Genetic Structure of Field Populations of Begomoviruses and of Their Vector *Bemisia tabaci* in Pakistan

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ABSTRACT

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The genetic structure of field populations of begomoviruses and their whitefly vector *Bemisia tabaci* in Pakistan was analyzed. Begomoviruses and *B. tabaci* populations were sampled from different crops and weeds in different locations in Punjab and Sindh provinces, in areas where cotton leaf curl disease (CLCuD) occurs or does not occur. Phylogenetic analysis based on nucleotide sequences of the intergenic region in the viral DNA-A provided evidence of two clusters of isolates: viruses isolated from species in the family *Malvaceae*, and viruses isolated from other dicotyledon families. Analysis of the capsid protein (CP) open

reading frame grouped isolates into three geographical clusters, corresponding to isolates collected in Punjab, Sindh, or both provinces. Random amplified polymorphic DNA analyses of the *B. tabaci* population showed that intrapopulation diversity was high at both the local and regional scales. Sequence analysis of the mitochondrial cytochrome oxidase I (mt COI) gene showed that the *B. tabaci* population was structured into at least three genetic lineages corresponding to the previously described Indian, Southeast Asian, and Mediterranean-African clades. The Indian clade was present only in Punjab, the Mediterranean-African only in Sindh, and the Southeast Asian in both provinces. *B. tabaci* haplotypes of the Indian clade were found only in the Punjab, where CLCuD occurs. Hence, the geographical distribution of virus and vector genotypes may be correlated, because similar phylogenetic relationships were detected for the viral CP and the vector mt COI genes.

The emergence of new diseases caused by begomoviruses has been associated with changes in the incidence, the area of distribution (6,8–10,47), and the genetic structure (7,35,48) of the populations of their whitefly vector, *Bemisia tabaci* (Gennadius). In addition, a number of new recombinant *Begomovirus* spp. have been associated with outbreaks of a number of diseases (44,45,50,53,54). Because the fate of a new genetic variant in the virus population may depend on different selection pressures, it is important to understand the factors involved in the evolution of begomoviruses to explain the emergence of new diseases caused by them (28,38). The characterization of the genetic structure of the population of an organism may provide information important to understand its evolution. For vector-transmitted viruses, it would be desirable to have information on the genetic variability and structure of both the vector and virus population.

An important example of an emerging plant disease is cotton leaf curl disease (CLCuD), which has become the main threat to cotton production in Pakistan since it was first reported in the Punjab province in 1967 (1,24). CLCuD has severely affected more than 1 million ha in the Punjab province, and in the north of the Sindh province. Interestingly, CLCuD did not occur in most of the cotton-growing areas in Sindh until very recently (Fig. 1). A *Begomovirus* sp., *Cotton leaf curl virus* (CLCuV), recently reclassified as a group of different species (*Cotton leaf curl Alabad virus* [CLCuAV], *Cotton leaf curl Kokhran virus* [CLCuKV], and

Cotton leaf curl Multan virus [CLCuMV] (17), has been associated consistently with CLCuD-affected plants (5,55). For these viruses, only a DNA-A, but not a DNA-B, component has been identified (5,36). CLCuV is a helper virus for a satellite-like DNA (DNA β), and infection by both CLCuV and DNA β is necessary to cause the symptoms of CLCuD (4). DNA β is encapsidated in CLCuV particles and, presumably, also is transmitted by *B. tabaci* (4). Mixed infections between CLCuV species, and recombinants among them, have been described from diseased cotton and other malvaceous and nonmalvaceous species in cotton-growing areas of Pakistan (49,50,55).

