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IDENTIFICATION OF NEW GENETIC VARIANT OF *BEMISIA TABACI* FROM PAKISTAN

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A B S T R A C T

New genetic variant of *Bemisia tabaci* {Gennadius, (Hemiptera: Aleyrodidae)} Asia-II-7 (Cv biotype) was found and is reported for the first time in Pakistan. Samples were collected randomly from vegetable cultivated fields in Rawalpandi and Islamabad regions. Previously all the reported sequences are from the southern and central Punjab and samples were taken from cotton and other vegetables hosts. A phylogenetic tree was constructed showing all the sequences generated by this study were grouped into two genetic clades Asia-II-7 and Asia-II-1 (K, P, PCG-1, PK1, SY and ZHJ2 biotype). A report suggests that Asia-II-7 genetic variant adapts readily to ornamental plants as compared to vegetables. The sampling area (Rawalpindi) from where this genetic variant was found was a large area harboring both vegetables and ornamental plants. One reason for this genetic variant not being previously reported from Pakistan may be that the previous reports concentrated on fiber and food crops not on the ornamentals. A more detailed study involving extensive work, including large sample areas and from multiple hosts and locations is needed to fully elucidate the genetic variation of *B. tabaci* in Pakistan.

Keywords: *B. tabaci*, phylogenetic analysis, new genetic variant, Asia-II-7.

INTRODUCTION

Bemisia tabaci {Gennadius, (Hemiptera: Aleyrodidae)} is a cosmopolitan pest that is found throughout the world in all tropical subtropical and moderate agricultural areas (Mound & Halsey, 1978, Ahmed *et al*., 2010). It is haplo-diploid insects that feeds upon plant sap and belongs to the group of insects generally known as whiteflies (De Barro, 1995, De Barro, 2005). It is reported that due to this insect 20 to 100 % losses happen in crops yield every year (Brown & Bird, 1992, Pan *et al*., 2012). Not all the biotypes/genetic variants of *B. tabaci* are equally efficient in the transmission of viruses (Shoorcheh *et al*., 2008). The classification of whitefly is very problematic due to its close similarity in morphological traits, and difficult to identify in genera and species (Gill, 1992, Lopez-Avila, 1986, Mound & Halsey, 1978). During the last decade a number biochemical, molecular and DNA finger printing techniques have become available for identification of the different genetic groups of *B. tabaci* that are otherwise indistinguishable morphologically (Boykin *et al*., 2007, Brown, 2000, Cervera *et al*., 2000, Rosell *et al*., 1997, Shoorcheh *et al*., 2008). All the methods used for the classification of *B. tabaci* give different results and therefore different names were assigned to the same entity (Ahmed *et al*., 2011, Costa *et al*., 1991). Now a days *mt*CO1 marker is greatly used for the identification of the genetic variability of *B. tabaci* and by the advancement of *mt*CO1 marker, *B. tabaci* population can be differentiated in a better way and classification among the biotypes and dissimilar genotype groups has become easier (Berry *et al*., 2004, Legg *et al*., 2002, Maruthi *et al*., 2004, Sseruwagi *et al*., 2005). To date 41 populations of *B. tabaci* are reported, out of these 24 are labeled (named) and 17 of which are still unlabeled (Ahmed *et al*., 2011, Perring, 2001). Boykin *et al*., (2007) has classify these cryptic species in 12 genetic groups on the basis of *mt*CO1 gene using Bayesian analysis. Three years later Dinsdale *et al*.,

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(2010) further evaluate Boykin classification and classify it in 24 groups on >3.5% divergence.

