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Genetic diversity of *Bemisia tabaci* (Genn.) Populations in Brazil revealed by RAPD markers

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Abstract

Bemisia tabaci (Genn.) was considered a secondary pest in Brazil until 1990, despite being an efficient geminivirus vector in beans and soybean. In 1991, a new biotype, known as *B. tabaci* B biotype (=*B. argentifolii*) was detected attacking weed plants and causing phytotoxic problems in *Cucurbitaceae*. Nowadays, *B. tabaci* is considered one of the most damaging whitefly pests in agricultural systems worldwide that transmits more than 60 different plant viruses. Little is known about the genetic variability of these populations in Brazil. Knowledge of the genetic variation within whitefly populations is necessary for their efficient control and management. The objectives of the present study were to use RAPD markers (1) to estimate the genetic diversity of *B. tabaci* populations, (2) to study the genetic relationships among *B. tabaci* biotypes and two other whitefly species and (3) to discriminate between *B. tabaci* biotypes. A sample of 109 *B. tabaci* female individuals obtained from 12 populations in Brazil were analyzed and compared to the A biotype from Arizona (USA) and B biotype from California (USA) and Paraguay. *Trialeurodes vaporariorum* and *Aleurodicus cocois* samples were also included. A total of 72 markers were generated by five RAPD primers and used in the analysis. All primers produced RAPD patterns that clearly distinguished the *Bemisia* biotypes and the two other whitefly species. Results also showed that populations of the B biotype have considerable genetic variability. An average Jaccard similarity of 0.73 was observed among the B biotype individuals analyzed. Cluster analysis demonstrated that, in general, Brazilian biotype B individuals are scattered independently in the localities where samples were collected. Nevertheless, some clusters were evident, joining individuals according to the host plants. AMOVA showed that most of the total genetic variation is found within populations (56.70%), but a significant portion of the variation is found between crops (22.73%). The present study showed that the B biotype is disseminated throughout the sampled areas, infesting several host plants and predominates over the A biotype.

Key words: whitefly, molecular markers, UPGMA, AMOVA.

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Introduction

Bemisia tabaci (Genn.) (Hemiptera: Aleyrodidae), known as the California strain, cotton strain, A-strain and A biotype (Perring, 1995), has been detected in Brazil since 1928 (Bondar, 1929). Before 1990, this species was considered an occasional pest and a vector of phytovirus in beans, soybeans and tomato (Costa *et al*., 1977). The Brazilian *B. tabaci* population also known as the *B. tabaci* biotype BR (Lima *et al*., 2000) occurs on several native and cultivated host plants and is thought to be widely distributed in the country. This biotype may represent an indigenous biotype of *B. tabaci* as it does not feed on cassava, contrary to A biotype and is a vector of only three geminiviruses: the bean golden mosaic virus, the bean dwarf mosaic virus and the tomato golden mosaic virus.

In the last three decades, a new biotype has colonized the Americas, causing heavy losses in different agroecosystems. This new biotype was first detected in poinsettia in Florida (USA) and received the common names of Florida strain or poinsettia strain. It has also been known as B-strain, B biotype or type B, to distinguish it from the indigenous species presented in the United States (A biotype). Results of allozyme and RAPD analyses, insecticide applications, and mating studies have led some authors to conclude that A and B biotypes differ genetically and should be considered as separate species (Wool *et al*., 1993; Perring *et al*., 1993; Bellows *et al*., 1994). The name, *Bemisia argentifolii* (Bellows *et al*., 1994) and the common name of silverleaf whitefly (Perring *et al*., 1993) have been suggested for this biotype. This distinction is not widely accepted (De Barro, 1995; Williams *et al*., 1996) and the taxonomic classification of *Bemisia* is still controversial. In

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Australia, the presence of hybrids derived from crosses between the silverleaf whitefly and the indigenous whitefly in cotton crops suggests that *B. tabaci* may be a species-complex undergoing evolutionary change (Drost *et al*., 1998; Franzmann *et al*., 2000).