B. tabaci can be thought of as a species complex containing numerous genetic and biological variants (2,7,8,10,46). This variability is manifest by numerous variants, which have been recognized using several different genetic markers (12,14,16,20,22). Sequence data of the mitochondrial cytochrome oxidase I gene (mt COI) has revealed that the analyzed *B. tabaci* populations from different locations worldwide are distributed in at least six well-supported lineages or clades that are strongly correlated with specific geographic areas on the continental scale (7,18,33,34). Although slightly less informative, the analysis of the sequence of the internal transcribed spacer 1 (ITS1) region of rDNA produces a phylogeny consistent with that derived from mt COI (16). Previous studies have shown the presence, in Pakistan, of a *B. tabaci* biotype not reported elsewhere, type K (2). Hameed et al. (25) reported the presence in Pakistan of the B biotype. Finally, the analysis of mt COI sequences of samples from three *B. tabaci* populations collected in Pakistan from cotton (7) showed them to belong to two different phylogeographical groups, the Indian and Southeast Asia clades. This indicates a high variability of *B. tabaci* in Pakistan.

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Here, we report on an analysis of the genetic structure of *B. tabaci* populations in cotton-growing areas of Pakistan, and compare it with a genetic analysis of the *Begomovirus* population.

MATERIALS AND METHODS

Virus isolates and *B. tabaci* populations. Cotton and other dicotyledonous crops and weeds showing *Begomovirus*-like symptoms (mainly leaf curl, vein thickening and enation, yellow vein, and mosaic) were sampled in a north-south transect of the cotton-growing area in the provinces of Punjab and Sindh, in Pakistan, in the locations indicated in Figure 1A. Samples were collected in autumn 1995 and spring 1997. Here, we define a virus isolate as the geminiviral DNA polymerase chain reaction (PCR) amplified from an individual plant. The analyses in this work also include isolates from samples collected in the Punjab province in 1996 by D. J. Robinson, Dundee, UK (isolates oymv201, clc62, and clc804 in Table 1) (55). *B. tabaci* adults were sampled in the same area, in the locations indicated in Figure 1B, in spring and fall 1997 and in spring 1998. Here, we define a population of *B. tabaci* as the set of whitefly individuals (≈ 100 adults) collected at a certain location in a single field. One whitefly population, sampled in Multan before 1995 and maintained at the SCRI, Dundee, UK (population PK4); a population sampled in Multan in 1995 and maintained in the NARC, Islamabad (Pj 95.1); and the laboratory populations of biotype B, collected in Almeria (Spain) and biotype K, collected in Pakistan (2, 22), also were included in some of the analyses. *Begomovirus* isolates and whitefly populations are shown in Tables 1 and 2, respectively, which include information on their origin.

Characterization and analyses of *Begomovirus* isolates. DNA purification from plant samples, primers for PCR amplification of begomoviral DNA, PCR procedures, and nucleotide sequence determination of PCR-amplified fragments have been described (27,49). Two genomic regions were PCR amplified. For the first one, including the intergenic region (IR), primers were annealed to nucleotides 316 to 298 and 2,309 to 2,339, yielding a predicted fragment of ≈ 710 nucleotides (nt). For the second region, including most of the capsid protein (CP), primers were annealed to nucleotides 990 to 972 and 199 to 218, yielding a predicted fragment of ≈ 745 nt (nucleotide numbering for primer positions according to sequence of CLCuMV, accession no. AJ002459). Although the nucleotide sequence of the primers was based on that of DNA-A of CLCuV-a (an isolate of CLCuMV) (17), as reported by Nadeem (40), they directed the amplification of DNA fragments from other *Begomovirus* spp. (49,50).

Except for the CP open reading frame (ORF) of isolate p12 and the IR of a second variant from sample p26 (isolate p26b), sequences analyzed in this work have been previously reported (49,50,55). Accession numbers in the EMBL data bank for all sequences are indicated in Table 1. DNA sequences were aligned by the program CLUSTALW (52) and genetic distances were estimated by Kimura's two-parameter method (32). Phylogenetic analyses were done using the neighbor-joining and maximum likelihood methods using the PHYLIP Package (version 3.6; J. Felsenstein, Seattle, WA).

