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POPULATION DYNAMICS, GENETIC DIVERSITY AND OCCURRENCE OF *WOLBACHIA* IN *AMRASCA DEVASTANS* POPULATION FROM DIFFERENT DISTRICTS OF PAKISTAN

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ABSTRACT

Among all species of jassids, cotton jassid (*Amrasca devastans*) is a widely-distributed and key pest with wide host range including many economically important cash crops. *WoIbachia* is a typical, prevalent and maternally transmitted group of endosymbiotic bacteria that have been found in 76% insect species. In the present study, cotton jassids were collected from three provinces, Punjab (Faisalabad, Lahore and Multan), Khyber Pakhtoonkhwa (Peshawar), Sindh (Hyderabad), reared on natural as well as artificial diets in the laboratory and semi-field conditions. Few jassid specimens from each collection site were preserved in 90% ethanol for molecular studies. Statistical analysis showed that the maximum population of jassids transpired in July and August during the years (2015-2017) and more population of *A. devastans* on a natural diet as compared to an artificial diet. Among the natural host plants, *A. devastans* were more abundant in cotton, primary host for this pest in Pakistan. *Wolbachia pipientis* was detected in *A. devastans* populations through PCR by using *wsp* general primers (*w*sp 81F and 691R) and genetic diversity through COI and S12 primer sets. However, Faisalabad, Hyderabad and Multan populations showed a high density of *Wolbachia* as compared to the jassid populations in Peshawar. Gene Runner, Clustal X and Mega 5 programs were used for phylogenetic analysis. The COI based phylogenetic tree showed that the *A. devastans* genotypes from Pakistan are diverse from the genotypes reported from other countries

Keywords: : *Wolbachia*, *Amrasca devastans*, population dynamics, genetic diversity, phylogeny

INTRODUCTION

Jassids (Homoptera; Ciccadelidae) are an extremely wide-ranging group of destructive agricultural pests and distributed throughout the world. Among other insect pests of vegetables, fruits and crops, jassids are extremely polyphagous (Babar *et al*., 2013). These are not only able to feed and breed on the cotton crop; it can also damage other vegetables as well as remain active around all the year (Akbar *et al*., 2012; Khan and Khaliq, 2004; Akram *et al*., 2011). According to an estimate, sixty-five percent cotton crop is blemished due to the attack of this pest every year (Razaq *et al.*, 2014). *Wolbachia* is a complex group of intracellular, gram-negative bacteria that can transmit maternally. Most commonly founded this alpha-proteobacteria observed in numerous species of arthropods (Inaki *et al*., 2011; Ravi kumar *et al*., 2011; Dobson *et al*., 2002). Generally, *Wolbachia* is occurred in the reproductive tissues of the host induce a range of reproductive abnormalities such as male-killing, parthenogenesis, feminization and most commonly cytoplasmic incompatibility (Werren *et al*., 2008; Zeh *et al*., 2005; Jiggins *et al*., 2001).

The current research work was conducted during the years (2015-2017) in lab and semi-field conditions to investigate and achieve the following aims: 1) Studies on the population dynamics of *A. devastans* in different districts of Pakistan along with hosts and diet preference. 2) Molecular characterization of *Wolbachia* in *A. devastans* populations of Pakistan 3) COI based genetic diversity and phylogenetic characterization among the populations of *A. devastans* 4) Encourage environment-friendly techniques to control insect pest populations.

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MATERIALS AND METHODS

This study was carried out to detect common endosymbiont bacteria *Wolbachia* in *A. devastans* populations in Pakistan. The experimental work was conducted in Molecular/ Entomology Laboratory, Department of Zoology, Government College University, Faisalabad during the years 2015-17. Five districts (Faisalabad, Lahore, Multan, Peshawar and Hyderabad) of Pakistan were selected for *A. devastans* collection (Fig.1 and 2).

Jassids were collected by installing traps in the field. The most commonly used trap for jassid collection is sticky yellow bands for adult collection. After twenty-four hours, each trap was checked regularly. The other methods used for jassids collection were handpicking and by using hand nets. The specimens were preserved in 90% ethanol and were kept at -20°C. The collected populations of jassid were reared in semi-field conditions under standardized conditions (for field 35 - 40 ° C and 65-75 % R.H and in the laboratory 25°C and 65 % R.H) on natural hosts (cotton, okra, potato and brinjal) and artificial diets (formulation in supplementary data). Different quantities of various nutrients, amino acids and vitamins were tested and the most suitable diet was used to rear *A. devastans*. The populations of *A. devastans* were reared in the green house of GCUF. In the lab, jassids were reared in insect rearing cages on an artificial diet. Data were analyzed statistically using SASS software (Steel and Torrie, *et al*., 1960).