The B biotype is biologically distinct from the A biotype (Costa *et al*., 1993). The B biotype is more fecund than the A, produces greater amounts of honeydew, has a broader host plant range, exhibits significantly higher levels of insecticide resistance and induces several different phytotoxic disorders in certain plant hosts (Costa and Brown, 1991; Liu *et al*., 1992; Anthony *et al*., 1995). However, morphological markers cannot reliably distinguish these two biotypes.

In Brazil, *B. tabaci* biotype B was introduced in the early 1990s, probably through the international trade of ornamental plants (Lourenção and Nagai, 1994). It was first detected in the state of São Paulo. In the last three years it has spread to all regions of the country and has been estimated to cause losses greater than US$ 2.0 billion dollars (Oliveira and Faria, 2000). The main crops attacked are melon, watermelon, cotton, beans, soybeans, okra, tomato, and cabbage. Insecticides have been used weekly on crops in some areas to control the insect populations. The ability to colonize over 600 different plant species and vector over 60 different plant viruses is cause for great concern within agricultural regions of the world (Secker *et al*., 1998).

Knowledge of the genetic variation within whitefly populations is necessary for their efficient control and management. Random Amplified Polymorphic DNA (RAPD) markers provide a powerful tool for the investigation of genetic variation. RAPD does not require prior genomic information, and is simpler, less costly, and less labor intensive than other DNA marker methodologies. In *Bemisia*, RAPD markers have been used for analysis of genetic variation (Gawel and Bartlett, 1993; De Barro and Driver, 1997; Guirao *et al*., 1997; Moya *et al.,* 2001), for taxonomic studies (Perring *et al*., 1993), biotype identification and geographical distribution (Lima *et al*., 2000; Martinez *et al*., 2000).

In the past four years, whitefly samples have been collected on native and cultivated plants from several geographical locations in Brazil (Lima *et al*., 2000). No assessed crop was found to be exclusively attacked by biotype BR of *B. tabaci.* Rather, the survey has shown the dominance and broad host range of the B biotype in this country.

In spite of this, little is known about the genetic variability of the Brazilian populations. The main objectives of the present study were to use RAPD markers to (1) estimate the genetic diversity of *B. tabaci* populations, (2) study the genetic relationships among *B. tabaci* biotypes and two other whitefly species, and (3) discriminate between *B. tabaci* biotypes.

Material and Methods

Insect populations

Adult whiteflies were collected from host plants using a hand-held aspirator and preserved immediately in 100% ethanol. Samples were maintained in a refrigerator, at 4 °C, until DNA extraction (maximum period of 15 days). A total of 109 *B. tabaci* biotype B individuals were collected in 12 localities in seven Brazilian States (Figure 1) and on seven distinct host plants (Table I). All strains used in this study were first identified as *B. tabaci*, based on the morphology of the pupal stage and RAPD patterns. We also included an indigenous biotype from Brazil (*B. tabaci* biotype BR), biotype A from Arizona (USA), biotype B from California (USA) and Paraguay, and two other whitefly species: *Trialeurodes vaporariorum* (Westwood) and *Aleurodicus cocois* (Curtis).

DNA extraction

A single step DNA extraction method was used according to a protocol described originally by Aljanabi *et al*. (1998) with some modifications. Each individual female whitefly was homogenized in a 1.5 mL microcentrifuge tube with 60 µL of lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.30% Triton X-100, 60 µg/ mL proteinase K). The homogenate was then incubated at 65 °C for 15 min. Samples were boiled for six minutes to inactivate the proteinase K and stored at -20 °C.

Figure 1 - Map of Brazil indicating the localities where *Bemisia* individuals were collected. Map numbers correspond to the populations indicated in Table I.

Table I - Host plants, sites where whitefly populations were collected, *B. tabaci* biotypes (or insect species) population codes used in the analyses and number of individuals analyzed per population. Numbers following the localities correspond to the numbers indicated in the map (Figure 1).

