Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

Original Research Article **DOI: 10.26479/2019.0501.36**

MOLECULAR IDENTIFICATION OF A PARASITOID FROM *PULVINARIA FLOCCIFERA* INFESTING KANGRA TEA OF HIMACHAL PRADESH, INDIA

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ABSTRACT: Cottony camellia scale, *Pulvinaria floccifera* (Westwood, 1870) (Hemiptera: Coccidae) is one of the important soft scale species infesting more than 80 host plant species in the tropical and sub tropical environment in the world. It is observed as a major pest of tea in Kangra Valley, Himachal Pradesh, India. These scales are parasitized by some parasitoids. Therefore, a simple and quick molecular approach was developed to detect and identify a parasitoid of *P. floccifera* within the host. About 658 bp fragment of mitochondrial COI was amplified using universal primers. The blast analysis showed its 99% similarity with the parasitoid, *Lysiphlebia japonica*, the most important natural enemies of aphids. However, interestingly, in the present study, *Lysiphlebia* has been observed and reported from *P. floccifera* infesting tea.

KEYWORDS: Parasitoid, *Lysiphlebia japonica,* scale insect, *Pulvinaria floccifera*, tea, mitochondrial COI.

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1.INTRODUCTION

Scale insects (Hemiptera: Coccoidea) are phytophagous insects that infest shrubs and trees. These suck the sap with the help of their piercing-sucking mouth parts and are the major pests that cause serious damage to ornamental plants, horticulture and other economically important crops including tea [1]. Scale insects have been reported as serious pests attacking a huge number of host plants

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around the world [2, 3, 4, 5, 6, 7, 8]. These excrete honeydew that results in the development of sooty mould [9, 10]. Severe infestation cause yellowing or drooping of leaves, unthrifty appearance of the plants and eventually death of all or part of the plant resulting in qualitative and quantities losses. Besides pesticides, scale insects are managed by different natural enemies including coccinelids, lacewings, and parasitoid wasps [11]. The most important groups of natural enemies that have been used in biological control of scale insects belongs to the parasitoids families viz. Aphelinidae, Encyrtidae, Eulophidae, Eupelmidae, Pteromalidae and Signiphoridae (Hymenoptera: Chalcidoidea) [12, 13]. In the management of scale insects, it is very important to know and study the parasitoid species infesting scale insects, so that they can be utilized in maintaining scale insect populations below the economic injury level [14]. Identification of parasitoid species is essential for their mass multiplication and utilization in biological control and integrated pest management [15]. Cottony camellia scale, *Pulvinaria floccifera* (Westwood) is reported as a major pest of tea in Kangra Valley, Himachal Pradesh, India. It has been observed to be infested with the parasitoids. Correct identification of species is essential for studies of biological control and integrated pest management [15, 16, 17]. Misidentification of parasitoids used in biocontrol may result in serious economic losses [18, 19, 20, 21]. In order to confirm the infestation by the parasitoid, one has to wait the emergence of the parasitoid from its host. Coccidae and their parasitoids are considered difficult groups to identify at the species level because of their small size and high degree of similarity [22]. Therefore, an alternative method is needed because dissection of different life stages of host insect for identifying the parasitoid is difficult. In this experiment, a simple and quick molecular approach was developed to detect and identify a parasitoid of cottony camellia scale, *P. floccifera* within the host.

2. MATERIALS AND METHODS

Sample collection

Adults of cottony camellia scale, *Pulvinaria floccifera* were collected along with the tea twigs from the CSIR-IHBT experimental tea gardens located at Banuri (32.1090 N, latitude; 76.549 0 E longitudes; 1290 m amsl), Palampur, Himachal Pradesh, India. Some of them were kept in plastic jars under the laboratory conditions (25±1 ºC; 60±10% RH; 16: 08 light and dark hrs) for studying the emergence of parasitoids and some were stored at -80 ºC.

DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA of scale insects suspected to be infested with parasitoids was extracted from the scale insects kept at -80 ºC using DNeasy Blood & Tissue Kit (QIAGEN Inc.) as per the protocol provided with the kit with some modifications. Briefly, 20 mg scale insects sample was taken and washed with distilled water 2-3 times. 180 µl of prewarmed ATL buffer was added to the eppendorf containing washed sample. After mixing, 50µl of RNAase was added, and the sample was kept at 30 ºC for 30 min. 20 µl of proteinase K was added to the sample, and was kept at 56 ºC for 2 hours.

