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MOLECULAR CHARACTERIZATION OF *WOLBACHIA* IN RUGOSE SPIRALLING WHITEFLY *ALEURODICUS RUGIOPERCULATUS* AND ITS PARASITOID *ENCARSIA GUADELOUPAE*

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ABSTRACT

***Wolbachia* is a common maternally inherited bacterial endosymbiont of insects. Many species of whiteflies and their endoparasitoids exhibit interspecific variations/ species complexes which can be analysed through the variations between the host and the parasitoid using molecular markers. This study characterized the occurrence of *Wolbachia* in the whitefly *Aleurodicus rugioperculatus* and its parasitoid *Encarsia guadeloupae*. The *Wolbachia* surface protein(wsp) (450-600 bp) sequenced from the host-parasitoid collected from the field populations at Karnataka was analysed. The comparison of these sequences in the NCBI database through BLAST and ORF analysis revealed the presence of two new sequences infected with *Wolbachia* from the samples collected on the coconut palms (*Cocos nucifera*). The genetic relatedness of these has been brought out using the wsp gene through PCR.**

Key words: *Wolbachia*, *Aleurodicus rugioperculatus*, *Encarsia guadeloupae*, wsp, host, parasitoid, species, transitions, transversions, genetic relatedness, BLAST, ORF and phylogeny.

Wolbachia is a highly diverse group of intracellular, maternally inherited endosymbionts belonging to the α-Proteobacteria (Jeyaprakash et al., 2000; Prakash and Puttaraju, 2007), and these infect a wide range of arthropods (Hilgenboecker et al; 2008). These bacteria are known for resulting in reproductive anomalies in their arthropod hosts (Stouthamer et al., 1999; Saridaki and Bourtzis, 2010). Most notably the reproductive manipulations result in an increased proportion or fitness of *Wolbachia*-carrying females (Werren et al., 2008). *Wolbachia* is an obligate nutritional symbiont in filarial nematodes and bed bugs (Hosokawa et al., 2010) and in other cases where it is facultative, may contribute to the fitness of hosts by enhancing pathogen resistance or nutrient provision (Hedges et al., 2008; Brownlie et al., 2009). *Wolbachia* has attracted much interest for its role in biological, ecological and evolutionary processes (Kambris et al., 2009). The rugose spiralling whitefly *Aleurodicus rugioperculatus* Martin has been recently reported from India (Shanas et al., 2016; Martin et al., 2004; Selvaraj et al., 2017). It is highly polyphagous with 118 hosts belonging to 43 plant families (Francis et al., 2016). It mainly infests coconut palms and other broad-leaved hosts in its native range (Mayer et al., 2010).

Interspecific transmission of secondary symbionts has been inferred from phylogenetic data (Kraaijeveld et al., 2011; Ahmed et al., 2013). By natural means, interspecific symbiont transmission has been documented between parasitoids developing in the same host (Duron et al., 2010) and between hosts and the parasitoids developing on them (Chiel et al., 2009). One of the promising routes to determine transmission between hosts and endoparasitoids is through molecular identification, and phylogenetic analyses confirm this (Vavre et al., 1999; Zchori-Fein et al., 2004). Parasitoids of the genera *Encarsia guadeloupae* feed and develop in *A. rugioperculatus* nymphs and are potentially vulnerable to symbiont infections. Therefore, the most informative method to determine the *Wolbachia* infection at the molecular level in *A. rugioperculatus* and its parasitoid *E. guadeloupae* is by using the molecular marker and sequencing the amplicons generated from the *Wolbachia* surface protein (wsp) gene (wsp) gene (Chiel et al., 2014) by using the consensus sequences through blast search and later submitting in NCBI database to assign genetic relatedness between interspecies (Pradeeksha and Puttaraju, 2020). Analysis of the wsp gene greatly improves the understanding of the genetic diversity of the whitefly parasitoid species complex (Bosco et al., 2006; Dinsdale et al., 2010; Ellango et al., 2015; Francis et al., 2016). Identification of the genetic diversity between the two insect species accurately determines whether the *Wolbachia* present in these are pests or vectors (Brown, et al., 1995). The present study characterizes the interspecific genetic diversity of the whitefly parasitoids collected from different locations in Karnataka.