Host plant	Locality - State	Biotype or species	Population code	Number of individuals
Cabbage	Goianira, Goiás (GO) - 1	B	CBGO	10
	Tianguá, Ceará (CE) - 2	B	CBCE	10
Cotton	Guanambi, Bahia (BA) - 3	B	CTBA	10
	Campina Grande, Paraíba (PB) - 4	B	CTPB	10
Grape	Campinas, São Paulo (SP) - 5	B	GRSP	4
Melon	Aracati, Ceará (CE) - 6	B	MECE	10
	Boa Vista, Roraima (RR) - 7	B	MERR	10
Melon	California - USA	B	USAB	5
Soybean	Miguelópolis, São Paulo (SP) - 8	B	SBSP	10
	Guaiuba, Ceará (CE) - 9	B	SBCE	10
Squash	Vassouras, Rio de Janeiro (RJ) - 10	B	SQRJ	10
	Tabuleiro do Norte, Ceará (CE) - 11	B	SQCE	10
Tomato	Paraguay	B	TOPY	5
Tomato	Pacoti, Ceará (CE) - 12	B	TOCE	5
Melon	Arizona - USA	A	USAA	5
Weed	Rondonópolis, Mato Grosso (MT) - 13	BR	Weed	5
Cashew	Mossoró, Rio Grande do Norte (RN) - 14	*Aleurodicus cocois*	AleRN	5
Melon	Brasília, Distrito Federal (DF) - 15	*Trialeurodes vaporariorum*	TriDF	5

RAPD assays

RAPD analyses were carried out according to Aljanabi *et al*. (1998) with some modifications. Amplification reactions were performed in a 30 µL reaction mix, containing a final concentration of 6.0 mM Tris-HCl pH 8.8, 0.2 µM dNTPs, 0.4 µM of 10-base primer (Operon Technologies Inc.), 2.5 U of *Taq* DNA polymerase and 10-15 ng DNA (usually 4 µL), overlaid with a drop of mineral oil to prevent evaporation. Amplifications were performed in a PTC-100 thermal cycler (MJ Research). Reaction products were then analyzed by 1.5% agarose gel electrophoresis. Gels were photographed under UV following ethidium bromide staining. A negative control, without DNA, was included in all reactions. Some standard procedures were adopted to ensure the reproducibility of the results: the use of the same termocycler, *Taq* DNA polymerase from the same producer, the same amount of all the mix components in all the experiments; the inclusion of only strong bands in the analysis and the inclusion of standard DNA samples.

Data analysis

PCR amplification products of the 139 samples were scored as presence (1) or absence (0) of bands. The data matrix was used to calculate Jaccard's similarity coefficient (Sneath and Sokal, 1973) which does not consider the joint absence of a marker as an indication of similarity. A dendrogram was constructed using the unweighted pair-group method analysis (UPGMA). The frequencies of the RAPD fragments were estimated for each of the 15 *Bemisia* populations and for the two other whitefly species. The resulting matrix was used to calculate Manhattan distances between all pairs of populations or species. These analyses were performed using NTSYS-pc software, version 2.0 (Rohlf, 1993).

Analysis of Molecular Variance (AMOVA) (Excoffier *et al*., 1992) was used to analyze the partition of the total genetic variation between the sampled crops, between and within Brazilian biotype B populations. A total of 41 RAPD markers (with less than 5% of missing data) was used for this analysis, performed using Arlequin software, version 1.1 (Schneider *et al*., 1997).

Results and Discussion

Five of the twenty-seven primers screened produced clear bands on the RAPD amplifications and were used for analysis. These primers (OPA-02, OPA-04, OPA-05, OPA-10, and OPA-13) amplified a total of 72 markers. The total number of clear bands obtained from each primer ranged from 4 (OPA-02) to 20 (OPA-10), with an average of 14.4 bands per primer. Forty bands (55.5 %) out of 72 were polymorphic for B biotype populations (8.0 polymorphic markers per primer). The size of amplification products ranged from 300 bp to 1500 bp. An example of RAPD amplification patterns is shown in Figure 2. All primers produced identical RAPD patterns for the selected bands in the primer screening step and in the final analysis, and in the several replicated experiments that were done.

