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Molecular identification of scale insect (*Eulecanium giganteum*) in *Hibiscus rosa-sinensis*

Suganthi M[1], Logeshwaran R[1], Abirami G[1], Rupa Shree B[1], Anandaraj P[1], Senthilkumar P[2*]

[1]Department of Biotechnology, Vels Institute of Science, Technology & Advanced Studies (VISTAS), Pallavaram 600117, TamilNadu, India
[2]Department of Genetic Engineering, SRM Institute of Science and Technology, Kattankulathur 603203, Chennai, TamilNadu, India

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ABSTRACT

Hibiscus rosa-sinensis is a widely grown evergreen valuable medicinal, ornamental species planted in India. Scale insects are small herbivorous insects found on all continents and they are serious sap sucking pests of many ornamental plants. These scale insects are undetectable due to their tiny size, basic morphology, and polyphagous feeding nature. Hence, the management of these tiny insects become a serious concern across the globe. To afford a prospective solution to the problem, an accurate, simple, and developmental-stage-independent identification method is required, hence this study attempted the molecular identification of scale insect in *Hibiscus rosa-sinensis* using mitochondrial gene Cytochrome Oxidase Subunit I (mtCOI) sequencing. The experiment was carried out by isolating insect DNA using a modified CTAB method. Through two or three rounds of error-prone PCR followed by a steady procedure to amplify a mtCOI region. This region of mtCOI has been used as a standard DNA barcode for a diverse array of taxa. The confirmation has been done by sequencing of mtCOI which suggest the highest similarities with *Eulecanium giganteum*. This study addresses the questions of biodiversity and molecular characterization of scale insects. Further, the information obtained in this study provides baseline data for future crop improvement programs and integrated pest management strategies.

* Corresponding author
E-mail: mpsenthilkumar@gmail.com (Senthilkumar Palanisamy)

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1 Introduction

Soft scales (Hemiptera: Coccoidea: Coccidae) are the most invasive group of pests and the largest family of scale insects in India, causing a notable loss in fruiting and ornamental crops. The species come under the genus Eulecanium and these are important economic pests distributed worldwide (Ben-Dov and Hodgson 1997). Usually, scale insects feed on the plant sap and excrete a sticky substance and induce sooty mold growth which causes an enormous economic loss in woody ornamentals and fruit trees (Li et al. 2002; Xie et al. 2006). Efficient management of these scale insects are limited due to the small size and inefficacy in morphological identification. Species-level identification of scale insects is often difficult and challenging due to their tiny size, reduced morphology, and high similarity in their immature stages (Watson and Kubiriba 2005). Recent studies reported that pine scales excrete non-metabolized insecticides in honeydew which has the potential to negatively affect organisms that feed on tainted honeydew such as predators, parasitoids, and pollinators (Quesada et al. 2020). *H. rosa-sinensis* known colloquially as china rose belongs to the family Malvaceae with important medicinal properties for treating diabetes, inflammation, wounds, cough, and fever, and also reducing the infections caused by bacteria and fungi (Kanthesh and Geethanjali 2021). Further, it is mainly used to prevent hair loss and gastric ulcers in several tropical countries (Missoum 2018). Due to their soothing properties, the flowers and leaves of Hibiscus have been traditionally used to treat medical conditions *viz*., gall bladder damage, cancer, to relieve dry coughs, lower blood pressure, and also to treat skin afflictions (Shashi et al. 2013).

A severe infestation of scale insects was observed in the stems of the Hibiscus plants. Scale insects vary dramatically in appearance, from very small organisms (1-2 mm) that grow beneath a wax cover of the stem to shiny pearl-like objects (about 5mm), covered with mealy wax. Usually, adult females are always immobile and attached to the stem. They secrete a waxy coating for defense, making them resemble fish scales, and so-called scale insects (Xie et al. 2006). Scale insects mainly damage the plant by sucking sap from leaves, stems, and trunks, further severely colonizing the stem and destroy which leads to the falling of leaves and stunted growth. Stems of hibiscus plants were found to be widely affected by scale insects (Chua 1997). However, there is little information on the molecular identification and species composition of scale insects infesting hibiscus in India. Generalizing the life cycle and biology of soft scales seems to be difficult since variations exist even among the subspecies.

