Pakistan Entomologist

Journal homepage: www.pakentomol.com

DETECTION AND GEOGRAPHICAL DISTRIBUTION OF *WOLBACHIA ENDOSYMBIONT* IN THE NATURAL POPULATIONS OF COTTON LEAFHOPPER, (*AMRASCA DEVASTANS)* IN PAKISTAN.

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ARTICLE INFORMATION

Received: May 25, 2016

Received in revised form: November 06, 2016

Accepted: November 20, 2016

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ABSTRACT

Wolbachia a maternally transmitted endosymbiont is found in the reproductive and steroidogenic tissues of arthropods and nematodes. The cotton leafhopper, *Amrasca devastans* (Distant) (Cicadellidae: Homoptera) is sap feeding pests of malvaceous and solanaceous crops and is widely distributed throughout the cotton growing provinces, Sindh and Punjab.Therefore,there is need to work on the identification, diversity and molecular characterization of secondary endosymbiont isolated from cotton leafhopper from Pakistan. In the present study, we employed a *Wolbachia* specific *16S rRNA* PCR assay to investigate the presence of *Wolbachia* in live field specimens of *A. devastans* collected from 8 different cotton field locations from Punjab province Pakistan. *Wolbachia* endosymbiont detection percentage was 23.5%. Sequence comparisons revealed that the *16S rRNA Wolbachia sequence from A.devastans* had the highest sequence identity to the *Wolbachia* strain of whitefly reported from India and China. This study is first evidence of *wolbachia* endosymbiont incidence in cotton leafhopper using *16S rRNA* PCR .By using this technique manipulation of that endosymbionts help to control the the sap feedind pest in future.

Keywords: Wolbachia, *Amrasca devastans*, biodiversity, endosymbiont, Pakistan, cotton leafhopper

INTRODUCTION

The cotton leafhopper, *A. devastans* (Distant) (Cicadelleidae: Homoptera) is one of the major sap feeding pests of malvaceous and solanaceous crops and is broadly distributed throughout the cotton growing provinces, Sindh and Punjab in pakistan (Shabbir, 1958; Babar *et al*., 2013). Cotton leafhopper is also known as cotton jassid, okra leafhopper, eggplant leafhopper, green jassid and Indian cotton leafhopper. Host plants other than cotton includes okra, holly hock, brinjal, potato, maize, sorghum, groundnut, pigeonpea, sunflower, beetroot, mulberry, amaranthus, marigold and cucurbits (Caasit-Lit, 1989; Mitsuhashi *et al*., 2002; Akbar *et al*., 2012). The pest has been recorded as damaging the cotton and okra crops in countries such as Pakistan, India, Thailand and other South East Asian countries (Srivastava, 1993; Gariepy *et al*., 2014; Tembe *et al*., 2014). Twenty five species of *Amrasca* attacking cotton plant have been recorded throughout the world (Afzal and Ali, 1969). *A. devastans* nymphs and adults suck plant sap resulting in downward leaf curling, burning, drying, and growth retardation and shedding of buds and bolls of cotton plants (Srivastava, 1993; Rashid *et al*., 2002; Pontarotti, 2010). *A. devastans* infestation damage to plant may account for 18.78 percent loss of cotton yield in Pakistan (Anonymous *et al*., 1992).

Wolbachia (Alphaproteobacteria: rickettsiales) is a maternally and cytoplasmically transmitted endobacteria which is found in the reproductive and steroidogenic tissues of arthropods and nematodes (Bordenstein and Rosengaus, 2005; Hoerauf and Rao, 2007; Negri *et al*., 2010; Pontarotti, 2010; Ahmed *et al*., 2010; Henke *et al*., 2013; Kennedy *et al*., 2013).