DNA extractions from the collected samples were performed through DNA extraction kits (Qiagen). Quantification of DNA was performed by nanodrop UV Spectrophotometer. The *wsp* primers 81F (5'tggtccaataagtgatgaagaaac-3') and 691R (5'-aaaaattaaacgctactcca-3') were used to investigate the samples for *Wolbachia* infection. The reactions were set up with 25 µl volumes containing, 1x NH_4 buffer (Fermentos), 2mM $MgCl_2$, 100 µM dNTPs, 0.2 µM of each primer, 0.5 U Taq polymerase (Fermentos) and 2 µl of the template DNA. PCR was started for 2 minutes at 95°C and followed by 32 cycles at 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 1 minute and a final extension at 68°C for 15 minutes. PCRs with mitochondrial primers were steered to explore the COI and S12 genes. The products of the PCR with mitochondrial primers were purified following a slightly adapted Fermentos DNA extraction-purification kit. The purified DNA samples were sent or sequencing. Sequences were aligned using Clustal X (Thompson *et al*., 1994). Strain-specific restriction sites were searched using Gene Runner version 3.0. The sequence data were compared with published mitochondrial sequences by running a BLAST search (Altschul *et al*., 1997). The sequences from other *A. devastans* of other countries from gene bank were aligned to construct the phylogenetic tree in the EMBL Nucleotide Sequence database and phylogenetic tree was based on COI gene using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (Tamura *et al*, 2007) used to infer the phylogenetic tree..

RESULTS AND DISCUSSION

The overall mean results depicts that *A. devastans* population were observed increasing trends and reaching maximum from mid-weeks of June to August up to mid weeks of September and later on due to unfavorable conditions like scarcity of food and temperature fluctuation, populations started to decline in 3rd week of September (2015-2017; Fig. 3 & Table 1).

Amrasca devastans populations were reared on their different hosts e.g. cotton, brinjal, potato and okra. Cotton is the primary host for this pest in Pakistan, while others are considered as secondary hosts and among these hosts more abundant on the cotton crop than okra, brinjal and potato. The low population of *A. devastans* was recorded on okra because of its thick hair on leaves, stem and fruit. Moreover, brinjal and potato received the low paroxysm of this pest than cotton but higher as compared to okra (Fig. 4). The maximum population was observed on natural hosts as compared to the artificial diet. But in the mid of June and the first three weeks of July, *A. devastans* population was relatively high on artificial diet because of high temperature in semi-field conditions which decreased the population in the field (Fig. 5 and Supplementary data).

During this study samples were collected through most commonly used attractants for different species of jassids, yellow sticky band and by hand nets from various locations similar to the method adopted by Sun *et al*., 2007; Klein *et al.,* 2001. Jassid density was varied with different locations and time which might be due to environmental factors or age of the host plants. Jassid (*A. devastans*) population was found on different host plants e.g. cotton, brinjal, potato and okra. Among all host plants, cotton acts as the primary host for this pest, okra, brinjal and potato serve as secondary host for the same pest. Therefore, the population of *A. devastans* was higher on cotton crop than okra, brinjal and potato. The low population of *A. devastans* was recorded on okra because of its thick hair on leaves, stem and fruit (Akbar *et al*., 2012; Akram *et al*., 2011). In this research work, a chemically modified artificial diet by adjusting the balance of required nutrients was developed for the growth of *A. devastans*. Then the most standardized diet for the growth of *A. devastans* was chosen and collected samples were reared on this defined artificial diet (Fu *et al*., 2001).

In the current study, the results of population dynamics showed that the population of *A. devastans* was highest in July and August because of suitable temperature, high humidity and availability of host plants. Among five districts of Pakistan population of *A. devastans* was maximum in Faisalabad, Multan, Hyderabad and Lahore respectively. In district Peshawar, population of *A. devastans* was quite low due to low temperature and scarcity of food.