DNA barcoding is a recent taxonomic supporting tool that uses a short gene as a marker in an organism's DNA to identify a particular species (Javal et al. 2021). DNA barcoding has several advantages over morphological identification-based taxonomy, being a relatively quick and easy identification process that uses small tissue samples and is not limited by developmental stages or gender. Insect mitochondrial DNA (mtDNA) is a small circular molecule (~16 kb in size) and has a fast mutation rate, which results in notable variation in mtDNA sequences between insect species and little variation within insect species (Wilson et al. 1985). A fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) is a conserved region and is considered a promising barcode in animals (Rodrigues et al. 2017). Molecular characterization was done using isolating the genomic DNA from the scale insect and amplifying the partial mitochondrial gene called COI . It was observed from several studies that the COI region (~710bp) can be used as a DNA barcoding tool for insect species identification. This region has provided accurate species identification even between the subspecies. The cytochrome c oxidase1 COI is used for species identification because of its high mutation speed and the highly conserved sequence of the species through which species can be easily identified even between the subspecies (Hebert et al. 2003).

In this study, a single insect was taken for insect DNA isolation. The protocol of DNA extraction is time-saving as well as economic with available laboratory chemicals, consumables, and basic equipment. Similar to the plant DNA extraction procedure, insect DNA extraction relies on a nonionic detergent like CTAB (Cetyl Trimethyl Ammonium Bromide) to lyse the insect cuticle (Saghai-Maroof et al. 1984). The amplification of the full-length sequence of the mtCOI gene was done and the sequencing of that region was performed. The present study initiated to identify the species of the *Eulecanium* genus. The resultant information may help for the insect management programs by allowing the implementation of mtCOI sequencing strategy on the insect DNA.

2 Materials and Methods

2.1 Identification and Collection of Scale insect

Scale insects were found on the stem of the *H. rosa-sinensis* plant in the area T. Nagar Chennai, Tamil Nadu. Insects were observed as thick scales and looked like a settlement of a waxy layer on the stem due to severe colonization. Adult scale insects were collected and stored in Eppendorf tubes containing 70% ethanol. Ethanol was added to the tubes to avoid bacterial or fungal formation on the insect. The tubes were stored in the refrigerator until used in further studies.

2.2 DNA isolation from the scale insect

Individual scale insect was taken and the abdomen was used to prepare the genomic DNA by following the modified cetyl tri

methyl ammonium bromide buffer (CTAB) method (Saghai-Maroof et al. 1984). The abdomen was ground by adding 1.0 mL of 2% CTAB, 1.4 M sodium chloride, 100 mM Tris-HCI (pH 8.0), and 20 mM ethylenediamine tetraacetic acid (EDTA), 0.1% of 2-mercaptoethanol and suspended in the same buffer. The suspension was incubated at 65° C for 2 hrs and chloroform: isoamyl alcohol (24:1) was added in equal volume. The suspension was centrifuged at 10,000 rpm for 10 min at 8°C. The supernatant was transferred to a fresh microcentrifuge tube without disturbing the middle protein interface. An equal volume of ice-cold 95% ethyl alcohol was added to precipitate the DNA. Further, centrifugation was carried out at 10,000 rpm for 5 min to get the precipitated DNA as a pellet. The resultant DNA pellet was washed with 70% ethanol and dissolved in 50 µL of TE buffer. Isolated DNA was further purified by adding 10 µg/100 µL of RNase mainly to remove RNA contaminants. Insect genomic DNA was resolved in 1% agarose/ ethidium bromide (EtBr) gel, visualized under UV transilluminator, and quantified using a spectrophotometer (Amersham Biosciences).

2.3 PCR amplification of COI gene

The Folmer fragment of the 5' region of COI was amplified using PCR procedure by using forward primer LCO (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). PCR amplification was carried out in 25 µL reaction mixture containing 10X PCR buffer (contains 25mM magnesium chloride), 2 µL dNTP mixture (0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP), 1µL of forward and reverse primer (10µmol each), 1 µL template DNA (50-150 ng), 0.3 µL Taq DNA polymerase (3U/µl), and final volume made with 16 µL distilled water. The mt COI regions were amplified in a Thermal Cycler (Eppendorf) with the PCR cycle program comprising 4 min of pre-denaturation at 94°C, followed by 35cycles of amplification (30 sec of denaturation at 94°C; 1 min of annealing at 46°C for COI; 45 sec of extension at 72°C), and final extension at 72°C for 10 min. The amplified PCR products were resolved in 1.5% agarose gel/ EtBr (0.5 µg/mL) and documented using a gel documentation system (Syngene).