Up till now Wolbachia has been reported as most abundant and widely spread endosymbiont in the insects, about 75% of insect species are estimated to be infected by *Wolbachia* (Hilgenboecker *et al.,* 2008). *Wolbachia* has gained attention

Cite this article as: Shafiq, M., S. Arif, A. Bibi, M. Ali, M.S. Haider, M. Ashfaq, S. Ahmad, M.T. Manzoor, M. Shahzad, A. Tanveer and A. Ahmad, 2016. Detection and geographical distribution of wolbachia endosymbiont in the natural populations of cotton leafhopper, (*Amrasca Devastans*) in Pakistan. Pak. Entomol., 38(2):141-146.

as parasitic symbionts all around the world among entomologist, molecular biologist, plant pathologist and ecologist due to its unique ability to cause different reproductive manipulations in arthropods such as cytoplasmic incompatibility, feminization of genetic males, parthenogenesis induction and killing of genetic males. *Wolbachia* is diverse enough to establish itself in all kind of environment and ecosystem (Bandi *et al*., 1998). Due to this diversity of *Wolbachia* scientists have classified bacteria into different "super groups" from "A to K" depending upon different species and type of primer used mostly *ftsZ* and wsp (Ros *et al*., 2009). This is the first report of *Wolbachia infection* from cotton leafhopper (*A. devestans*) using 16S rRNA markers in Pakistan.

Materials and methods

This experiment is carried out in postgraduate central lab, Institute of Agriculture science, university of the Punjab Lahore. Eighteen sample of *A. Devestans* were collected from Tehsil of different distric of Punjab, Pakistan (as shown by map in Figure.1) from cotton fields by hand-held aspirator in 2012 and were preserved immediately in 95% ethanol to avoid the decomposition of soft body insect. The incidence of *Wolbachia* was determined in *A. devastans* population from different geographical location of Punjab Pakistan (Table 1). Total nucleic acids were extracted from single leafhoppers by using CTAB method of (Doyle *et al*., 1990) as adapted by (Marzachi *et al*., 1998). DNA extracted from *A. devestans* was tested by PCR for the presence of *Wolbachia*, using a pair of primers wol16sF (5'-GCATGAGTGAAGAAGGCC-3') and wol16s R (5'-AGATAGACGCCTTCGCCA-3' (Li *et al*., 2007) that can amplify partial 16S rDNA PCR product was cloned pTZR/T vector (Fermentas Canada) and sequenced (Macrogen Korea). Sequences were assembled and analyzed with the aid of Laser Gene Software (DNAStar Inc., Madison, WI, USA). Multiple sequence alignment was carried out using MUS-CLE software implemented in CLC DNA Workbench 6.9.1 (CLC - Sequence viewer). Neighbour joining method was used to infer phylogenetic relationships as shown in figure 2

RESULTS AND DISCUSSIONS

Distribution of infection

We assayed for the presence of wolbachia endosymbiont in 16 individual *A. devastance* (Table1, Figure 2). For *Wolbachia*, 4(25%) single infections were observed (as ascertained through direct sequences of PCR products that were easily readable without messy peaks). The sequenced clones from present study are named as SA7 (HG315627), SW2 (LN626608) and SW3 (Ln717257).