The molecular study was conducted to explore genetic diversity of naturally occurring endosymbiont *Wolbachia* in *A. devastans* populations of Pakistan through mitochondrial CO I gene. The possible occurrence of *W. pipientis* was observed on the mitochondrial genome of *A. devastans* and PCRs were steered to explore the CO I and S12 genes amplification. Optimization of PCR condition and *Taq* concentration showed 80-90% amplification of *A. devastans.* PCR with COI mitochondrial primer illustrated 90%

amplification of *A. devastans* population from Faisalabad, Multan, Lahore and Hyderabad districts. While Peshawar districts demonstrated 80% amplification with the mitochondrial COI primers (Table 3). Similarly, S12 mitochondrial primer showed 90% amplification of *A. devastans* in all districts (Table 3). After PCR with mitochondrial primers and purification of the samples sequences were generated. *Wolbachia* was detected from *A. devastans* populations in different districts of Pakistan. The populations of Faisalabad and Multan demonstrated a high degree of infection than population of Lahore (25%, 25% and 15%). The jassid population from Peshawar and Hyderabad were found *Wolbachia* infected with low-density and lower infection rate of 10% and 15% (Fig. 9: Table 4). Nine samples of Jassids population from Peshawar and twelve from Hyderabad exhibited no sign of *Wolbachia* infection (Fig. 8). PCRs with five housekeeping genes (*Wolbachia* preserved genes) i.e. *gatB, coxA, hcpA, ftsZ* and *fbpA* was carried out for confirmation of the infection status and found overall low density in *A. devastans* population of Pakistan.

CO I based sequence analysis showed that the Pakistani populations of *A. devastans* genotypes were diversely reported from other countries. The Pakistani genotypes showed more relatedness with Chinese genotypes if *A. devastans* as compared to other reported from Australia and Europe. The neighbor-joining tree and bootstrap support are clustered with *A. devastans* from China, France, Australia and Russia. Bootstrap showed that the *A. devastans* from China is 75-95% resembled with Pakistani *A. devastans*. Bootstrap support beared a resemblance upto 65% with France, 70% with Australia and 80% with that of Russian populations. The phylogenetic tree showed that these were closely associated with each other (bootstrap support >75%; Fig. 8). Total nine sequences from other *A. devastans* populations compared for comparison of the targeted population (Supplementary data)

Molecular identification of *Wolbachia* provides a promising and valid tool for the identification of *Wolbachia* species and development of phylogenetic relationships among species, further finding of new *Wolbachia* strains may open a new channel for the exploration of new and environment-friendly tools of *Amrasca devastans* pest management and control. *Wolbachia* can alter the reproductive capacities of its host and due to this ability in most of the countries huge efforts have been efficacious on the role of *Wolbachia* as a biological control agent in many arthropod species. We surveyed and studied for the first-time *A. devastans* population for the mitochondrial diversification which may help for a detailed study of possible infection of the endosymbiont *Wolbachia* through the CO I mitochondrial gene in the selected populations of *A. devastans. Wolbachia* and mitochondrial DNA have the same passage of transmission i.e. cytoplasmic pathway. *Wolbachia* influences the host mitochondrial DNA evolution and results in its diversification. Previously (Singh *et al*., 2012) and present work is promising on the determination of phylogenetic analysis of jassids carrying *Wolbachia* infection for incorporation in future pest management strategies.

In this study, *Wolbachia* general primers (*w*sp primers) were used for detection of *Wolbachia.* Potential of selected *wsp* primers for strain characterization was found limited due to frequent recombinations (Malloch and Fenton, 2005; Werren and Bartos, 2001). The *Wolbachia* infection rate was quite low may be due to high density of its associated bacteriophages. (Rasool, 2011; Arthofer *et al*., 2009) reported the high *Wolbachia* diversity in *Rhagoletis cerasi* from eight European countries by the amplification of the host`s COI mitochondrial gene and similarly in stored grain *T. castaneum* population of Pakistan (Rasool, 2019). Recently, a MLST system based on five housekeeping genes was introduced to overcome the limitation of *w*sp primers. Through MLST loci quantification the integrity of *Wolbachia* was confirmed in *A. devastans* populations (Baldo *et al*., 2006). *Wolbachia* has ability to lessen the suitability of their host and change the reproductive strategies of its hosts and this phenomenon could be used as another method to control the agricultural insect pests (McMeniman *et al*., 2009; Moreira *et al*., 2009; Zabalou *et al*., 2009). Researchers have focused on the possible application of *Wolbachia* through biological control programmes (Zabalou *et al*., 2009; Rasool *et al*., 2017) and incorporation in integrated pest management of insect pest including agricultural pests and vectors of human diseases. Different strategies have been projected including discharge of properly raised transinfected males directly in the field to reduce the natural population of insect pest or using these symbionts to transfer the genes of desire all over the pest population (Saridaki and Bourtzis, 2010)

Fig. 1
Sampling site of districts in Pakistan.