Characterization and analyses of *B. tabaci* populations. Two different approaches were used for the genetic characterization of *B. tabaci* populations. First, random amplified polymorphic DNA (RAPD)-PCR analysis was applied to evaluate the intrapopulational genetic diversity of 13 *B. tabaci* populations. Nine males were analyzed individually from each of 13 populations (total of 117 insects). Three populations were from the Punjab (Pj 98.5, Pj 98.8, and Pj 98.9), six were from Sindh (Sd 97.1, Sd 97.2, Sd 97.3, Sd 97.4, Sd 98.2, and Sd 98.6), two were the laboratory populations PK4 and Pj 95.1, and two were reference populations of biotypes B and K. RAPD-PCR analyses also were done on 24 individual male whiteflies from the populations collected in 1997

and 1998 (Table 2). Of these male whiteflies, 12 were randomly sampled from the Punjab populations and 12 from those from Sindh. DNA from individual insects was extracted in 200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate and precipitated with one volume of isopropanol (11), and RAPD-PCR amplification was performed according to a procedure previously described (22). Six 10-mer primers were selected based on preliminary experiments and used in PCR:

Fig. 1. Map of Pakistan, indicating the sites where **A**, *Begomovirus* isolates and **B**, *Bemisia tabaci* populations were sampled. Information on the genetic type of the capsid protein of virus isolates (I to III) and on the phylogenetic clade of whiteflies (In: Indian; MA: Mediterranean-African; SA: Southeast Asia) (7) are indicated between parenthesis. The main cotton-growing areas (gray) and the area where cotton leaf curl disease occurs (dark gray) are indicated.

OPB13, OPC4, OPC10, OPC15, OPO6, and OPO18 (Operon Technologies, Alameda, CA). PCR-amplified fragments were resolved by electrophoresis in 1.4% agarose gels in Tris-borate-EDTA buffer, pH 7.0. Presence and absence of RAPD bands was used to define haplotypes, and genetic distance between pairs of haplotypes was estimated (42) by $d_{ij} = 2n_{ij}/(n_i + n_j)$, where d_{ij} is the genetic distance between haplotypes i and j , n_i and n_j are the number of RAPD bands in haplotypes i and j , respectively, and n_{ij} is the number of bands common to haplotypes i and j . Genetic distances were used to estimate within-population diversity. Diversity for population k , D_k , was computed as $D_k = n_k/(n_k - 1) \sum x_i x_j d_{ij}$, where n_k is the number of analyzed individuals, x_i and x_j are the sample frequency of haplotypes i and j , respectively, in population k , and d_{ij} is the genetic distance between haplotypes i and j (41).

A second approach, sequencing of mt COI, was done on a set of 25 adult whitefly males randomly sampled from all the local populations in Table 2. A PCR product of ≈ 700 bp of the mt COI gene of the samples described in Table 2 was amplified with the primers C1-J-2195 (5'-TTG ATT TTT TGG TCA TCC AGA AGT-3') and L2-N-3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') (51), according to a previously described protocol (18). The DNA sequence of the amplified fragments was obtained using an ABI Prism 310 automatic sequencer (Applied Biosystems, Foster City, CA). Four sequences were selected from the GenBank (AF342779, AF164667, AF164668, and AF0110708) (7) in order to assign the obtained sequences to known *B. tabaci* clades. Genetic distances among mt COI gene sequences of whiteflies were estimated by Kimura's two-parameter method (32), as explained for *Begomovirus* DNA sequences. Genetic distances so estimated were used to estimate within-population diversity and the coefficient of genetic differentiation (G'_{ST}) (41). Phylogenetic analyses were performed by the neighbor-joining

and maximum likelihood methods as described for *Begomovirus* sequences.

RESULTS

Genetic structure of the *Begomovirus* population. The primers used to PCR amplify *Begomovirus* DNA were designed from the sequence of a single isolate of CLCuMV, but directed the amplification of fragments from other species of CLCuV and other *Begomovirus* spp. (50). Thus, isolates from different *Begomovirus* spp. can be compared, to the extent that their DNA can be amplified with these sets of primers. The analyzed sequence set in this work included all the available sequences of isolates collected from various host plant species from Sindh and a similar number of sequences of isolates from Punjab. The Punjab sequences were randomly sampled within the larger set of sequences available for this province. There were 26 available sequences for the IR and 22 for the CP ORF; 19 of the isolates had both IR and CP sequences (Table 1).

