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Contrasting genetic diversity and intra-population polymorphism of the invasive pest *Henosepilachna vigintioctopunctata* (Coleoptera, Coccinellidae): A DNA barcoding approach

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ABSTRACT

Henosepilachna vigintioctopunctata (Coleoptera: Coccinellidae) is one of the most important pests attacking Solanaceae crops in Asian countries. Despite its agricultural importance, little information is available on the molecular ecology and evolution. In this study, we used DNA barcoding to explore the genetic diversity and intra-population polymorphisms of *H. vigintioctopunctata*. Haplotype analysis of the partial COI gene sequences of *H. vigintioctopunctata* from four localities in South India and other globally available haplotypes revealed two genetically distinctive lineages. The TCS haplotype network with the main clusters G1, G2, G4 and G6 represented intra-population polymorphism among the Indian lineages, while other clusters were differentiated with unique mutational steps. High substitutions in the 1st and 3rd codon suggested the dominance of synonymous substitutions with significant p-values < 0.05, that inferred for purifying selection. The genetic diversity indices Fu's Fs and neutrality index Tajima-D had negative values that implied for recent population expansion and low frequency of polymorphism. Further, high genetic divergences among certain haplotype comparisons evidenced the influence of micro-evolutionary pressures. The threshold value was 1.26%, which was supported by the postulates of 10× rule. Overall, the study firstly reported the genetic diversity and intra-population polymorphism of *H. vigintioctopunctata* populations attacking Solanaceae species in Asian countries.

Introduction

The Coccinellidae family comprises a number of important biological control agents, which feed on aphids, scales, Lepidoptera larvae and insect eggs. On the other hand, some coccinellids are pests of economic importance. Among these, the most prominent are the subfamily Epilachninae, which are plant eaters (Seago et al., 2011). *Henosepilachna vigintioctopunctata* (Fabricius) (Coleoptera: Coccinellidae), known in Asia as Hadda beetle, it is also one of the most important pests attacking *Solanaceae* crops in Asian countries (Bhagat and Munshi, 2004; Rahaman et al., 2008; Islam et al., 2011). The destructive potential is high at both the adult and larval stages, which feed on the epidermal tissues of leaves, flowers and fruits, causing yield loss (Ghosh and Senapati, 2001). The affected leaves of the plant become skeletonized, gradually dry and drop down. The larvae confine their attack to the lower surface while adult beetles usually feed on the upper surface of the leaves (Khan et al., 2000).

Several studies on the phylogenetic relationships reported considerable genetic differences comparable with the congeneric species (Kobayashi et al., 2000). Due to prevalence of intense debate over sympatric speciation (Howard and Berlocher, 1998), extensive efforts have been conducted to characterize cryptic phytophagous species occurring in the same geographic area (i.e. sympatric species), whereas little research considered allopatric or parapatric cryptic species.

To the best of our knowledge, no molecular studies on the genetic patterns among the worldwide populations of *H. vigintioctopunctata* have been conducted. The unique genetic and structural characteristics of mitochondrial DNA are the chief reasons to employ it in phylogenetic and evolutionary inferences facilitating for the identification of individuals or groups within a species. Interestingly, mutations in the CO1 gene could result in a number of ailments (Lin and Beal, 2006) or fluctuations in life-history traits that could have on the impact on the factors of

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insect fitness such as development, lifespan, and resistance to abiotic stress such as pesticides (Pichaud et al., 2012). Fu and Zhang (2005) conducted the first research on molecular systematic analyses of Coccinellidae, by sequencing partial COI gene region to infer the higher taxonomic level relationships of sixteen species belonging to four subfamilies. Molecular studies on Coccinellidae contributed to clarify the hidden relationships among species, which could not be resolved by the phenotypic studies or behavioral studies. Besides this, 16S rDNA gene has been shown to be useful for examining insect relationships from the genus level to the family level (Ribera et al., 2003). Aruggoda et al. (2010) investigated the sequences of the 16S rDNA gene of eight tribes belonging to six subfamilies of Coccinellidae as identified by the current phenotypic classification. However, little is known with regards to the haplotype variation that exists among *H. vigintioctopunctata* populations, hindering the further exploration into its evolutionary lineages. In this scenario, our study employed DNA barcoding with sequence analysis of COI gene marker to shed light on the genetic diversity and intra-population polymorphisms of *H. vigintioctopunctata*.

