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Short Communication

Diversity analysis of *Bemisia tabaci* biotypes: RAPD, PCR-RFLP and sequencing of the ITS1 rDNA region

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Abstract

The *Bemisia tabaci* complex is formed by approximately 41 biotypes, two of which (B and BR) occur in Brazil. In this work we aimed at obtaining genetic markers to assess the genetic diversity of the different biotypes. In order to do that we analyzed *Bemisia tabaci* biotypes B, BR, Q and Cassava using molecular techniques including RAPD, PCR-RFLP and sequencing of the ITS1 rDNA region. The analyses revealed a high similarity between the individuals of the B and Q biotypes, which could be distinguished from the BR individuals. A phylogenetic tree based on ITS1 rDNA sequence was constructed. This is the first report of the ITS1 rDNA sequence of *Bemisia tuberculata* and of the BR biotype of *B. tabaci*.

Key words: whitefly, ITS rDNA, genetic diversity, phylogenetic analysis.

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Bemisia tabaci (Gennadius) (Hemiptera, Aleyrodidae) is considered one of the most destructive pests in agriculture, causing severe losses in crops around the world, including tropical and subtropical regions. The *B. tabaci* complex is formed by approximately 41 morphologically indistinguishable biotypes (De Barro *et al.*, 2005).

Brazilian populations of *B. tabaci* were first reported in Bahia in 1929 and by the early 1960s they had become a pest of important cultures (Lima *et al.*, 2002). Further studies, using molecular analyses, were performed and the Brazilian population was designated the BR biotype (Lima *et al.*, 2000). The B biotype was first detected in the USA in the beginning of the 1980's (Costa and Brown, 1990, 1991) and it was introduced in Brazil in the beginning of the 1990's (Lourenção and Nagai, 1994). In the last few years, this biotype has spread to several states in Brazil, causing severe losses (Lima *et al.*, 2002). Another biotype which is attracting an increasing interest worldwide is the Q biotype. This biotype is exotic to Brazil and is of extreme importance due to its similarity to the B biotype. These biotypes coexist in some areas around the world (Moya *et al.*, 2001), which raises some interesting questions regarding their biological and phylogenetic relationships and the possibility of viable hybrids.

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Due to limitations in morphological traits for the identification of the diverse biotypes of *B. tabaci*, the classification of the populations of *B. tabaci* complex is still controversial and molecular techniques have been employed. Initially, esterase locus polymorphisms were used as molecular markers (Brown *et al.*, 1995) and more recently DNA-based techniques such as DNA:DNA hybridization and PCR provided new prospects (Khasdan *et al.*, 2005). RAPD-PCR analysis has been considered a useful technique for the distinction of closely related populations, especially to detect inter and intraspecific differences among *Bemisia* spp. (Gawell and Bartlett, 1993; Perring *et al.*, 1993; De Barro and Driver, 1997; Khasdan *et al.*, 2005). Other techniques, such as AFLP and RFLP, have also been used to successfully distinguish populations of *B. tabaci* (Cervera *et al.*, 2000). Analyses of the conserved ribosomal and mitochondrial DNA have also been used to infer phylogenetic relationships among *B. tabaci* biotypes (De Barro *et al.*, 2000; Abdullahi *et al.*, 2004).

Studies of the native Brazilian *B. tabaci* biotype (BR) are scarce and only a few molecular analyses have been performed (Lima *et al.*, 2002). In the present study, we evaluated the genetic diversity of exotic and native Brazilian biotypes in an attempt to establish methods to help to control the arrival of new biotypes.

Seven different populations of *Bemisia tabaci*, representing the B, BR, Q and Cassava biotypes were analyzed (Table 1). The BR population was pre-classified based on

Table 1 - *Bemisia tabaci* populations used in this study.

Biotype/species	N	Origin	Culture	Identification code/individual	Accession number
B	5/5/1	USA, California	Melon	61/1	EF173281
B	4/5/1	Brazil, Balsas, Maranhão	Soybean	140/1	EF173278
B	5/5/1	Brazil, Brasília, Distrito Federal	Tomato	288/1	EF173280
Q	5/5/1	Morocco	Cucumber	7/1	EF173284
Cassava	4/5/2	Nigeria	Cassava	8/1 8/2	EF173282 EF173283
Q	5/5/1	Spain	Tomato	14/1	EF173279
BR	5/5/1	Brazil	Beans	45/1	EF173285
A	4/5/1	USA	Cotton	62	-
Bemisia tuberculata	0/0/1	Brazil, Vale do Ivinhema, Mato Grosso do Sul	Cassava	58	-
B*	-	USA, Arizona	Cotton	-	AJ 315821
B*	-	USA, Florida	Tomato	-	AF 216072
Q*	-	Spain	Cassava	-	AJ 315795
A*	-	USA, Arizona	Cotton	-	AJ 315796
A*	-	USA, Arizona	Cotton	-	AF 216068
Cassava*	-	Nigeria	Cassava	-	AJ 315819
Cassava*	-	Congo	Cassava	-	AJ 315809

N: Number of individuals used in RAPD/ PCR-RFLP/sequencing. *Sequences obtained from GenBank.