2.4 COI sequencing and data analysis

The amplified bands of the COI gene were excised and purified from the gel using Gel Extraction Kit (Qiagen, Inc., Germany) by following the manufacturer's protocol. The purified PCR products were sequenced with an automated DNA sequencer with COI gene-specific primers at Barcode Biosciences, (Bangalore, Karnataka). Sequences were edited and aligned using BioEdit 7.0. The BLASTn program (http://www.ncbi.nlm.nih.gov/blast/) was used to identify the similarities between the sequences obtained and their homology in the public database. Multiple sequence alignments of the sequences were performed using CLUSTAL W and a phylogenetic tree was constructed using the Neighbor-joining tree.

3 Results

3.1 Identification and collection of scale insects from *Hibiscus* plant

Soft scales are tiny pests with a cottony, waxy surface that damage the plant by sucking sap from leaves, stems, and trunks thereby destroying the plant by reducing its growth. Scale insects were observed in the stem of *H. rosa-sinensis* plant (Fig. 1) which severely colonized the stem of the *Hibiscus* plant (Figures 1 & 2) and at the severe infection stage, it reached to leaves and lead to leaves falling and stunted growth. Figure 3 shows the stunted plant growth due to the scale insect comparing the normal growth of the plant without the insect. Insect sample was collected and transferred into 2ml of Eppendorf tube containing 70% ethanol.

Figure 1 Scale Insect identification in the *Hibiscus rosa-sinensis* plant

Figure 2 Scale Insect colonization in the stem- Close view

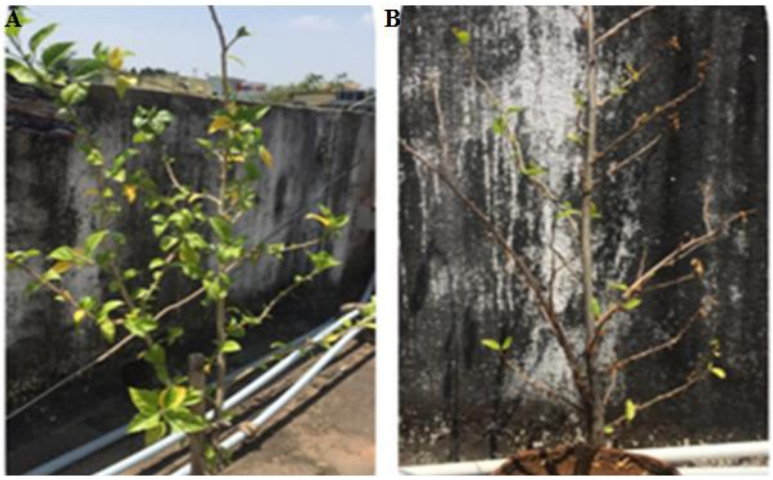

Figure 3 Comparison of the healthy plant (A) and plant affected by the scale insect (B)

3.2 Genomic DNA Isolation & PCR amplification of mtCOI gene

Genomic DNA was successfully isolated from the single-scale insect by using a modified CTAB method and the quality check was done by agarose gel electrophoresis which showed a good yield of genomic DNA under the Gel Documentation system (Figure 4). A manual CTAB method that provides high quantity and high yield DNA was used. Though the presence of RNA contamination and DNA degradation were seen, the yield quality of the DNA was good enough for the PCR amplification of the COI gene. Mitochondrial COI is a standard barcode that was used for species identification. The forward primer LCO1490 and reverse primer HCO2198 were used for the amplification of the COI gene. The PCR product was run on 1.2% Agarose gel and a 1Kb DNA ladder was used to identify the random size of the COI gene. The PCR amplification of the mtCOI gene yielded a 709 bp fragment (Figure 5).

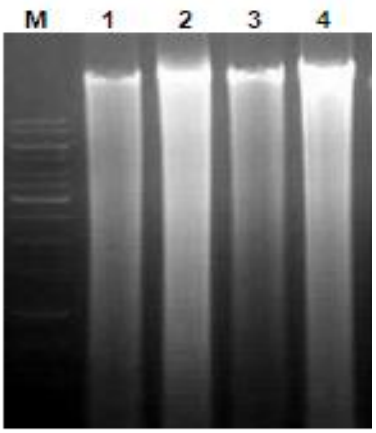

Figure 4 Genomic DNA isolation from *E. giganteum* (Lane M - DNA Marker, Lane 1-4 - Scale insect DNA with replication)