For begomoviruses, it has been shown that sequences within the IR and the CP ORF can be useful markers to analyze diversity (7). Thus, using the IR and CP sequences described above, genetic distances between each pair of isolates were estimated and phylogenetic relatedness was estimated for these two genomic regions. In the case of the IR, all virus isolates clustered into one of two groups, referred to as IR I and IR II (Fig. 2). Isolates in cluster IR I were from malvaceous hosts, whereas most (7/9) isolates in cluster IR II were from nonmalvaceous hosts (Table 1; Fig. 2). Hence, analysis of the IR showed a significant clustering ($P < 10^{-4}$ in a contingency analysis) of *Begomovirus* isolates according to the host plant from which it was detected (i.e., from the family *Malvaceae* or from another host). Sequences in cluster IR I grouped with CLCuV species from Pakistan (49,50,55) (i.e.,

TABLE 1. Origin of *Begomovirus* isolates used in this work

Virus	Place	Year	Host plant (symptom) ^a	Accession no. ^b
	Punjab			
oymv 201	Faisalabad	1995	<u>Okra</u> (yellow vein mosaic)	AJ002451
olc 1	Faisalabad	1995	<u>Okra</u> (leaf curl)	AJ228575 , <i>AJ228585</i>
clc 8	Faisalabad	1995	<u>Cotton</u> (leaf curl)	AJ228581 , <i>AJ228594</i>
clc 12	Faisalabad	1995	<u>Cotton</u> (leaf curl)	AJ228576 , <i>AJ228586</i>
clc 49	Sahiwal	1995	<u>Cotton</u> (leaf curl)	AJ228577 , <i>AJ228590</i>
clc 60	Bahawalpur	1995	<u>Saklari</u> (leaf curl)	<i>AJ228591</i>
clc 62	Bahawalpur	1995	<u>Cotton</u> (leaf curl)	AJ002447
clc 75	Multan	1995	<u>Cotton</u> (leaf curl)	AJ228579 , <i>AJ228592</i>
clc 78	Multan	1995	<u>Cotton</u> (leaf curl)	AJ228580 , <i>AJ228593</i>
clc 82	Multan	1995	<u>Cotton</u> (leaf curl)	AJ228582 , <i>AJ228595</i>
clc 83	Multan	1995	<u>Cotton</u> (leaf curl)	AJ228583 , <i>AJ228596</i>
clc 85	Multan	1995	<u>Cotton</u> (leaf curl)	AJ228584 , <i>AJ228597</i>
olc 90	Multan	1995	<u>Okra</u> (yellow vein mosaic)	<i>AJ228599</i>
clc 804	Alabad	1996	<u>Cotton</u> (leaf curl)	AJ002452
p 1	Multan	1997	Tomato (yellow leaf curl)	AJ270852
p 10	Ahmad Pur	1997	<u>Cotton</u> (leaf curl)	AJ270853
	Sindh			
p 12	Mirpur Mathelo	1997	<u>Hollyhock</u> (leaf curl)	AJ270854 , <i>AJ496519</i>
p 17	Mohenjo Daro	1997	<u>China rose</u> (yellow vein)	AJ270855
p 19	Nawabshah	1997	<i>Solanum nigrum</i> (leaf curl & mosaic)	AJ270856
p 20	Shahdadpur	1997	Goar (yellow mosaic)	AJ270857
p 24	Mirpur Khas	1997	Weed 1 (yellow leaf curl)	AJ245458 , <i>AJ270895</i>
p 25	Mirpur Khas	1997	Weed 2 (yellow vein)	AJ245459 , <i>AJ270896</i>
p 26	Hyderabad	1997	<u>Hollyhock</u> (leaf curl)	AJ270860 , <i>AJ245497</i> AJ496520
p 27	Hyderabad	1997	Tobacco (leaf curl)	AJ270861 , <i>AJ245498</i>
p 28	Tando Muhammad Khan	1997	Tomato (yellow leaf curl)	AJ270862 , <i>AJ245499</i>
p 29	Tando Muhammad Khan	1997	Bottlegourd (yellow mosaic)	AJ270863 , <i>AJ245500</i>
p 30	Tando Muhammad Khan	1997	Watermelon (severe stunt)	<i>AJ245501</i>
p 31	Karachi	1997	<u>China rose</u> (leaf curl)	AJ270864