Phylogenetic analysis of *Wolbachia*

Blast analyses for 16S rRNA SA7, SW2 and SW3 clone sequence was 95-99% similar to other *Wolbachia* sequences submitted to NCBI database. Phylogenetic tree was constructed using 16s rDNA sequences of *Wolbachia* from other leafhoppers species, whitefly and other insects that are being reported around the world. *Wolbachia* is also reported from Pakistan from leafhopper but cannot be included in this study because of different primer used for its amplification. It is evident from maximum likelihood analysis that the clones of endosymbiont of leafhopper SA7, SW2 and SW3 clusters independently thus forming separate clade (Figure 2). The present study is the first evidence of *Wolbachia* incidence in cotton leafhopper (cicadellidae: *A. devastans*). The endosymbiont *Wolbachia* from present study was amplified using 16S rDNA, Previously *Wolbachia* has been reported from leafhoppers *Eupteryx decem- notata Rey* and *Eupteryx melissaein* Germany (Henke *et al*., 2013), mulberry leafhopper *Hishimonoides sellatiformis* and *Hishimonus sellatus* in Japan (Mitsuhashi *et al*., 2002), and European leafhopper *Zyginidia pullula*ns Italy (Machtelinckx *et al*., 2012; Machtelinckx *et al*., 2009). In blast analysis the clones, SA7, SW2 and SW3 shows homology about 95% to 99% with the *Wolbachia* strain of *B. tabaci*, which is being reported from India and China (Singh *et al*., 2012b). The clones from this study has no co-relation with any of these leafhoppers, instead these clones' clusters separately forming new clade. *Wolbachia* has also been reported from *B. tabaci* in Pakistan from Khenwal, Kabirwala and PirMuhal (Ahmed *et al*., 2013). The *Wolbachia* strain reported from Pakistan belong to mostly super group B (Ahmed *et al*., 2013). But the reported *Wolbachia* strain cannot be included in present study for phylogenetic analysis because the *Wolbachia* strains were amplified using wsp (*Wolbachia* surface protein) primers and present study used 16S rDNA gene primer. *Wolbachia* identified from *A. devastans*, is most similar to the previously identified strains of this *Wolbachia* related with *B. tabaci from* different regions although they don't fall in same clade. Study related to *B. tabaci* from India and its neighboring regions has revealed that *Wolbachia* in South Asia are different from the *Wolbachia* reported from *B. tabaci* from rest of the world and clusters separately but alignment results show little variance (Singh *et al*., 2012a). The present study suggest that *Wolbachia* distribution is quite complex and there is probability that *Wolbachia* strain reported in this study has ancestor different from those in other insects (Gueguen *et al*., 2012).

The first most probable reason for SA7, SA2, and SA3 in cotton leafhopper showing homology with strains of *B. tabaci* (reported from India and China), suggest that both organism may share common ancestor. Clones from both regions show homology about 99% indicating that ancestoral host was common and then Wolbachia diverge with little divergence. The other probable reason for presence of *Wolbachia* in cotton leafhopper similar that from *B. tabaci* can be the different ways of horizontal transfers since both cotton leafhopper and whitefly are present in cotton field. SA1 showed only 77% homology with other strains of *Wolbachia* leaving it to being under more research. Horizontal transmission is very important feature of *Wolbachia* that has earned it the diversity and abundance in insect world (Saha *et al*., 2012; Weisburg et al., 1989; Weisburg *et al*., 1991). There are studies that suggest more than one way of horizontal transmission of secondary endosymbiont *Wolbachia* (Singh *et al*., 2012b). In one such study a parasitoid was found to transmit *Rickettsia* to *B. tabaci* (Chiel *et al*., 2009). Up till now there is no record of horizontal transfer of *Wolbachia* from parasitoid to host insect, but there is probability that parasitoid can transfer *Wolbachia* to host.

The factor other supporting horizontal transfer can be multiple infection of *Wolbachia*, interactions between the host and plant, interactions with parasitoid and environmental factors ultimately making it very difficult to analyze the precise reason for the prevalence of *Wolbachia* in host (Hain *et al*., 1990). Some reports have suggested that host plant for whitefly act as source for transmitting *Wolbachia* to other insects present in the field (Sintupachee *et al*., 2006), since *B.*