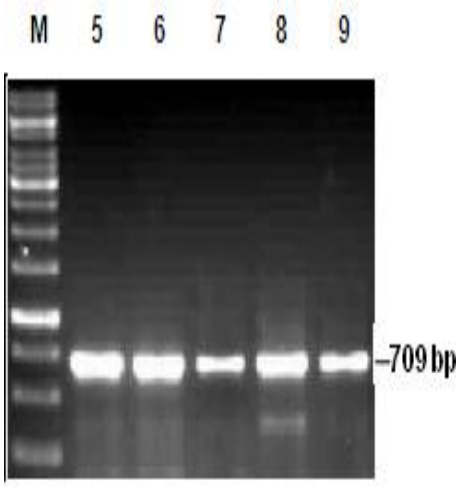

Figure 5 PCR amplification of COI gene

3.3 Sequencing analysis & PCR amplification of mtCOI gene

The sequencing of the purified PCR product was done using Sanger sequencing and a good chromatogram was obtained. mtCOI sequence analysis is essential to elucidate the presence of cryptic insect species. Using National Center for Biotechnology (BLAST) search, the mtCOI sequence was analyzed to find the regions of sequence similarity between the COI gene sequence of *Eulcaneum* and database sequences. Sequence analysis of the collected scale insect identified as *E. giganteum*. The BLAST search revealed that these insects have the highest similarity with *E. giganteum* mtCOI sequences.

Neighbor-joining phylogenetic tree (1000 bootstrap replications) constructed using mtCOI sequence of *E. giganteum* and other Genbank available mtCOI sequences. The phylogenetic tree was computed by using the Kimura-2 parameter method. Bootstrap values of more than 25 are shown. Bootstrap values are specified at each branching point. The scale bar point out the estimated genetic distance (Figure 6). Gaps are treated as missing data which are eliminated from the dataset during tree construction (Tamura et al. 2007).

4 Discussion

DNA barcoding is a major tool in the bio-surveillance of insect pests which allows rapid identification of an unknown insect