^a Host plants are: Bottlegourd = *Luffa cylindrica*, China rose = *Hibiscus rosa-chinense*, Cotton = *Gossypium hirsutum*, Guar = *Cyanopsis tetragonoloba*, Hollyhock = *Althaea rosea*, Okra = *Abelmoschus sculentus*, Saklari = *Hibiscus tiliaceus*, Tobacco = *Nicotiana tabacum*, Tomato = *Lycopersicon esculentum*, Watermelon = *Citrullus lanatus*. Hosts in the family *Malvaceae* are underlined.

^b Accession numbers for intergenic region sequences are in bold, and those for capsid protein genes are in italics.

CLCuAV, CLCuKV, and CLCuMV) (data not shown). Comparison of the analyzed sequences with those in the databases showed that the IR of p27 is most related (93% similarity) to that of *Papaya leaf curl virus* from India (accession no. Y15934), and

TABLE 2. Origin of *Bemisia tabaci* populations analyzed in this study

Population	Place	Year	Host plant
	Punjab		
PK4	Multan	<1995	Unknown
Pj 95. 1	Multan	1995	Cotton
Pj 97. 1	Multan	1997	Eggplant
Pj 97. 2	Multan	1997	Eggplant
Pj 97. 4	Ahmad Pur	1997	Eggplant
Pj 98. 5	Multan	1998	Eggplant
Pj 98. 6	Multan	1998	Eggplant
Pj 98. 7	Kloor Kot	1998	Mung bean
Pj 98. 8	Nattak	1998	Mung bean
Pj 98. 9	Kroore	1998	Mung bean
Pj 98.10	Fateh Pur	1998	Cotton
Pj 98.11	Chak 99	1998	Mung bean
	Sindh		
Sd 97.1	Mirpur Mathelo	1997	Hollyhock
Sd 97.2	Ubaro	1997	Eggplant
Sd 97.3	Shahdadpur	1997	Melon
Sd 97.4	Tando Muhammad Khan	1997	Eggplant
Sd 98.1	Khairpur	1998	Cotton
Sd 98.2	Kot Digi	1998	Cotton
Sd 98.3	Larkana	1998	Okra
Sd 98.4	Sanghar	1998	Unknown
Sd 98.5	Moro	1998	Cotton
Sd 98.6	Sumar Chanar	1998	Zucchini squash with silverleaf phenotype

that sequences in cluster IR II are related (up to 98% similarity) to *Tomato leaf curl New Delhi virus* (accession no. U15016) (data not shown).

A similar analysis was done with the CP ORF. Analysis of the CP ORF sequences for the 19 isolates for which IR sequences were available showed three significant clusters (Fig. 3). Isolates from malvaceous hosts grouped in clusters CP I and CP II, and those from nonmalvaceous hosts in cluster CP III ($P = 0.004$ in a contingency analysis). Sequences from malvaceous hosts in cluster CP I grouped with reference sequences of CLCuMV (e.g., clc62, accession no. AJ002447), whereas some of the sequences of the cluster CP II belonged to CLCuAV and *Okra yellow vein mosaic virus* (e.g., clc804, accession no. AJ002452, and oymv201, accession no. AJ002451) (17) (Fig. 3). Geographical origin also was a significant factor for clustering ($P = 0.004$ in a contingency analysis). Isolates in cluster CP I all were from the Punjab, those in cluster CP III all were from Sindh, and those in cluster CP II were from both regions. Similar results were obtained when the analysis was done with the 22 CP sequences indicated in Table 1 (data not shown).