Genetic relationships between populations are shown in Table II. Considering only the B biotype populations, the average genetic distances were 0.201. The smallest average

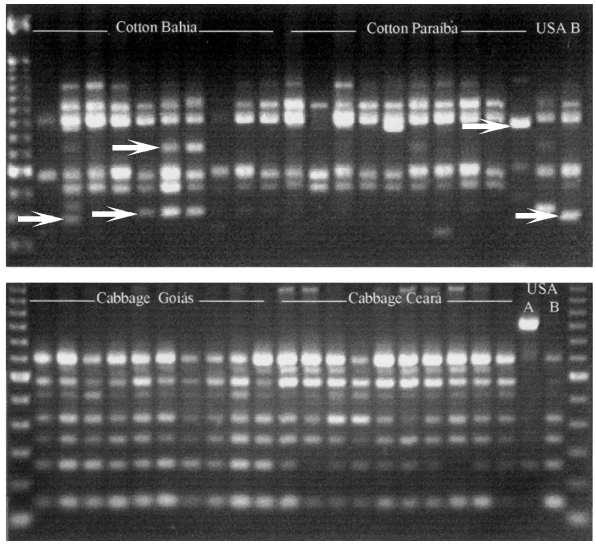

Figure 2 - Example of RAPD patterns generated by primers OPA-05 (A) and OPA-13 (B). Arrows indicate the polymorphic fragments used in the analysis. Lanes 1 in panel A and lanes 1 and 24 in panel B are 100 bp DNA ladder.

distance (0.08) was between populations TOPY (tomato from Paraguay) and GRSP (grape from Campinas, São Paulo) while the highest (0.31) was between TOCE (tomato from Pacoti, Ceará) and SBSP (soybean from Miguelópolis, São Paulo). In general, higher distances were observed between biotype B populations and the other two biotypes (A and BR) and species (*T. vaporariorum* and *A. cocois*), with values ranging from 0.29 to 0.57.

An UPGMA dendrogram based on Jaccard's similarity coefficient was constructed for the 139 samples analyzed (Figure 3). Four major groups were evident. The first group contained all populations of *B. tabaci* biotype B from different localities. This group is basically divided into four subgroups. The first subgroup consists of individuals collected on soybean from Ceará, the second includes cabbage, cotton, and squash from different localities and soybean from São Paulo, the third group consists of melon from Ceará and Roraima States, and the fourth subgroup includes grape and tomato populations and B biotype samples from the USA. The second major group included populations of *B. tabaci* biotype A and BR collected from populations in Arizona, the USA and Brazil, respectively. The third and the fourth major groups contain the two other whitefly species: *T. vaporariorum* and *A. cocois*.

Cluster analysis showed that, in general, biotype B individuals are scattered independently in the localities where the samples were collected, especially samples from the CTBA (cotton, Bahia), CTPB (cotton, Paraíba), and SBSP (soybean, São Paulo) populations. Nevertheless, some clusters were evident, joining individuals according to the host plants. This result suggests that a differentiation of populations has already occurred, mainly according to the host plant, instead of the geographical region where populations are localized. No grouping of samples collected on different crops in the same State was obtained. Individuals collected on three different crops (tomato, melon, and squash) and from the same state (Ceará), for example, did not cluster according to the locality, but to the crop (Figure 3). But individuals collected in two very distant States (Ceará and Roraima) (Figure 1), but on the same crop (melon), were joined in the dendrogram. Similar results were obtained for populations collected on cotton crops in Paraíba and Bahia States and on squash from Ceará and Rio de Janeiro. Results of the AMOVA partitioning of genetic variation of *B. tabaci* populations (Table III) corroborated these findings, by showing that a considerable proportion of the total genetic variation (22.73%) was found between crops ($p < 0.00293$). AMOVA also showed that 20.56% ($p < 0.00001$) of the total genetic variation was found between populations and 56.70%, within populations. These results indicated that some differentiation of Brazilian B biotype populations has occurred, despite their recent introduction to Brazil. The cluster analyses of the B biotype populations showed the heterogeneous set of groups and

Table II - Manhatan's distance matrix between *B. tabaci* populations and two other whitefly species. Population codes are listed in Table I.