As a pest *B. tabaci* was reported for the first time in north India in late 1920s which is now part of Pakistan (Hussain & Trehan, 1933, Misra & Lamba, 1929). About the genetic status of *B. tabaci* there are only two relatively recent reports are available, both by the same author (Ahmed *et al*., 2010, Ahmed *et al*., 2011). One of these reports is based on the analysis of ITS1 (Ahmed *et al*., 2010) and in other *mt*CO1 sequences were used for phylogenetic analysis (Ahmed *et al*., 2011). Results of the study based on ITS1 sequence alignment indicated that all the *B. tabaci* Pakistani populations were quite similar to each other and belong to unresolved biotype PCG1 of the Asia-II genetic group described by Boykin *et al*. (2007). The other study based on *mt*CO1 sequences that support the presence of previously reported three cryptic species, Asia-1, Asia-II-1 and MEAM-1 in the country based on the Dinsdale *et al*. (2010) classification. These three genetic groups (cryptic species) were found not to be uniformly distributed in the country. Asia-1 was the only genetic group found in both Sindh and Punjab province. Asia-II-1 was recorded from Punjab only, whereas MEAM-1 was found only in Sindh (Ahmed *et al*., 2011).

The previous study about the genetic status of *B. tabaci* is not clear due to insufficient data. The work was done in patches and the sample size and sampling area was very small. The samples in previous studies were only collected from cotton plants while vegetables and ornamental crops are also the host of *B. tabaci*. So to know the exact genetic status of *B. tabaci* in the country need a large sampling size and area and also include vegetables and ornamental plants.

MATERIALS AND METHODS

Samples collection: Samples of *B. tabaci* were randomly collected from vegetables cultivated fields in Chak Shazad (Islamabad; capital city of Pakistan) and Satellite town (Rawalpindi; metropolitan city adjacent to Islamabad). Both the regions are about 10 km apart from each other. From each region 40 samples were collected. Some flies were freshly used for DNA extraction and others were preserved in 85 % ethanol for later use.

DNA extraction: Protocol followed by Czosnek *et al*., 1988 was used for the DNA extraction with some modification. A single fresh/preserved fly was taken in 1.5 ml centrifuge tube and crushed with a micropipette-molted tip. After crushing 100 µl of (0.4% w/v) sodium dodecyl sulfate was added followed by the addition of 10 µl (1 %) Proteinase K (Fermentas). The reaction samples were incubated in thermo cell mixing block (MB-201, Bioer) at 55 ^{0}C for 1 hour. After an hour 100 µl of concentration (24: 1 v/v) isoamyl alcohol (A156 Roti®) was added and centrifuged (Eppendorf 5452 Mini Spin Centrifuge) for 10 minutes at 12,100 xg rcf. The supernatant was transferred to a new tube and the pellet was discarded. The DNA was precipitated by adding double amount of 95 % ethanol and 35 µl, (3M) sodium acetate. The samples were allowed to cool down by keeping at -20 ^{0}C for 30 minutes and then centrifuged for 5 minutes at 12,100 xg rcf. Ethanol was discarded and the pellet was washed with 70 % ethanol. Then the pellet was kept on room temperature for 30 minutes to dry. In the dried pellet 20 µl of ddH_2O was added and used directly for PCR reaction or stored at -20 ^{0}C for later use.

PCR amplification and sequencing: Thermocycler (Amplitronyx ATC 201, NyxTechnik, USA) was used for PCR amplification. PCR program was as, pre-denaturation for 5 min at 94 °C, followed by 35 cycles of denaturing for 30 sec at 94 °C, annealing for 30 sec at 50 °C, and extension time of 45 sec at 72 °C. Final extension for 10 min at 72 °C was set. *mt*CO1 gene sequence (879 bp) was amplified via PCR using forward primer {wfco1879F, (TTGATTTTTTGGTCATCCAGAAG)} in combination with reverse primer {wfco1879R, (TATAAATCTTAAATTTACTGCA)}. Each PCR reaction was performed in a volume of 25 µl containing 2.5 µl template DNA, 0.25 µl (5 U/µl) Taq polymerase (Fermentas, USA), 2.5 µl (10 X) Taq buffer (Fermentas, USA), 1.5 µl (25 mM) $MgCl_2$ (Fermentas, USA), 2.5 µl (2 mM) dNTPs (Fermentas, USA), 0.5 µl (10 pMol) forward and reverse primer and 14.75 µl ddH_2O. Amplified products were resolved electrophoretically in 1 % agarose gel and ethidium bromide stained were recorded by using Gel-doc. The amplified products were then sequence commercially (Macrogen, Soul, Korea) with forward primer.