For both the IR and the CP, the phylogenetic relationships among isolates was the same when analyses were done using the neighbor-joining (Figs. 2 and 3) or the maximum likelihood (data not shown) methods.

Genetic structure of the *B. tabaci* populations. The RAPD-PCR reaction produced the amplification of numerous random DNA fragments (Fig. 4) for all the individuals analyzed from all populations. Every individual analyzed was of a different haplo-

Fig. 2. Phylogenetic tree generated with the nucleotide sequence of the intergenic region (IR) and the neighbor-joining method for 26 *Begomovirus* isolates from Pakistan. *Tomato leaf curl Karnataka virus* (U38239) was used as an outgroup. One thousand bootstrap replications were done and all branches below 60% were collapsed; bootstrap values for the main nodes are indicated.

Fig. 3. Phylogenetic tree generated with the nucleotide sequence of the capsid protein (CP) open reading frame and the neighbor-joining method for 19 *Begomovirus* isolates from Pakistan. *Tomato leaf curl Karnataka virus* (U38239) was used as an outgroup. One thousand bootstrap replications were done and all branches below 60% were collapsed; bootstrap values for the main nodes are indicated.

type, as defined by the presence or absence of the RAPD-PCR polymorphic bands, although some differed by only a few fragments (Fig. 4). Genetic distance between haplotypes was used to estimate within-population diversity. Intrapopulation diversity for all field populations was high, 0.15 to 0.23 (Table 3). Laboratory populations were less diverse. The population diversity estimates for the RAPD-PCR analysis of 24 individuals randomly sampled from all field populations in Table 3 (i.e., those collected in 1997 and 1998) yielded a value of 0.3049. Hence, the whitefly population from Punjab plus Sindh was not much more diverse than for the most diverse local populations (compare values of 0.30 for the entire population and 0.23 for Pj 98.8 and Sd 97.3 in Table 3). A cluster analysis of the data for each of the nine individual whiteflies from seven of the populations in Table 3 showed three distinct clusters. One cluster contained the reference population of biotype B collected in Spain, Sd 98.2 and Sd 98.6. A second cluster contained populations PK4 and Pj 95.1. A third cluster contained populations from biotype K from Pakistan (2,15) and Pj 98.5. The nine individuals from each population clustered together, with the exception of those of population Sd 98.6. Six individuals from population Sd 98.6 formed a cluster sister to that of population Sd 98.2, one individual clustered with the nine individuals of population Sd 98.2, and two other formed an independent cluster within the larger one including population B from Spain, Sd 98.2, and Sd 98.6 (data not shown).

B. tabaci population structure also was examined using the mt COI gene sequence obtained from 25 whiteflies randomly sampled from the entire set of local populations. Phylogenetic analyses indicated that the Pakistan population of *B. tabaci* comprises three main genetic types (Fig. 5). Types I and III were found only in the

Fig. 4. Random amplified polymorphism DNA-polymerase chain reaction analysis of whiteflies. Results of amplification for primer OPC-4. Lanes 1 and 20: molecular weight marker, 123-bp ladder (Invitrogen, PA); lanes 2 to 10: nine individuals of reference population of biotype K; lanes 11 to 19: nine individuals from population Pj 95.1.

Population	<i>d_{ij}</i>
B	0.06
K	0.08
PK4	0.14
Pj 95.1	0.16
Pj 98.5	0.18
Pj 98.8	0.23
Pj 98.9	0.16
Sd 97.1	0.13
Sd 97.2	0.19
Sd 97.3	0.23
Sd 97.4	0.22
Sd 98.2	0.15
Sd 98.6	0.16

^a Population diversity was estimated from random amplified polymorphic DNA data from individual adult insects.