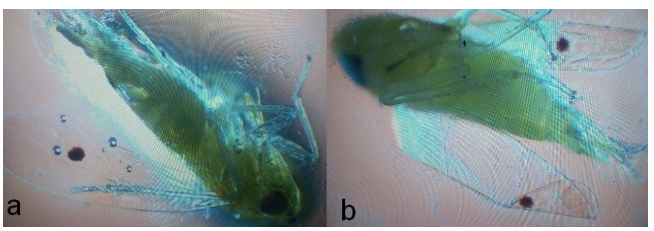

Fig. 2
Adults of Cotton Jassid (*Amrascadevastans*) Photo @Zoology, GCUF.

Table 1
Month x District x Week interaction means.

District	Week	Months				Mean
		June	July	August	September	
Faisalabad	W1	50.00±7.77n-u	270.67±7.84d-g	331.33±10.84a-e	133.00±5.51j-p	196.25±33.61EF
	W2	72.00±8.14l-u	305.67±19.38a-f	351.00±8.74a-e	105.00±5.13j-t	208.42±36.98C-F
	W3	178.00±14.64g-j	353.00±12.49a-e	382.67±19.20ab	70.00±4.04l-u	245.92±39.09ABC
	W4	276.00±7.81def	344.67±16.80a-e	372.00±10.79abc	42.00±6.08p-u	258.67±39.45A
Lahore	W1	16.33±4.33stu	314.00±17.35a-f	386.67±16.25a	113.00±6.08j-r	207.50±45.27C-F
	W2	44.33±6.01o-u	285.00±23.07c-f	352.00±19.66a-e	119.33±13.04j-q	200.17±37.93DEF
	W3	175.67±16.95h-k	353.33±14.99a-e	342.00±11.36a-e	63.00±6.43m-u	233.50±36.90A-E
	W4	236.00±19.86f-i	296.67±31.95a-f	350.00±20.98a-e	34.00±7.51q-u	229.17±37.27A-E
Multan	W1	22.00±5.86r-u	316.00±21.28a-f	287.00±13.28c-f	117.67±10.17j-q	185.67±36.99F
	W2	47.00±7.23o-u	274.33±13.69def	304.33±58.97a-f	87.00±2.31j-u	178.17±36.35F
	W3	150.00±11.68i-m	291.00±29.46b-f	346.00±46.52a-e	51.00±5.86n-u	209.50±37.03B-F
	W4	264.00±19.86e-h	297.00±37.40a-f	345.00±11.85a-e	16.00±2.65stu	230.50±39.47A-E
Peshawar	W1	23.00±3.06r-u	136.33±9.49j-o	143.00±6.08i-n	23.00±2.31r-u	81.33±17.78G
	W2	22.67±4.26r-u	159.33±12.24i-l	171.00±16.77h-k	15.00±3.06stu	92.00±22.58G
	W3	63.00±3.46m-u	159.00±7.94i-l	114.00±16.52j-r	11.67±0.88u	86.92±17.10G
	W4	83.33±3.76k-u	106.00±5.57j-s	92.00±7.64j-u	9.00±1.15u	72.58±11.54G
Hyderabad	W1	12.00±2.89tu	362.00±12.29a-d	352.00±14.57a-e	137.00±9.85j-o	215.75±44.88B-F
	W2	33.00±5.20q-u	348.00±12.17a-e	324.00±16.74a-f	97.00±7.00j-u	200.50±41.77DEF
	W3	175.00±15.82h-k	370.00±23.90abc	385.00±8.89a	63.00±6.11m-u	248.25±41.31AB
	W4	262.67±20.30e-h	341.00±9.29a-e	317.67±16.83a-f	33.00±2.65q-u	238.58±37.28A-D