	CBGO	CBCE	CTBA	CTPB	SBSP	SBCE	MECE	MERR	SQRJ	SQCE	GRSP	TOPY	USAA	USAB	TOCE	Weed	TriDF
CBCE	0.140																
CTBA	0.120	0.120															
CTPB	0.160	0.150	0.100														
SBSP	0.120	0.130	0.150	0.120													
SBCE	0.170	0.180	0.180	0.220	0.190												
MECE	0.240	0.220	0.230	0.210	0.230	0.260											
MERR	0.230	0.210	0.200	0.190	0.220	0.240	0.110										
SQRJ	0.160	0.160	0.180	0.170	0.140	0.200	0.200	0.200									
SQCE	0.150	0.130	0.170	0.150	0.100	0.220	0.240	0.230	0.140								
GRSP	0.250	0.170	0.220	0.220	0.240	0.210	0.200	0.200	0.180	0.260							
TOPY	0.260	0.210	0.240	0.240	0.260	0.240	0.210	0.200	0.230	0.270	0.080						
USAA	0.520	0.520	0.540	0.530	0.570	0.520	0.490	0.460	0.520	0.560	0.430	0.440					
USAB	0.270	0.210	0.240	0.250	0.280	0.270	0.240	0.210	0.250	0.290	0.120	0.090	0.420				
TOCE	0.280	0.220	0.240	0.270	0.310	0.290	0.250	0.220	0.250	0.290	0.110	0.130	0.400	0.080			
Weed	0.480	0.490	0.520	0.520	0.510	0.470	0.510	0.460	0.510	0.530	0.390	0.390	0.150	0.380	0.380		
TriDF	0.450	0.430	0.450	0.490	0.480	0.410	0.480	0.450	0.450	0.510	0.330	0.360	0.290	0.300	0.290	0.220	
AleRN	0.500	0.480	0.500	0.510	0.530	0.440	0.500	0.460	0.510	0.530	0.370	0.410	0.310	0.390	0.380	0.290	0.280

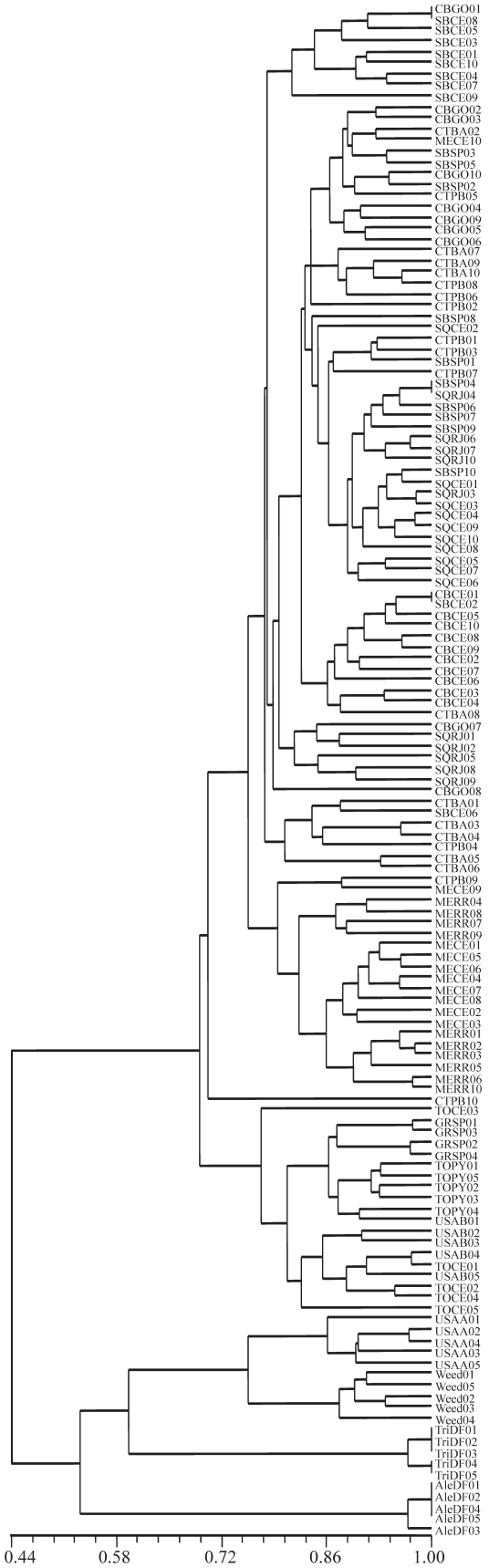

Figure 3 - Dendrogram for the 139 whitefly samples, generated by UPGMA. Population codes are listed in Table I.