Sequence retrieval: For this study all the available sequences of *B. tabaci* from GenBank (NCBI) were retrieved in FASTA format. It was found that a total 83 *mt*CO1 sequences of *B. tabaci* were present. Out of these 83 sequences 18 sequences were unreliable and removed from further analysis. The rest 65 mtCO1 sequences were used for the phylogenetic tree construction.

Sequence analysis: All the sequences were named as

Accession number-province name-district name (GU585369-Punjab-Faisalabad)
http://www.ncbi.nlm.nih.gov/nuccore/GU585369.
Then all the sequences along with the consensus sequences generated by Dinsdale *et al*., (2010) kindly provided by Paul J. De Barro were aligned by using Clustal W (Thompson *et al*., 1994). The aligned sequences were then trimmed from both ends to a specific length (657 bp) that was used by standard international publications. MEGA 5 was used for phylogenetic tree construction. Evolutionary analysis was conducted in MEGA 5 based on Neighbor Joining method. Applying 1000 bootstrap and positions containing gaps and missing data were eliminated. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed (Tamura *et al*., 2011). *Bemisia afer* (GQ139515) sequence was used as an out-group. A map was constructed that shows the distribution of different genetic variants in the country. For map construction, CorelDRAW X4 was used.

RESULTS

There are four provinces in Pakistan other than federally administered areas. The deposited sequences were reported only from two provinces Punjab and Sindh. In Punjab the reported sequences were from 12 districts. All the sequences were reported from central and eastern Punjab where cotton is cultivated on a large scale. In Sindh from 10 districts, the sequences were reported mostly from cotton growing districts. The detail of sequences distribution in the country is enlisted in Table 1.

Table 1.Status of the previously reported sequences from Pakistan.

Locality	No of sequences	Genetic variants			Host		
		Asia-1	Asia-II-1	MEAM-1	Cotton	vegetables	Unknown
Punjab	37	6	31	0	13	4	20
Sindh	23	5	0	18	3	7	13
Unknown	5	4	1	0	1	1	3

The table shows the locality, genetic variants and host from where the samples were collected. A total 65 sequences were present in Gene Bank till February 2012.

As a result of this analysis we have found that in our country Asia-II-1 clade is restricted to the province of Punjab only and is found commonly in the districts of this province. MEAM-1 clade in Pakistan is limited to only Sindh province. The third genetic variant Asia-1 is the only clade that is reported from both provinces. This clade is reported from a total of 7 districts of the country (4 districts of Punjab and 3 districts of Sindh). The distribution of genetic variants in the country is presented in Fig 1.

Out of 19 analyzed sequences, generated by this study 11 sequences were from the specimens that collected from Rawalpindi and 8 sequences were from that specimens sampled from Islamabad. Sequences of specimens that were collected from Rawalpindi, 10 sequences out of 11 were grouped into Asia-II-7 clade. This Asia-II-7 was not reported from the country and is reported for the first time in this study. Only 1 sequence from Rawalpindi clustered in Asia-II-1clade (Ahmed *et al*., 2011). The genetic variants of this clade was already reported from Pakistan and found commonly in the districts of Punjab province. The remaining 8 sequences that were sampled from Islamabad found to belonged Asia-II-1 clade that present very commonly in Punjab province. The phylogenetic tree is shown in Fig 2.

The sequences of this project were also analyzed and compared with the sequences that were reported earlier. The sequences that were collected from Islamabad, grouped into Asia-II-1 clade that was already reported from the country. A total of 8 sequences from Islamabad put in analyses that are highlighted with green circles in front of sequences. The sequences of *B. tabaci* sampled from Rawalpindi grouped into two clades. One sequence fell into Asia-II-1 clade and the remaining 10 sequences grouped into a new clade Asia-II-7. The sequences of specimens that were sampled from Rawalpindi are marked by red circle in front of the sequence. Newly reported, Asia-II-7 clade is highlighted in the phylogenetic tree. A combine phylogenetic tree was constructed shown in Fig. 3.