Table 2
Analysis of variance table for the population of *Amrasca devastan* in five sampled districts.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
District (D)	4	717719	179430	241.75**
Month (M)	3	2579216	859739	1158.34**
Week (W)	3	49807	16602	22.37**
D x M	12	181314	15109	20.36**
D x W	12	31296	2608	3.51**
M x W	9	409297	45477	61.27**
D x M x W	36	83544	2321	3.13**
Error	160	118755	742	
Total	239	4170948		

** = Highly significant (P<0.01)

Table 3
Detection of infection of *Wolbachia* in *Amrasca devastans* samples through mitochondrial (COI and S12)

Sr. No.	Location	Number of samples	For CO1 primer		For S12 primer	
1	Faisalabad	10	+++++++++	-	+++++++++	-
2	Multan	10	+++++++++	-	+++++++++	-
3	Lahore	10	+++++++++	-	+++++++++	-
4	Peshawar	10	++++++++	--	+++++++++	-
5	Hyderabad	10	+++++++++	-	+++++++++	-

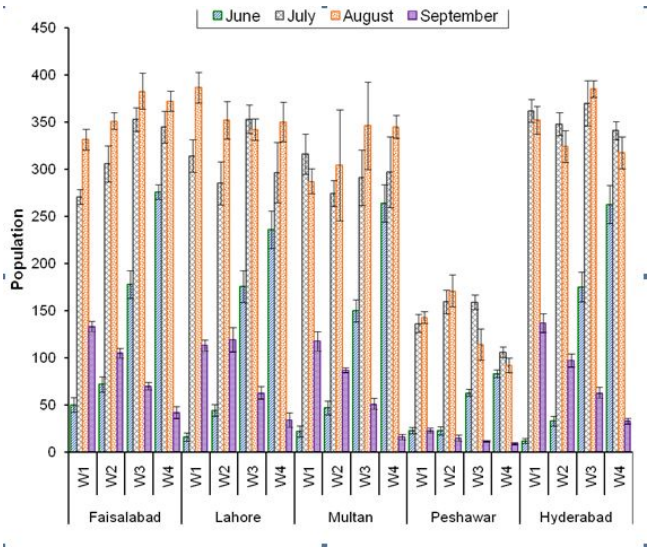

Fig. 3
Overall mean population of jassids in five districts during the months (2015-2017).

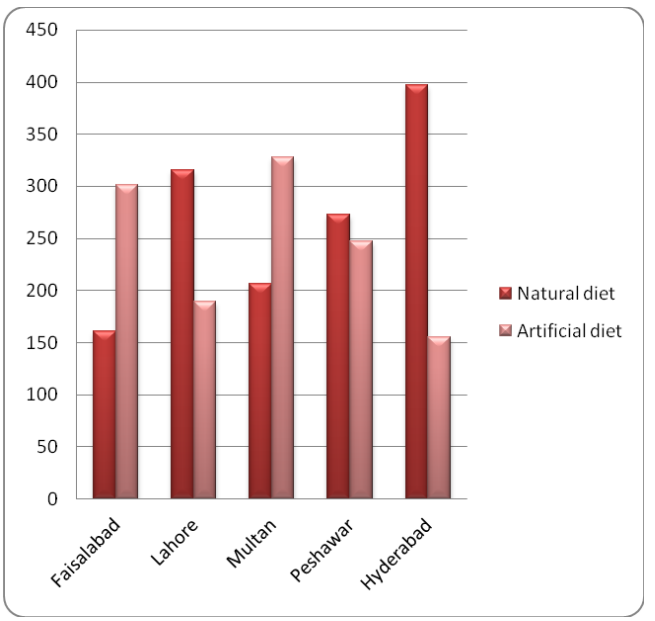

Fig. 4
The overall mean population of *Amrasca devastans* on natural and artificial diets.