sub-groups, probably due to differences between several nutritional and behavioral factors. The differentiation observed could also be explained by the introduction of B biotype populations in Brazil from several rather than a single founder population (Lourenção and Nagai, 1994), by the massive and crop-specific insecticide applications in these populations that could have affected the allele frequencies in different crops or by possible crossings between biotype B individuals and indigenous biotype individuals.

Genetic variation was detected in all the populations analyzed. Considering only B biotype populations, an average Jaccard similarity of 0.73 was observed between all individuals analyzed. Moreover, UPGMA showed the clustering of individuals by host crops instead of geographical region. These results suggest that management methods established for a host crop in one region would probably function for the same crop in other regions.

Individuals were randomly collected from 10 different populations in Brazil and morphologically identified as *Bemisia* sp. Samples of A and B biotypes from California (A) and Arizona (B) were included in the analysis as standards. RAPD analysis showed that the great majority of the populations analyzed were the B biotype instead of the A or BR biotypes and that the B biotype is disseminated throughout the sampled areas, even in localities or host plants, such as soybeans and cotton, where the indigenous biotype predominated previously (Lima *et al*., 2000). Effective monitoring of the spread of *B. tabaci* in Brazil and the ability to reliably distinguish non-B biotypes from the more important B biotype of this pest will assist in the forecasting of potential losses and improve the prospects for adoption of appropriate management strategies.

A surprising result produced by the cluster analysis (Figure 3) was the grouping of *T. vaporariorum* and *A. coccois* populations with *B. tabaci* biotypes A and BR. This indicates that these two whiteflies, from other genera, are more closely related to *B. tabaci* biotypes A and BR than to biotype B. All primers used in this study produced RAPD patterns that clearly distinguished biotypes A (USA) or BR (Brazil) from biotype B or any other distinct whitefly species analyzed. Some of these primers are routinely used to differentiate A and B biotypes (Lima *et al*., 2000). This fact may have lead to an overestimation of the genetic distances between the two biotypes. In spite of this, genetic distances between populations of A and B biotypes are considerably higher than distances between B biotype populations. These results give support to the preliminary studies based on allozymes (Perring *et al*., 1992; Bellows *et al*., 1994), RAPD, crossing experiments and mating behavior studies (Perring *et al*., 1993) that had led the authors to conclude that *B. tabaci* biotypes A and B could be different species. Although our data are insufficient to support the raising of any biotype to new species status, the fact remains that *B. tabaci* biotypes A and B are not the same genetically.

Table III - AMOVA of 109 *Bemisia* biotype B individuals collected in 12 populations in Brazil.

Source of variation	Degrees of freedom	Sum of squares	Variance components	% of variation	P-value
Between host crops	6	51.48	0.33	22.73	0.00293
Within host crops					
Between populations	6	21.45	0.30	20.56	0.00001
Within populations	101	83.50	0.83	56.70	0.00001

Recent phylogenetic studies using more effective markers, such as mitochondrial DNA (Frohlich *et al*., 1999; Kirk *et al.,* 2000) and ribosomal DNA markers (De Barro *et al*., 2000), showed that the renaming of the B biotype to *B. argentifolii* is premature. These studies suggest that the best way to view *B. tabaci* is as a complex containing geographically distinct populations that exhibit variation across a number of traits. More studies are necessary concerning molecular sequences (mitochondrial and nuclear genomes), host range phenotypes and mating compatibility among biotypes and sub-groups of *B. tabaci* to elucidate this controversy.

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