As a result of our analysis it was suggested that a total of 3 genetic clades (Asia-II-1, Asia-1 and Asia-II-7) have so far been found in the Punjab province. The Asia-II-1 genetic variant was reported very frequently.

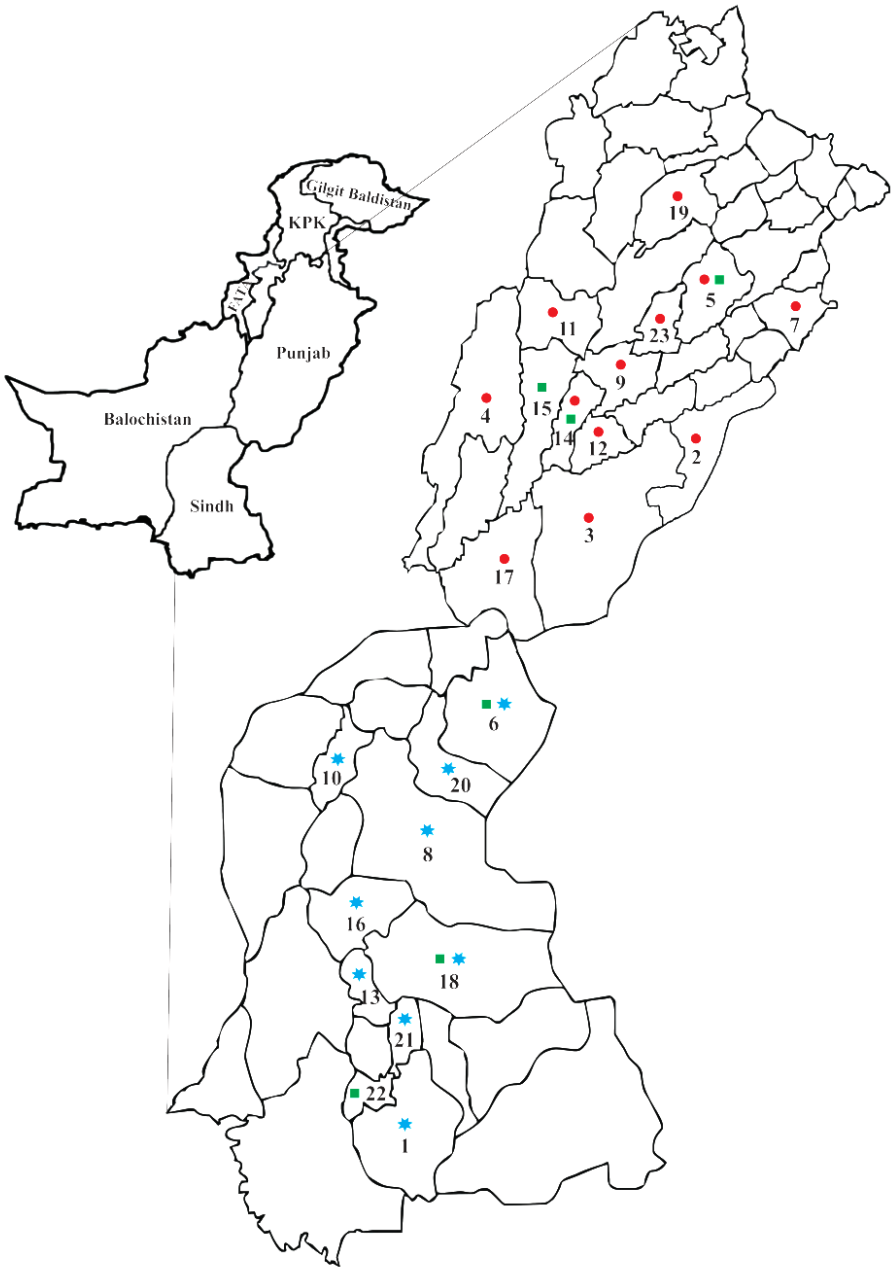

Figure1. Distribution of B. tabaci in different districts of Punjab and Sindh.