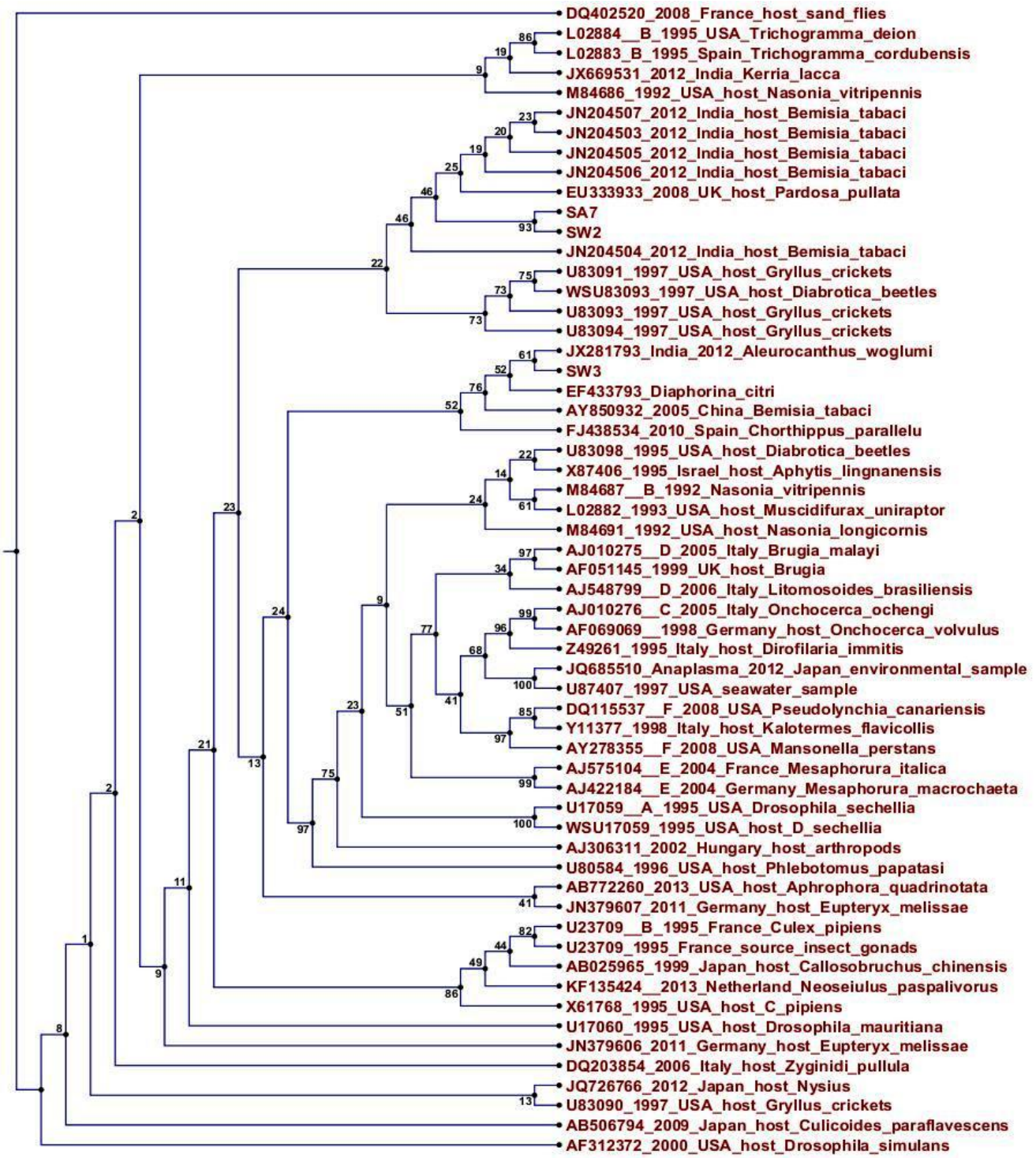

Fig. 1

Cluster analysis of *Wolbachia endosymbiont* based on 16S rDNA sequence. Neighbour joining method was used to infer phylogenetic relationships by using MUS-CLE software implemented in CLC DNA Workbench 6.9.1 (CLC Sequence viewer).