Material and methods

Collection and preservation

In South India, twenty haplotypes of *H. vigintioctopunctata* infested eggplants, *Solanum melongena* L. All samples were collected from four localities in Coimbatore state, namely Karamadai (11.16° N, 76.58° E), Pollachi (10. 40° N, 77. 01° E) Madampati (11.14° N, 77.08° E) and Palladam (10.98° N, 77.30°E). A total of 240 individuals were collected, each sample was represented by at least 15 adult individuals. All insects were collected from the dorsal side of the plant foliage by beating vegetation of *S. melongena*. The insects were examined over white paper sheets and transferred into clean glass collection vials by using an aspirator. The name of the collector, GPS coordinates of the location, as well as the date and time of sampling were noted (Supplementary Online Material Table S1). The study was conducted after obtaining due permission from the land owners, all individuals were identified at species level by the Zoological Survey of India, Regional Research Station of Chennai (India), then samples were preserved in 75 to 95% ethanol and kept at −20 °C in the entomological laboratory for molecular investigations.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was isolated from single whole insect sample using Qiagen DNeasy kit (Qiagen, Inc., Hilden, Germany) following the manufacturer's instructions. The amplified PCR product was visualized in 1% agarose gel electrophoresis (AGE) (GENEI, Bangalore) and the image was retained with the gel documentation (Medic Care, India). DNA amplification of COI gene was carried out by using ABI thermocycler with the following primers for COI gene forward (LCO1490: 5′-GGTCAACAAATCATAAAGATATTG-3′) and reverse (HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). Amplification was performed in a total volume of 50 μl containing 4 μl of DNA template, 20 pM of each primers, 400 μM of dNTP and 0.4 μl of Taq DNA polymerase (Qiagen). Thermo cycler conditions were as follows: 5 min at 95 °C for initial phase, then 35 cycles of 60 s at 95 °C for denaturation, 60 s at 52 °C for annealing, and 90 s at 72 °C for extension followed by 5 min at 72 °C for a final extension. Final PCR products were stored at −20 °C for further testing. The amplified products were resolved with 2% AGE and sequencing was done by using ABI 3500 XL Genetic Analyzer with manufacturer's protocol of Chromos Biotech, Pvt. Ltd., Bangalore (India). The sequences were trimmed and edited using Bio Edit v7.2.5 (Hall, 1999) and submitted to NCBI-GenBank. Multiple sequence alignment was done with T-COFFEE.

Species identification by DNA barcodes, composition of COI gene sequences and nucleotide divergence among haplotypes

The partial COI nucleotide sequence of each specimen was compared to barcode sequences on NCBI using BLASTn. Based on the availability in databases, seven of *H. vigintioctopunctata* haplotypes were retrieved from NCBI-GenBank and used for comparisons (Supplementary Online Material Table S1). The genetic divergence was calculated using the Kimura-2-parameter (MEGA.v.6) (Tamura et al., 2013). Overall AT bias and nucleotide sequence was computed using DnaSp.v.5.1 (Librado and Rozas, 2009).

Substitution rates and test of selection for the evolutionary divergence

The rate of transitions (TS) and transversions (TV) at the first, second and third codon positions were calculated and plotted against the F84 genetic using DAMBE 5.3.10 (Xia, 2013). Test of sequence saturation was performed to estimate the transition/transversion versus the genetic distance (F84). The codon based *Z*-test of selection was done to test the type of selection. The probability of rejecting the null hypothesis of neutral evolution in favor of the alternate hypothesis (positive/purifying selection) was done based on the *p*-values ($p < 0.05$). Analyses were conducted using the Nei-Gojobori method (Nei & Gojobori, 1986) for sequence pairs and all evolutionary analyses were carried out using MEGA v.6.

Genetic differentiation and haplotype network analysis

Genetic variability for COI marker was evaluated by the number of haplotypes (h), number of variable sites (S), average number of nucleotide differences (k), haplotype diversity (*Hd*) and nucleotide divergence using DnaSP v.5.0.The Tajima's D (Tajima, 1989) test was performed to test the hypothesis that all mutations were selectively neutral (Kimura, 1987) using DnaSP v.5.0.A TCS haplotype network was built to examine the haplotype relationship among 27 individuals to visualize relationships among haplotypes with default parameters using PopART software (http://popart.otago.ac.nz/index.shtml).

Phylogenetic analysis

Following the methods reported by Vadivalagan et al. (2015), the optimum substitution models were determined by the best-fit model test for the selection of the model to be applied for sequences. The T92 + G model was selected from 24 different nucleotide substitution models for the 10 original sequences of the selected species in the present study based on the Akaike Information Criterion (AIC) and lowest Bayesian Information Criterion (BIC) values. The maximum likelihood (ML) tree was constructed and the robustness of the clades of the tree was estimated using bootstrap analysis of 1000 replications with the elimination of all the codons containing gaps and missing data. The strength of the clades was assessed with the 10 random addition replicates and bootstrap analysis. Branch lengths were calculated by average pathway method and were presented as the units of number of changes over the whole sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood (−1834.6061) was shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 4.9847)). The analysis involved 27 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with <95% site coverage were eliminated. That