Different colored shapes shows the distribution of genetic variants in the districts of Punjab and Sindh.

- Asia-II-1
- Asia-I
- MEAM-I

All the districts are numbered alphabetically in the map and are as following,

1) Badin 2) Bahawalnaghar 3) Bahawalpur 4) Dera ghazi khan 5) Faisalabad 6) Ghotki 7) Kasur 8) Khair pur 9) Khanewal 10) Larkana 11) Layyaha 12) Lodhran 13) Metiari 14) Multan 15) Muzaferghar 16) Nawab shah 17) Rahim yar khan 18) Sanghar 19) Sargodha 20) Sukkar 21) Tando allahyar 22) Tando Muhammad khan 23)Toba tek singh.

This genetic group was present in 11 districts out of 13 reported. The second genetic variant that was reported from Punjab was Asia-1. It was found only in 4 district of the province. The third genetic clade Asia-II-7 is a new variant reported as a result of this study for the first time is reported from Rawalpindi district. The new situation of the genetic variation of *B. tabaci* including this new variant, in Punjab province shown in Fig 4.

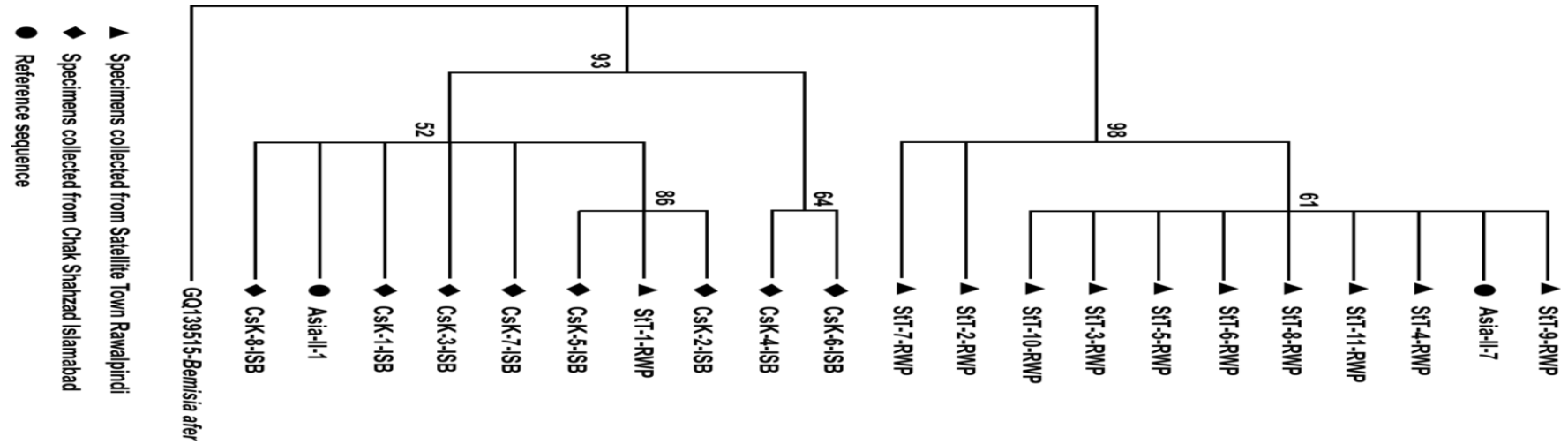

Figure 2. Phylogenetic tree of B. tabaci based on mtCO1gene.

Tree was reconstructed by maximum likelihood method in MEGA 5 (Bootstrap=1000) Dinsdale et al. (2010) consensus sequences were used as reference sequences, and Bemisia afer (GQ139515) as an outgroup.

