species collected from various geographical areas. Our study affirms the molecular variability of the *T.castaneum* species to be less. Hence, this set a ground for adopting similar management strategies for controlling these populations in the warehouses.

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