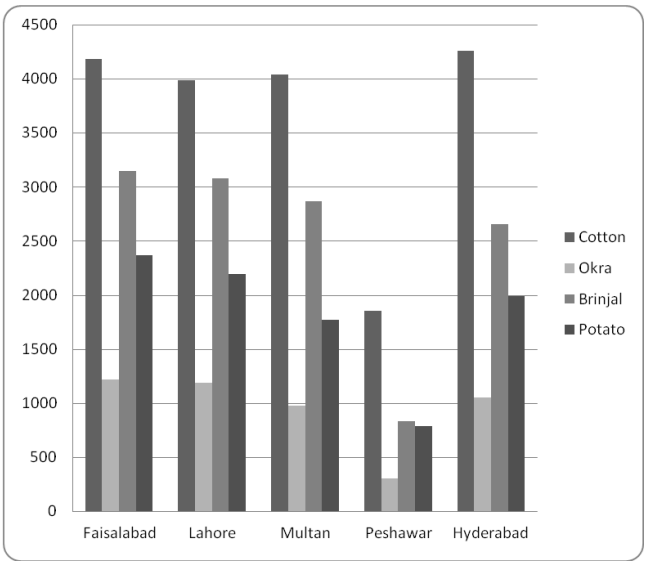

Fig. 5
The overall mean population of jassids on different host plants in five districts.

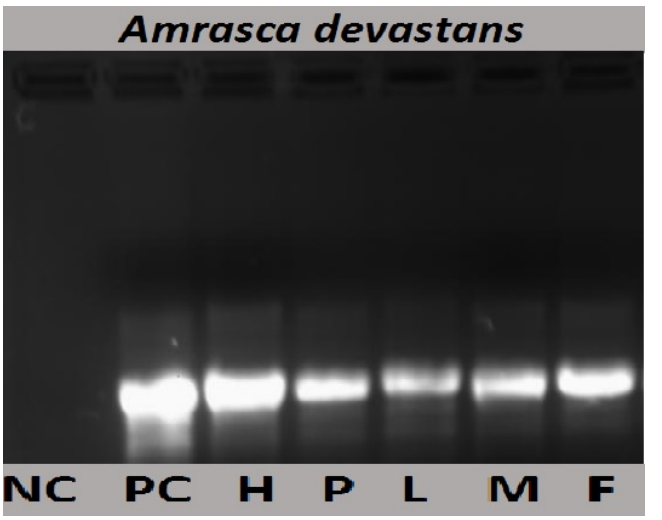

Fig. 6
PCR amplicons of individuals of *Amrascadevastans*from Faisalabad [F], Lahore [L], Multan [M], Peshawar [P] and Hyderabad [H] using Co1 mitochondrial Primer. Positive control [PC], Negative control [NC].

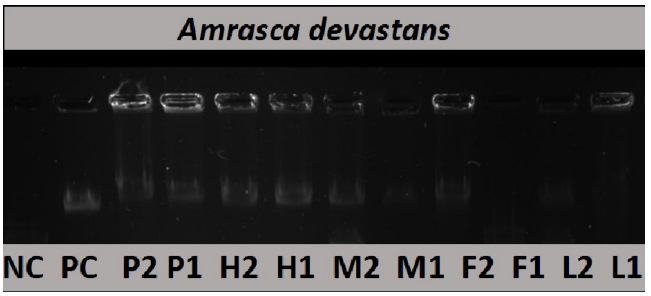

Fig. 7
PCR amplicons of individuals of *Amrasca devastans* from Faisalabad [F1,F2], Lahore [L1,L2], Multan [M1,M2], Peshawar [P1,P2] and Hyderabad [H1,H2] using Co1 mitochondrial Primer. Positive control [PC], Negative control [NC].

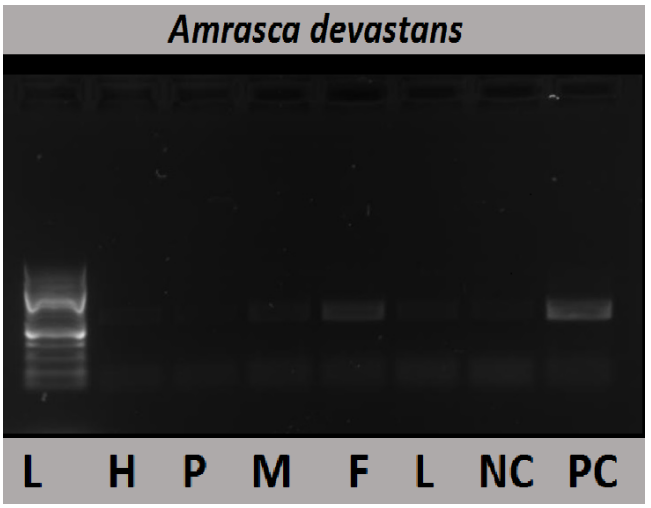

Fig. 8
PCR amplicons of individuals of *Amrasca devastans* from Faisalabad [F], Lahore [L], Multan [M], Peshawar [P] and Hyderabad [H] using *wsp* 81F and 691 R primers. Ladder [L] Positive control [PC] and Negative control [NC].