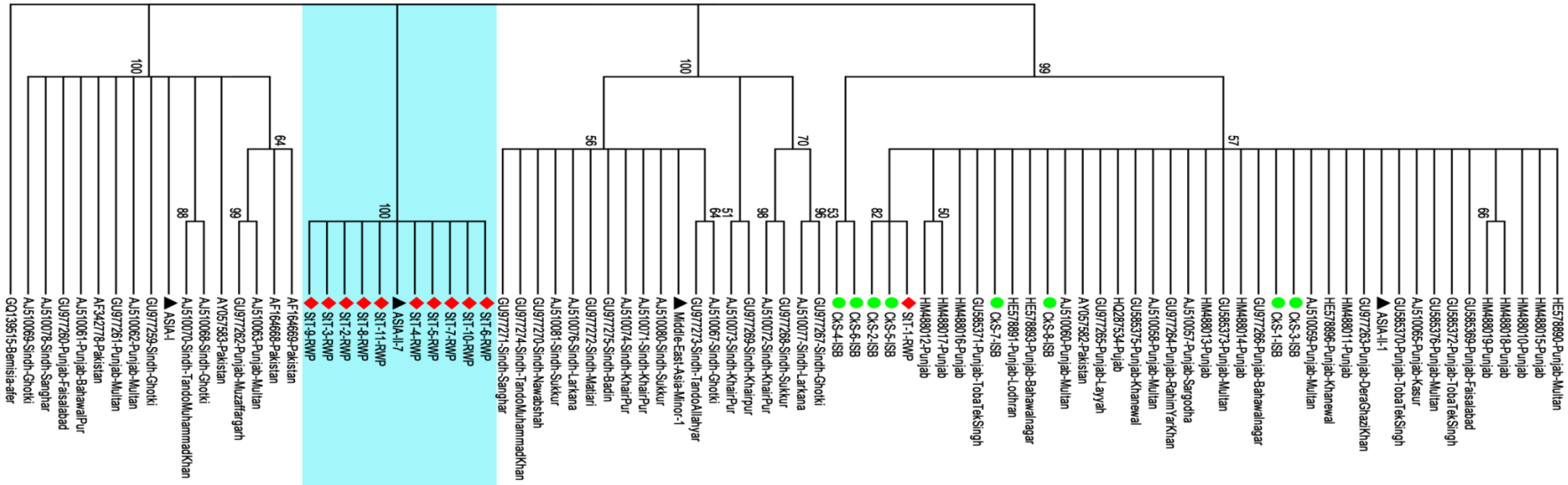

Fig 3. A combine phylogenetic tree of sequences generated by this study and sequences earlier reported from Pakistan

Tree was reconstructed by maximum likelihood method in MEGA 5 (Bootstrap=1000 (Dinsdale *et al.* 2010) consensus sequences were used as reference sequences, and *Bemisia afer* (GQ139515) as an outgroup