MATERIALS AND METHODS

Aleurodicus rugioperculatus nymphs were randomly collected from plantations crops at the Krishi Vigyan Kendra, Mangalore, Dakshina Kannada (12.86'N, 74.86'E). Collected specimens were brought to the laboratory and placd in closed petri dishes for the adult emergence of whiteflies or parasitoids. From these 50 from each were preserved in 100% ethanol, and stored at -80^{0}C prior to DNA extraction. DNA extraction and PCR analysis were carried out at the ICAR-National Bureau of Agriculturally Important Insect Resources (NBAIR), Bangalore. Extraction of DNA was carried out from the somatic tissues of the thorax and upper abdominal region of the single specimen of *A. rugioperculatus* and *E. guadeloupae* which is rich in mitochondria. The extraction process was performed using DNAase Qiagen kit method (catalogue no: 69504) based on the manufacturer's protocol. The quality of the extracted DNA was checked using the spectrophotometer. Thus, the crude DNA extracted was stored at -20^{0}C until it is subjected to PCR amplification. PCR amplification for the gene of interest was carried out using the standard protocol which involves the cocktail of reactions, using forward primer wsp-81F (5'TGG TCC AAT AAG TGA TGA AGA AAC) and the reverse primer wsp-691R (5'AAA AAT TAA ACG CTA CTC CA 3') (Puttaraju and Prakash, 2005). The reaction was carried out in flat topped PCR tubes of 200ul volume manufactured by Tarsons, Kolkata, India. The PCR reaction involves the cocktail of mixtures where the final volume is made up to 50 µl consisting of thermoscientific ® 5 µl of Taq buffer, 1µl of thermoscientific 10 mM dNTP mix, 1 µl of forward primer and reverse primer manufactured by Bioserves, Hyderabad, India, 1 µl of Thermoscientific Taq DNA Pol (1U/µl), 5µL DNA (50ng/µl) and 36 µl of Himedia molecular grade water, with the thermo-cycling profile consisting of initial denaturation at 94^{O}C for 5 min, followed by 36 cycles with denaturation at 94^{0}C for 1 min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 10 min. PCR reaction was performed using C1000TM thermocycler. Later, the quality of the amplicons was checked on 1.5% agarose gel electrophoresis (Sambrook and Russell, 2001). Followed by staining of the gel with 0.5 µg/ ml ethidium bromide prior to casting. Gel documentation was done by using UV transilluminator documentation system. Later, the sequencing of the amplified products was done by availing sequencing facility from Chromous Biotech, Bangalore.

Wolbachia infection was detected by PCR amplification of wsp fragments and the specificity of the amplicons was further verified by sequencing of the samples from single specimen of *A. rugioperculatus*, and its endoparasitoid *E. guadeloupae*. For this purpose, attempts were made to amplify the wsp gene through PCR and to sequence the amplified double stranded DNA directly using an automated fluorescent DNA sequencer. Bidirectional sequences of the amplified products of the *A. rugioperculatus* and its parasitoid *E. guadeloupae* were generated and analyzed for quality by using Bioedit 7.0.2 software, whereas similarities and ambiguities like insertions, deletions, stop codons, and frameshifts in these sequences were analyzed using proofreading tools like NCBI BLAST and ORF finder search tools (Altschul et al., 1990). Later, the respective accession numbers were generated by submitting the sequences to the GenBank database.

The pairwise and multiple sequence alignment of 11 sequences were performed, 9 sequences which were already present in NCBI database was downloaded and the remaining 2 new sequences were used for the analysis using the parameters like neighbour-joining, bootstrap method and Kimura-2 from MEGA X (Tamura et al.., 2011). Prior to the analysis, the sequences were aligned using CLUSTAL W software. Later, the tree was constructed based on a heuristic search approach using the above parameters. Accordingly, the branch lengths of the tree were calculated, by analysis of the base substitutions per site among all sequences, along with codon positions including 1st, 2nd, 3rd and non-coding regions, followed by the elimination of the codons with gaps and missing data from the total dataset. The total nucleotide content (A, T, G, C, AT, and GC) of all sequences was calculated using a computer program introduced in the bioinformatics lab at NBAIR. Residual position and pairwise distances were estimated using the Clustal W alignment matrix tool of MEGA X software with default settings of gap parameters, including gap opening penalty 15, a gap-extension 6.66 in pairwise and 6.66 in multiple alignments, transition weight of 0.50, sequence divergences were calculated. An NJ tree of distances was generated to provide a graphical representation of species divergences or closely relatedness to elucidate interspecies genetic relatedness through phylogenetic tree (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