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The eppendorf containing sample was mixed after regular interval and 200 µl of AL buffer and ethanol each was added to it and vortexed. The whole solution was added to the column and centrifuged at 8000 rpm for 1 min. The same procedure was followed with AW1 and AW2 buffer at 8000 and 14000 rpm for 3 and 1 min, respectively. The column was transferred to fresh eppendorf and added 50 µl of AE buffer which was centrifuged at 8000 rpm for 1 min. The DNA was then resolved on 0.8% agarose gel and stored at -20 ºC till further analysis. The partial sequence of mitochondrial cytochrome oxidase (COI) gene was amplified by polymerase chain reaction (PCR) using thermal cycler (ABI-Applied Biosystems, Veriti, USA) and universal primer pair, LCO1490 (5–GGT CAA CAA ATC ATA AAG ATA TTG G–3) and HCO2198 (5-TAA ACT TCA GGG TGA CCA AAA AAT CA-3) [23]. The PCR amplification of the processed samples were performed using following conditions for 25 µL amplification reactions: initial denaturation for 2 min at 94 ºC, followed by 35 cycles of 45 sec at 94 ºC, 45 sec at 55 ºC, and 1 min at 72 ºC and a subsequent final extension at 72 ˚C for 5 min. The amplified PCR products were then resolved on 1.2% agarose gel, stained with ethidium bromide and visualized in a gel documentation system (UVP).

Sequencing and sequence analysis

The amplified products were purified using Nucleospin® Gel and PCR clean-Up System kit according to the provided protocol. Briefly, for each 100 µl PCR product, added 200 µl of NT1 buffer. A column was then placed into a collection tube (2ml) which was loaded with the sample and was centrifuged at 11,000 rpm for 30s. Flow-through was discarded and 700 µl NT3 buffer was added to PCR clean-up column and centrifuged for 30s at 11000 rpm. Flow-through was discarded and the step was repeated for proper washing of the sample. 100 µl ethanol was added and centrifuged for 1 min at 11000 rpm to remove buffer NT3 completely. The column was placed in a fresh 1.5 ml microcentrifuge tube. 20 µl NE buffer was added in each column and incubated at room temperature (25±1ºC) for 1 min. The tubes containing columns were centrifuged at 11000 rpm for 1 min. The eluted DNA was preserved at -20 ºC. The eluted DNA was then checked on 1.0% agarose gel. The sequencing of the PCR product was done in both the directions by automated sequencer (3130xl Genetic Analyser, Applied Biosystems, HITACHI). The reaction was performed under the following conditions: initial denaturation at 96 ºC for 2 min followed by 24 cycles of denaturation at 95 ºC for 10s, annealing at 55 ºC for 5s and extension at 60 ºC for 4 min. Resulting chromatograms were analyzed using Chromas Lite and the homology search was done using NCBI-BLASTn programme (http://blast.ncbi.nlm.nih.gov). The blasted sequences were explored using MEGA 7.0 [24] to obtain conspecific and congeneric distances, while Neighbour-Joining (NJ) tree with 1000 bootstraps replicates and Kimura-2-parameter (K2P) substitution model was constructed [25, 26, 27].

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3. RESULTS AND DISCUSSION

DNA isolated from the cottony scale insect, *P. floccifera* suspected to be infested with parasitoid was resolved on 0.8% agarose gel. About 658 bp fragment of mitochondrial COI was amplified using universal primers and resolved on 1.2% agarose gel (Fig. 1). The visualized PCR product contained only discrete single band indicating that the sequence obtained is of mitochondrial DNA.

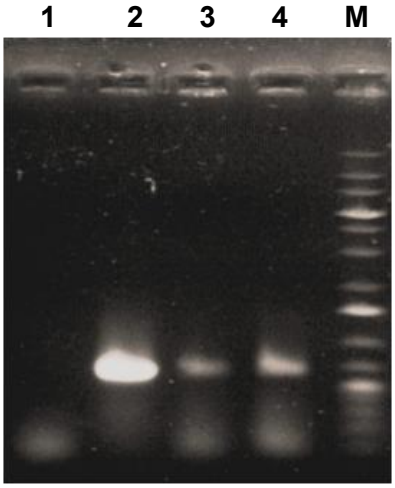

Fig. 1: PCR amplification of a mitochondrial cytochrome oxidase I (COI) gene fragment using DNA (isolated from scale insect). Lane 1: Blank; 2, 3 and 4-genomic DNA isolated from scale insect; M-DNA size marker (1 Kb DNA ladder)

The nucleotide sequences have been accessioned in NCBI, Bathesda, Maryland, US (IHBT SP, Accession No. MK430928). A phylogeny tree using N-J method revealed the percentage identity with its corresponding species (Fig. 2). NJ analysis allowed discrimination of species due to significant barcode gap. The sequence obtained in the present study was compared with the homologous sequences available in NCBI Blastn. The blast analysis showed its 99% similarity with the parasitoid, *Lysiphlebia japonica*, the most important natural enemies of aphids. It has been employed as a biocontrol agent for management of aphids and is reported to be imported by USDA from Japan against brown citrus aphid, *Toxoptera citricida* as a component of integrated pest management [28]. However, interestingly, in the present study, it has been observed and reported from the cottony camellia scale, *P. floccifera* infesting tea. The presence of this parasitoid in this scale insect was further confirmed, when parasitoid flies emerged from the scale insects kept under laboratory conditions collected from the tea field. In the present study, when DNA isolated from the host camellia scale insect that were infested with parasitoid was amplified using universal primers, no products of host scale insect were formed. Only the amplification of the parasitoid DNA appeared that was further confirmed with sequencing results. When the same template was amplified with fragment of D2 region of 28s rDNA using forward (5-CGT GTT GCT TGA TAG TGC AGC-3) and reverse (5-TTG GTC CGT GTT TCA AGA CGG–3) [29] primers, amplification of host DNA was achieved.