Table 1

A. devestans populations sampled for *Wolbachia.*

Location		Host plant	Host organism	Wolbachia infection
Distric	Tehsil			
Mianwali	Mianwali	Cotton	Cotton leafhopper	-
Bakhar	Kalorkot	Cotton	Cotton leafhopper	+
Bakhar	Mankera	Cotton	Cotton leafhopper	+
Bakhar	Daria khan	Cotton	Cotton leafhopper	-
Pakpattan	Pakpattan	Cotton	Cotton leafhopper	-
Pakpattan	Arifwala	Cotton	Cotton leafhopper	-
Vehari	Vehari	Cotton	Cotton leafhopper	-
Sahiwal	Sahiwal	Cotton	Cotton leafhopper	-
Sahiwal	Chichawatni	Cotton	Cotton leafhopper	-
Bahawalpur	Yazman	Cotton	Cotton leafhopper	+
Bahawalpur	Dera moza Izzat	Cotton	Cotton leafhopper	+
Bahawalpur	Ahmedpur	Cotton	Cotton leafhopper	-
Faisalabad	Tandlianwala	Cotton	Cotton leafhopper	-
Faisalabad	Sumandari	Cotton	Cotton leafhopper	-
Okara	Depalpur	Cotton	Cotton leafhopper	-
Miamwali	Piplian	Cotton	Cotton leafhopper	-
Jhang	Jhang	Cotton	Cotton leafhopper	-

+ Represents positive for Wolbachia infection in given sample

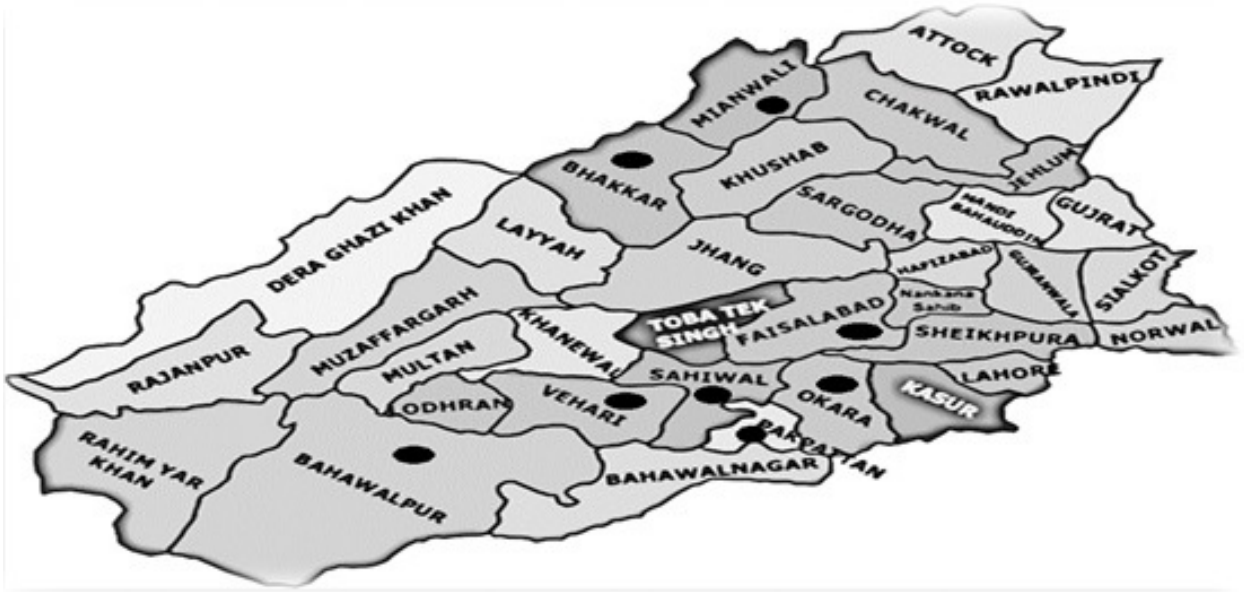

Fig. 2

Map of Punjab, dotted areas representing different locations of leafhopper sampling

tabaci and *A. devastans* feed on same cotton plant leaf, there is probability that *Wolbachia* was transmitted through the plant to leafhopper. But our studies has revealed that the *Wolbachia* needs to be small enough to pass through insect's salivary duct, but the endosymbiont *Wolbachia* is way too large to pass through the salivary duct (Moreira *et al.*, 2009). Hence making the host plant to be considered as one of the negative factor to transmit the *Wolbachia*to other insects. Further studies are required to detect in what type of phenotypic manipulation of *Wolbachia* is involved in leafhopper. It is very important to know the type of *Wolbachia* induced phenotypic alteration because it can serve as biological control of *Wolbachia's* population in field.

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