With morphological and molecular identification (Martin, 1987; Dubey and David, 2012; Heraty et al., 2008; Hoddle et al., 1998), DNA extraction and PCR analysis, the whitefly species and its parasitoid were identified. Amplified products from the PCR analysis of the whitefly-parasitoid samples were detected and checked for low quality bases at the ends on the

agarose gel through UV transilluminator (Whitworth et al., 2007; Wilson, 1989), and gene of interest in the range of 450-600 bp were classified as wsp amplicons (Fig. 1). The results revealed that whitefly-parasitoid species belong to *A. rugioperculatus* Mad (548bp) and *E. guadeloupae* (538bp). These were compared to the homologous sequences available in GenBank through BLAST search tool. Of all the 11 sequences used in the study, 9 were found to be similar with GenBank data (80- 100%); and remaining 2 sequences were observed to recorded for the first time at Karnataka in India on the coconut palms. Distribution of the whitefly-parasitoid complex revealed its distribution in Karnataka (Guruprasad et al., 2015; Fig. 2; Genbank accession numbers- RSW-K, MK550508; Eq-K- MK543951). These were used for phylogeny construction using MEGA software. The 11 sequences of 5 species get classified into two major clades from the node which consists of inter species belonging to different order and family which was analyzed using 1000 bootstrap values during the phylogeny test (Felsenstein,1985). The overall transition/ transversion bias is R = 0.25 and the transition/ transversion rate ratios are k1 = 0.08 (purines) and k2 = 0.901 (pyrimidines). There was a total of 621 positions in the final dataset, where transitions are more than transversions and there were two clusters (Fig. 3). The first cluster consisted of two subclades representing closely related whitefly species like *A. rugioperculatus,* parasitoid- *Encarsia guadueloupae* and *Bemisia tabaci* which originated from the similar ancestors along with another subclade consisting of *Encarsia* sp w *En* sp.1*, Encarsia* sp w En 1*, Wolbachia* sp w Ebim-2*, Encarsia* sp. w En_sp 2, *Wolbachia* sp. wE1*, Encarsia bimaculata* wEbim 1*, Eretmocerus* sp w Esp. 2 and *Eretmocerus mundus* wE mun 1. The second main cluster consisted of the characters from their ancestors which are closely related to the first clade, but shared a close relationship with the species of the first subclade. The present results reveal that the genetic relatedness of the whitefly -parasitoid species studied are more towards the south and eastern parts of India, particularly in coastal regions.

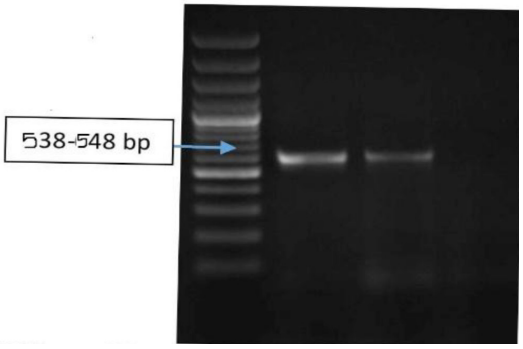

Fig. 1: PCR amplification of the whitefly species *A. rugioperculatus* and its endoparasitoid *E. guadeloupae*
Lane 1: Molecular weight marker (lkb); Lane 2: *E. guadeloupae*; Lane 3: *A. rugioperculatus*; Lane 4: Negative control

(a) (b)

Fig. 2. (a) *A. rugioperculatus* nymphal instar; (b) *E. guadeloupae* parasitized nymphal instars

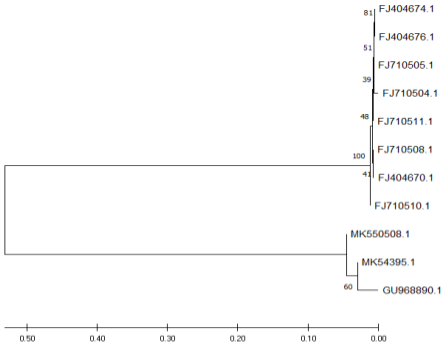

Fig. 3. Phylogenetic tree- neighbour joining method whitefly-parasitoid (wsp gene)

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