Sequences of specimens collected from Islamabad

Sequences of specimens collected from Rawalpindi

Reference sequences

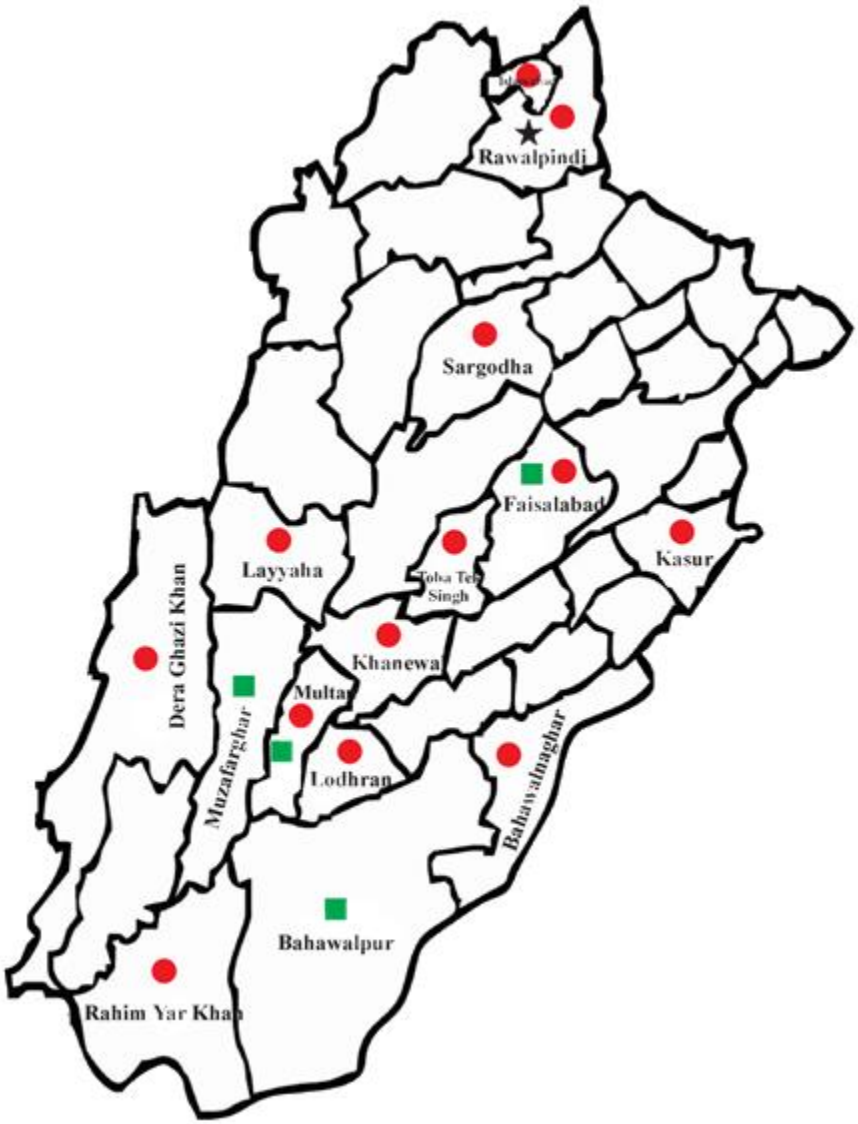

Figure 4. Shows distribution of B. tabci in different districts of Punjab. Colored shapes represent different genetic variants of *B. tabaci*

● Asia-II-1

◆ Asia-1

▲ Asia-II-7

DISCUSSION

B. tabaci is a species/biotype complex that is one of the most devastating agricultural pests worldwide. It is an agriculturally important pest in Pakistan as it affects a broad range of food, fiber and ornamental crops by its direct feeding and more importantly by vectoring Begomoviruses (Family: *Geminiviridae*).

Efforts to investigate genetic diversity of this insect from Pakistan so far were inconsistent and were based on small sample data. An ongoing debate on species/biotype status of this insect and multiple approaches to identify biotypes has made the situation further confusing. To provide a clear picture of the situation of the prevalence and genetic diversity of *B. tabaci* in Pakistan this study has reviewed all possible sequence data in the data base from the country. All available information was gathered and analyzed in comparison to standard international sequences. Phylogenetic analysis generated by this study supports that three genetic groups of this insect are predominant in Pakistan. These three genetic groups (cryptic species) are Asia-I, Asia II-1 and MEAM-1. Asia-1 was found in both Sindh and Punjab province. Asia-II-1 was recorded from Punjab only, whereas MEAM-1 was

found only in Sindh. Other than analysis of available data during this study samples of *B. tabaci*, from vegetable plots of Rawalpindi/Islamabad were collected and mtCO1 gene was amplified from single specimens for identification of genetic variant of this insect in this area. The PCR products were commercially sequenced and the results report a genetic variant/specie of *B. tabaci* that is not reported from the country previously. This study reports fourth putative specie Asia-II-7 from Pakistan. This variant is already reported from the neighboring countries like India and China but was not reported from Pakistan till now (Qiu *et al*., 2009).