Punjab or Sindh, respectively, whereas type II was found in both the Punjab and Sindh. For some populations, sequences for more than one individual were determined. In most cases, sequences belonged to the same type (i.e., type I for population PK4, type II for Pj 98.5 and Sd 97.2, and type III for Sd 98.1, Sd 98.2, Sd 98.3, and Sd 98.6), but individuals of different types also could be found within one population (i.e., individuals from population Sd 97.1 clustered in types II and III).

Some mt COI sequences from the EMBL data bank were included in the analysis. Type I sequences clustered with sequence AF342779 from Nepal, and composed a clade also reported to contain populations from India (7). Type II sequences clustered with sequence AF164668, belonging to the Southeast Asia–Far East clade (7). Finally, type III sequences clustered with sequence AF164667 from Israel (7), which represents the Mediterranean–African clade of *B. tabaci*. Because this sequence is of a population previously identified as the B biotype (7) and Sd 98.6, also placed in this group, was collected from squash plants exhibiting silverleaf, phenotype characteristic of B biotype (14), these results suggest that whiteflies in cluster III (Fig. 5) may be the B biotype. These results are consistent with the detection of *B. tabaci* biotype B in Pakistan (25). It also is interesting that Pj 98.5, which was in the same RAPD group as the biotype K con-

Fig. 5. Phylogenetic tree generated with 25 mitochondrial cytochrome oxidase I gene sequences and the neighbor-joining method for *Bemisia tabaci* from whiteflies from Pakistan. A sequence of *Trialeurodes vaporariorum* (AF0110708) (7) was used as outgroup. Reference sequences from the EMBL data bank (AF342779, AF164668, and AF164667) (7) also were included. One thousand bootstrap replications were done and all branches below 60% were collapsed; bootstrap values for the main nodes are indicated.

tol, belongs to the Southeast Asian clade. Phylogenetic analysis using maximum likelihood (data not shown) or neighbor-joining (Fig. 5) methods yielded the same results.

Within-population nucleotide diversity for *B. tabaci* was not significantly different for types I, II, and III (0.0220, 0.0259, and 0.0243, respectively, $P > 0.05$ in a Wilcoxon nonparametric test). Between-population diversity was much higher (0.1306 to 0.1466) and yielded a G'_{ST} (41) of 0.85, supporting differentiation of *B. tabaci* in Pakistan into at least three types.

DISCUSSION

Recombination and reassortment seem to have had an important role in the diversification of the family *Geminiviridae* (28,45). It has been shown experimentally that these mechanisms may account for the emergence of new *Begomovirus* forms after the introduction of DNA A and DNA B from related but different viruses into a common host (29,30). This likely has occurred also in nature, as shown in a number of instances (49,50,54,55). However, little is known of the factors that drive *Begomovirus* evolution in nature (i.e., what factors determine that a new genotype increases its frequency in, or is lost from, the virus population). An approach to gain insight into some of these factors is the analysis of the genetic structure of the virus population and its relationship with the environment. Major environmental factors include the host plant species and the whitefly vector, because they condition the viral distribution in crop ecosystems. Begomoviruses are transmitted by *B. tabaci*, a species complex (10) of temperate and tropical distribution. Changes in the number, distribution, and genetic structure of *B. tabaci* have been suggested to contribute to the emergence of new begomoviral diseases (7,8,33–35,47). The host often is considered in analyses of plant virus population structure (19), whereas joint analyses of the population structure of both virus and vector are less frequent (35).

We report here a parallel analysis of the genetic structure of begomoviruses and the whitefly vector from cotton-growing areas in Pakistan, including the provinces of the Punjab, where CLCuD is rampant, and Sindh, where CLCuD has only recently been reported in the northern part of the province (1,5,31). The virus isolates described in this work, and others collected in the same cotton-growing areas of Pakistan, have been reported previously (49,50,55). In these previous reports the extent of genetic diversity (49,55) and the role of recombination in the evolution of CLCuV-like begomoviruses (49,50) was analyzed. The set of isolates considered here has not been jointly analyzed before, nor has the role of the host plant and insect vector in their evolution been investigated.