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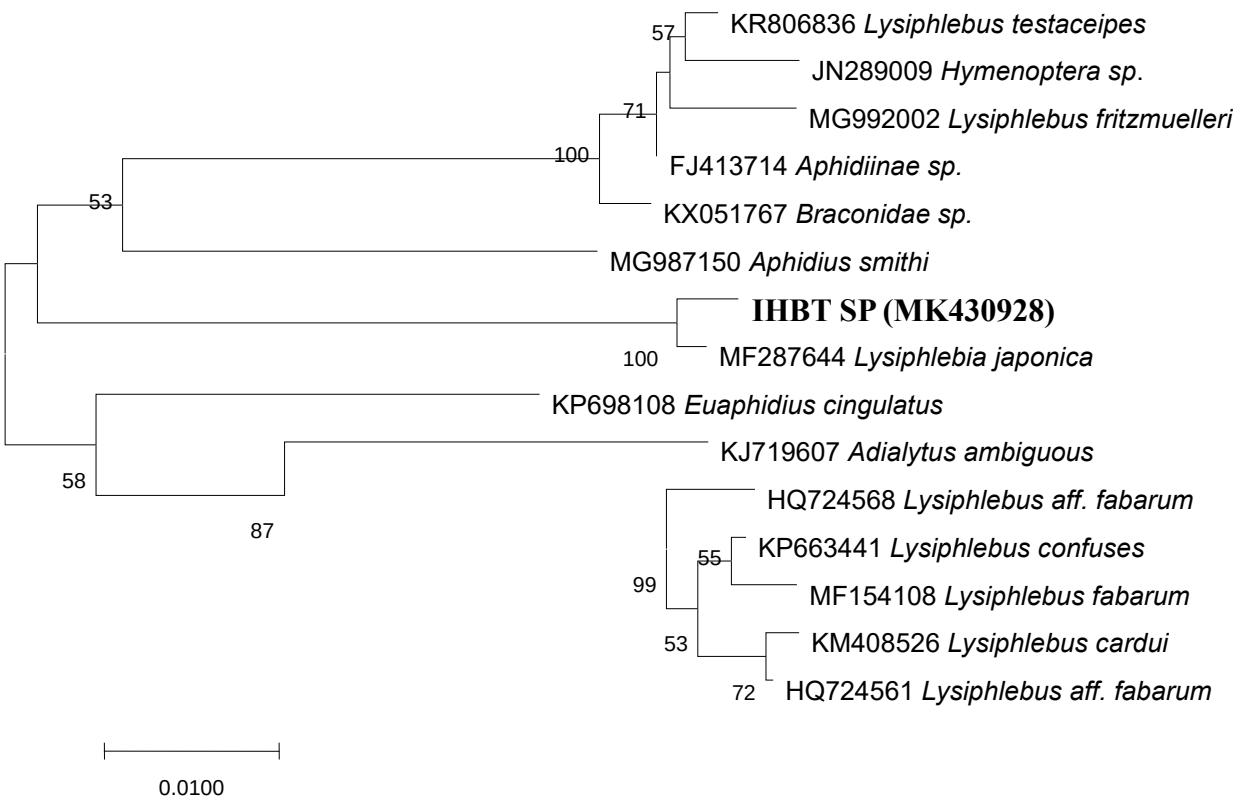

Fig. 2: Neighbour joining tree of mitochondrial COI based on Kimura 2-parameter. Number above branches indicate support of NJ based on bootstrap test with 1000 replicates

Similarly, rDNA sequences for ITS2 regions were used to discriminate host from parasitoid [30, 31]. The present method successfully detected and identified a parasitoid within its host, cottony camellia scale infesting tea plantation of Kangra Valley, HP. This method can alternatively be employed to increase the rate of parasitoid species identification within cottony camellia scale and other host scale insects.

4. CONCLUSION

A parasitoid, *Lysiphlebia* sp. is detected and identified within cottony camellia scale, *P. floccifera* using molecular approaches. COI barcoding sequence approach can be used to detect and identify different parasitoids within their hosts.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, CSIR-Institute of Himalayan Bioresource Technology (India), for providing necessary infrastructure, field and laboratory facilities. One of authors, Ms. Ruchi Sharma acknowledges to the CSIR, New Delhi. India for providing Senior Research Fellowship (SRF) ACK No.: 112663/2K11/1 and financial assistance. This manuscript represents CSIR-IHBT communication no. 4352.

CONFLICT OF INTEREST

The authors hereby declare there is no conflict of interest.

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