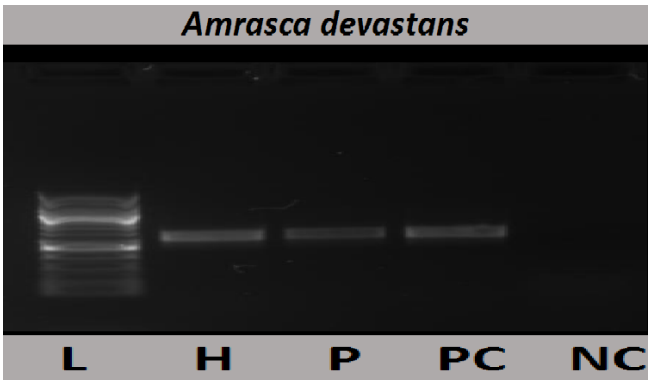

Fig. 9
PCR amplicons of individuals of *Amrasca devastans* from Peshawar [P] and Hyderabad [H] using *wsp* 81F and 691 R primers. Ladder[L], Positive control [PC] and Negative control [NC].

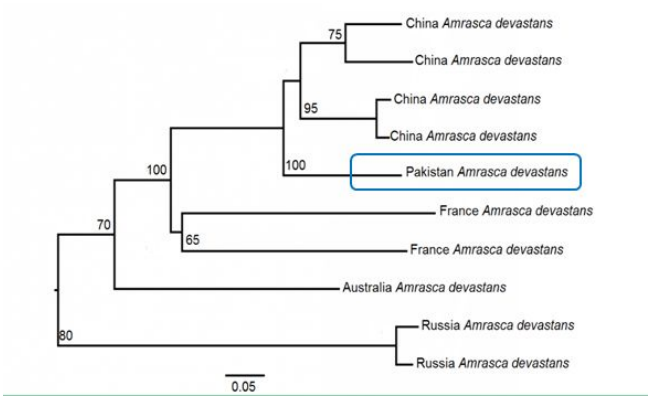

Fig. 10
Phylogenetic tree based on COI gene using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (Tamura *et al*, 2007) used to infer the phylogenetic tree.

Table 4
Amrasca devastans samples screened for *Wolbachia* infection.

Sr. No	Location	Selected Sites	No. of sampled individuals	Primer Used	Positive	Negative	% infection of *wsp* in the experimented samples
1	Faisalabad	Ayub research center	10	*wsp* 81F&*wsp* 691R	+++++	----------	25
		Chak no 38 JB	10	*wsp* 81F&*wsp* 691R			
2	Lahore	Mari pur	10	*wsp* 81F&*wsp* 691R	+++	----------	15
		Rot ghar	10	*wsp* 81F&*wsp* 691R			
3	Multan	Hassuwala	10	*wsp* 81F&*wsp* 691R	+++++	----------	25
		Chak no. 2	10	*wsp* 81F&*wsp* 691R			
4	Peshawar	Rahatabad	10	*wsp* 81F&*wsp* 691R	+	---------	10
5	Hyderabad	Hussainabad	10	*wsp* 81F&*wsp* 691R	++	--------	15
		Hussainabad	10	*wsp* 81F&*wsp* 691R	+	---------	

CONCLUSIONS

Jassids are well-known for their considerable economic loss and various molecular studies have described different microbes found in association. Among these microbes, *Wolbachia* causes a variety of reproductive abnormalities including feminization, male-killing, parthenogenesis and most commonly cytoplasmic incompatibility (CI).The main objectives of this study were, to investigate population dynamics, the exploration of *Wolbachia* and its role in host diversity and phylogenetic analysis of *A. devastans* population of Pakistan. This will further lead towards an environmentally well-coming tool to control jassid populations which may be integrated in future pest management strategies and open new horizons.The results of PCR with MLST showed that the density of *Wolbachia* infection in *A. devastans* population was relatively low which will be further explored in future experiments.

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