Mixed infections between different *Begomovirus* spp. and strains, and recombinants among them, have been shown to be frequent (49,50,55). Hence, it should not be unexpected that the genetic structure of the virus population examined in the present study differed according to the genomic region considered. For the IR, isolates clustered according to the host plant family (i.e., isolates collected from plants in the family *Malvaceae* [mostly cotton] or from other dicotyledon families). The IR has a role in mediating DNA replication by binding with the virus-encoded protein Rep. Rep interacts with the 5' half of the IR (23,26), and it has been suggested that host plant components may be a factor in the evolution of the IR (28). Our results are consistent with this hypothesis, but definitive experimental evidence needs to be obtained.

It also has been proposed that the main selection factor for geminivirus CP is the interaction with the insect vector (28). Our phylogenetic analysis of the CP ORF revealed three clusters correlated with geographical regions, comprising types found only in Punjab or in Sindh, and another type found in both regions. It is noteworthy that sequence analysis of the *B. tabaci* population also revealed three clusters with the same geographical distribution.

The similar genetic structure for the viral CP and the vector mt COI genes is suggestive of a relationship between both.

The population of *B. tabaci* in Pakistan is highly diverse. Three genetic types, representing the Indian, Southeast Asian, and Mediterranean-African phylogenetic clades (7), were identified in the analyzed region. The Indian and Southeast Asian clade types were detected in Punjab, and the Southeast Asian and Mediterranean African clade types in Sindh. It is possible that haplotypes belonging to the two clades present in one region are found together in the same population, as shown for population Sd 97.1 (Fig. 5). This would result in high genetic diversity at the local population scale, as shown in Table 3.

The presence in Pakistan of at least three genetic types of *B. tabaci* was shown in previous reports (7,25). However, results presented here from a larger sample size provide a view of the geographical distribution of these types. It is noteworthy that the Indian clade type appears predominant in the Punjab, and the Mediterranean African clade type in the Sindh; however, the reason for this is unknown at present, because no obvious geographical barrier occurs in the Indus Valley. The displacement of biotypes of *B. tabaci* by another in a region has been reported (3,15,35,39). However, the dynamics of this phenomenon may be complex, and different factors such as biological fitness in some hosts, resistance to insecticides, and susceptibility to parasitoids (10,14,15) can influence the distribution of *B. tabaci* genotypes.

Previous detailed analyses of the population structure of *B. tabaci* in a region have revealed limited diversity, with one or two dominant types prevailing (13,15,35,39). The higher genetic diversity of the population from Punjab and Sindh is compatible with the origin of *B. tabaci* in the Indian subcontinent, which also has been proposed based on the high diversity of parasitoids for this region (10,21,37). It is interesting to note that several sequences in cluster III (Fig. 5), including one from Sd 98.6 that was collected from squash with silverleaf symptoms (14), were highly similar (above 97% nucleotide sequence identity) to a sequence for the B biotype from Israel (AF164667) (7). This indicates that the B biotype was present in Pakistan at the time these samples were collected and suggests that it may be widespread in Sindh (Table 2; Fig. 1B).

The emergence of CLCuD was associated with the widespread use of a highly susceptible cotton cultivar, S129 (5,43), and it has been speculated that new begomoviruses of recombinant origin could have an increased fitness on this cultivar (55). CLCuD occurs where *B. tabaci* type I (the Indian phylogenetic clade type) is found. Thus, the distribution of CLCuD may have been influenced by the distribution of the Indian genotype of *B. tabaci*. It has been hypothesized that geographical distribution of virus genotypes and vector genotypes is highly correlated (7). Our data agree with this hypothesis for the cotton-growing area of Pakistan, because similar genetic structures were revealed for the viral CP and the *B. tabaci* mt COI genes. This may suggest that the insect vector is an important factor in virus evolution, an aspect that remains to be experimentally explored.

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