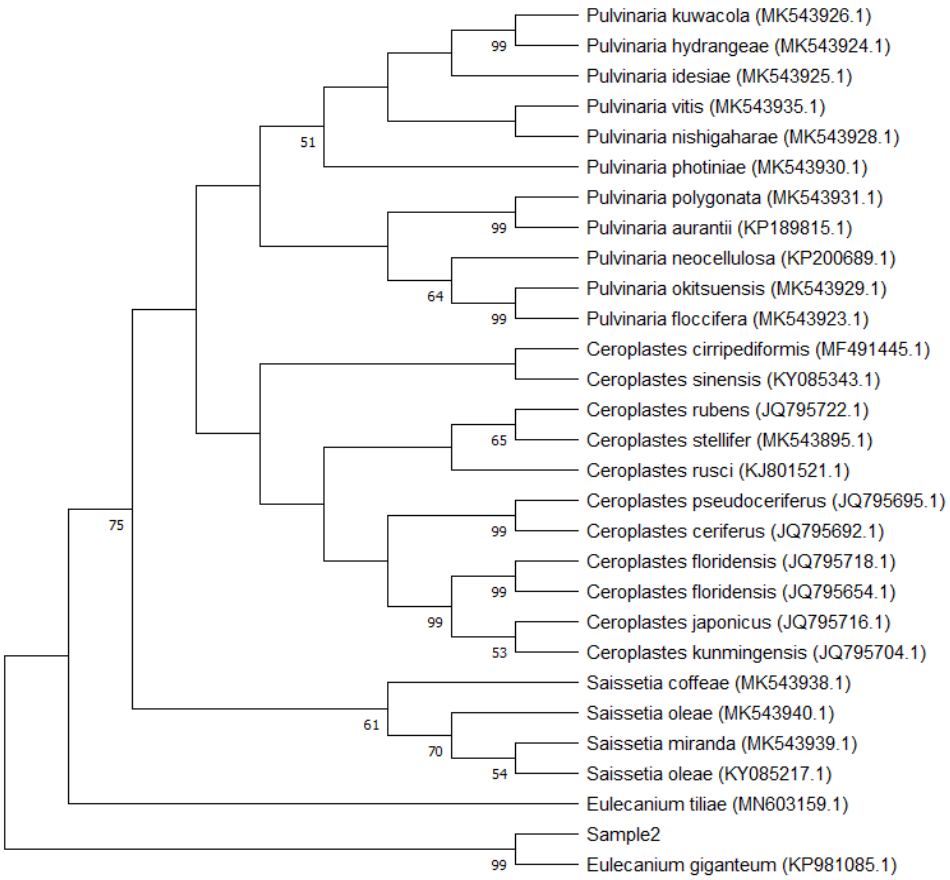

Figure 6 Phylogenetic tree analysis by using the neighbor-joining method

specimen independent of life stages (Javal et al. 2021; Hebert et al. 2003). Standardization of the PCR process for mtCOI gene amplification is the main principle in the DNA barcoding technique (Hollingsworth et al. 2011). The barcoding of an organism is done using standardized molecular techniques hence reducing any complications in morphological identification and easing the work of the researchers. The techniques that have been used for barcoding are DNA isolation, PCR amplification, and mtCOI gene sequencing (Seifert et al. 2007). DNA barcoding of insects is complicated because of the difficulty in isolating the DNA from the insect. Many successful manual methods were developed for getting a high quantity and high yield of insect DNA (Saghai-Maroof et al. 1984; Aljanab and Martinez 1997) by using tiny insect specimens. Recently, mtCOI sequencing was successfully used to identify the natural enemy of stored pests named *Xylocoris flavipes*, which promotes the effective utilization of *X. flavipes* in pest control for safe storage of grain (Zonglin et al. 2021).

In the previous study by the same author, a notorious pest of tea, the tea mosquito bug was collected from different tea plantations in India, and mitochondrial COI marker was successfully used to determine the species as *Helopeltis theivora*, which further leads to easy control measures (Suganthi et al. 2016). The tea scale (*Fiorinia theae* Green) is considered the most destructive pest of *Camellia* spp. in the southeastern U.S. (Borden and Dale 2020). Also in tea, cottony scale *Pulvinaria floccifera* was identified along with the parasitoid *Lysiphlebia* sp. by using this CO1 sequencing method (Sharma et al. 2019). In the present study, single species of *E. giganteum* was identified in the stem of the *Hibiscus* plant. Similarly, Engstrand et al. (2010) confirmed that avocado stem weevil, an important pest in avocado plantations is indeed one species named *Copturus aguacatae* using mtCOI sequencing and the result may support for successful implementation of biological control through pheromone synthesis. Higher genetic variation was observed between the species and relatively less variation within species using DNA barcodes (Hebert et al. 2003). Hence DNA barcoding has an advantage for identifying the tiny insect with less morphological polymorphism and different life stages. The mtCOI gene was used from the mitochondrial genome because it was found to be the most conservative sequence and successfully amplified for a different class of insect species (Folmer et al. 1994). In the present study, the scale insect mtCOI sequence was compared with NCBI available sequence. BLAST results showed that the sequence was matching 82% with *E. giganteum* (KP981085.1), matching 80.07% with *Aspidiotus excisus* (MK863028.1), 78.92% with *Aonidiella ensifera* (KY085356.1).

DNA barcoding was successfully utilized to identify three different thrips species *viz*., *Thrips tabaci, T. vulgatissimus*, and *T. palmi* (Karimi et al. 2010). Apart from mtCOI sequencing, the molecular diagnostic marker was developed by using the ribosomal DNA internal transcribed spacer 2 (ITS2), and this reliable molecular technique is used for the identification of various thrips species including *S. dorsalis* (Farris et al. 2010). In this study, the phylogenetic separation of *E. giganteum* (Figure 6) established the satisfactory resolution of the COI marker. In the phylogenetic tree, the mtCOI sequence of *E. giganteum* was matching 99% with already reported *E. giganteum* (KP981085.1), matching 70% with *Saissetia miranda* (MK543939.1), 65% with *Ceroplastes rubens* and *C. stelifer* (JQ795722.1 & MK543895.1) (Choi and Lee 2019; Deng et al. 2012) Hence DNA barcoding using mtCOI markers has a notable advantage where the morphological polymorphism is absent and identification of insects in various life stages.

Conclusion

In the present study, the mtCOI sequence marker was effectively used for the molecular identification of scale insects from the *Hibiscus* plant. Also, sequence analysis of the mtCOI gene confirmed that these scale insects belong to the genus *Eulcaneum* and species *giganteum*. Identification and characterization of common horticultural pests can be successfully applied in taxonomic classification. Using the DNA barcoding technique, future management of these scale insects can be more precisely implemented with accurate species identification. The study results might be useful to form the control measures since the *E. giganteum* is the notorious species of *Eulcaneum* infecting the Hibiscus.

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