The genetic diversity of the field populations of begomoviruses in relevance to biotype diversity of *B. tabaci* in Pakistan was analyzed by Simon and co-workers (2003). This study reported a parallel analysis of begomoviruses and *B. tabaci* populations sampled from cotton growing areas of Pakistan. Viruses as well as *B. tabaci* population showed three clades based on geographical distribution. Sequence analysis of *mt*CO1 gene showed that the *B. tabaci* population was structured into at least three genetic lineages corresponding to the previous reports.

There are only two relatively recent reports on the genetic diversity of *B. tabaci* in the country, both by the same author (Ahmed *et al*., 2010; Ahmed *et al*., 2011). One of these reports is based on the analysis of ITS1 (Ahmed *et al*., 2010) and in other mtCO1 sequences were used for phylogenetic analysis (Ahmed *et al*., 2011). Results of the study based on ITS1 sequence alignment indicated that all the *B. tabaci* populations from Pakistani cotton plants were quite similar to each other. This report provides very limited information as this report was based on small sample size (16 samples) and from Punjab area only. However it showed that the *B. tabaci* populations collected from cotton plants in Pakistan belong to an unresolved biotype PCG1 of the Asia II genetic group described in Boykin *et al*., (2007). Samples in this study were collected in year 2007 from cotton growing districts of Punjab. The other study based on *mt*CO1 sequences and including larger samples from both provinces also support the presence of previously reported three cryptic species, Asia-1, Asia-II-1 and MEAM-1 in the country (Ahmed *et al*., 2011).

We collected all available reliable sequences of *mt*CO1 of *B. tabaci* deposited in data base from Pakistan (unpublished) as well as the sequences reported in the papers described earlier and compared them with standard reference sequences from NCBI. Our analysis suggests the presence of Asia-I (H and M), Asia-II-1 (K, P and ZHJ2) and MEAM-1 (B and B2) in the country and their distribution in the two provinces is indicated. These three genetic groups (cryptic species) Asia-1, Asia II-1 and MEAM-1 were found not to be uniformly distributed in the country. Asia 1 was the only genetic group found in both Sindh and Punjab province. Asia-II-1 was recorded from Punjab only, whereas MEAM-1 was found only in Sindh. Available data was not sufficient to draw any conclusions about the relationship of genetic variation of *B. tabaci* and intensity of CLCuD in the two provinces of the country.

B. tabaci is generally considered to have originated from the Indian subcontinent, although little information has so far been collected on the molecular diversity of populations present in this region. If reports from India on the subject are reviewed same three genetic variants are present in India as in Pakistan. These three variants are Asia-I, Asia II-1 and MEAM-1 (Rekha *et al*., 2005; Boykin *et al*., 2007). Like in Pakistan Asia-I is widely distributed in India and is reported from almost all over the country. Asia-II-1 group is present in the Indian states close to Pakistan where it has been found previously (Simon *et al*., 2003) but is not present in other Indian States (Chowda-Reddy *et al*., 2012).

According to the previous reports (Ahmad *et al*., 2011), three putative species, Asia-1, Asia-II-1 and MEAM-1 are present in Pakistan. As a result of this study we are reporting fourth putative specie Asia-II-7. This genetic clad was first reported in 1998 in India (Ramappa *et al*., 1998), and then in China (Qiu *et al*., 2006). A report suggests that this genetic variant Asia-II-7 adopts readily to ornamental plants as compared to vegetables. The sampling area (Rawalpindi/ Rawalpindi) from where this genetic variant was found was a large area harboring both vegetables and ornamental plants. One reason for this genetic variant not being previously reported from Pakistan may be that previous reports concentrated on fiber and food crops and not ornamentals. A more detailed study involving extensive work, including large sample areas and from multiple hosts and locations is needed to fully elucidate the genetic variation of *B. tabaci* in Pakistan.

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