RAPD primers, as previously described by Lima *et al.* (2002). The A biotype (population code 62) was included in the RAPD analysis for comparison, since in previous studies this biotype was shown to be closely related to the BR biotype (Lima *et al.*, 2000). The Q and Cassava biotypes were provided by Dr. José Luis Caris (Murcia, Spain) and Dr. Ismail Abdullahi (University of Ibadan, Nigeria), respectively. For comparison purposes, one individual of *Bemisia tuberculata* (population code 58) was included in the sequencing analysis of the ITS1 rDNA region and was used as the outgroup in the phylogenetic analysis. The individuals of each population were named with the identification code followed by the numbers 1 through 5.

The populations were first sexed for the selection of female individuals and the DNA was extracted using the method described by Lima *et al.* (2000). Briefly, the samples were macerated in lysis buffer containing Tris-HCl 10 mM pH 8.0, EDTA 1 mM, Triton X-100 0,30%, proteinase K 60 $\mu g\ mL^{-1}$, and were incubated at 65 °C for 30 min and at 95 °C for 10 min. The samples were then stored at -20 °C until use.

For the RAPD-PCR analysis, a total of 33 individuals were used including four individuals of the biotypes B (140) and Cassava (8) and five individuals of the biotypes BR (45), B (61), B (288), Q (7) and Q (14). Four individuals of the biotype A (62) were also included for comparison. The amplifications were performed in a total volume of 30 µL containing 0.4 µM of primer, 0.2 mM dNTP, 1 U *Taq* DNA polymerase and 20 ng of DNA. The primers OPA04, OPA10, OPA11 and OPA15 (Operon Technologies, Inc.) were used in this study. The amplification reactions were performed with an initial denaturation at 94 °C for 3 min, followed by 45 cycles at 93 °C for 1 min, 35 °C for 1 min and 72 °C for 2 min. The RAPD amplification products were separated in 1.5% agarose gels in TBE 1X (Tris-borate 90 mM and EDTA 1 mM). The gels were stained with 5 $\mu g\ mL^{-1}$ ethidium bromide and the bands were visualized under UV using the EagleEye II still video system™ (Stratagene). The molecular marker Ladder 100 bp (Invitrogen) was used. At least three replicates were performed for each primer used.

The banding patterns observed were used to construct a dendrogram based on a similarity matrix obtained by the Jaccard's coefficient using the UPGMA (unweighted pair-group method analysis) program. The dendrogram was constructed with the NTSYS (Numerical Taxonomy and Multivariate Analysis System) program version 2.02 pc (Rohlf, 1993).

For the PCR-RFLP analysis of the ITS1 rDNA region, five individuals of each population of the biotypes B (61, 140 and 288), BR (45), Q (7 and 14) and Cassava (8) were used. The amplification of the ITS1 rDNA region was performed using the primers TW81 (Brust *et al.*, 1998) and 5.8R (De Barro *et al.*, 2000) as described by De Barro *et al.* (2003), with some modifications. The final volume of the reaction was 30 µL containing 2 µL of DMSO, $MgCl_2$ 50 mM, 5 µL of each primer TW81 and 5.8R (2 mM), dNTP 10 mM and 1U of *Taq* Polymerase (Pharmacia). The amplifications were performed with an initial denaturation at 94 °C for 5 min followed by the addition of *Taq* Polymer-

ase. The samples were incubated at 72 °C for 5 min and denatured at 95 °C for 3 min. A 30 cycle amplification was performed with an initial denaturation at 95 °C for 30 s, annealing at 54 °C for 1 min and extension at 72 °C for 1 min. The amplification product was electrophoresed in a 1.5% agarose gel and visualized after ethidium bromide staining (0,5 μL mL^{-1}). Ladder 100 pb (Invitrogen) was used as a molecular marker.

The amplified ITS1 rDNA region was digested with the restriction enzymes *Xho*I, *Bam*HI, *Eco*RI, *Pst*I, *Ava*I, *Dde*I and *Sau*3A. The digestions were performed according to the manufacturers instructions. The fragments obtained were separated by electrophoresis in a 2% agarose gel and visualized after ethidium bromide staining (0,5 μL mL^{-1}). Bands under 100 bp were not considered in the analyses.

The ITS1 rDNA of individuals representing each population (61, 140, 288, 7, 8, 14 e 45) and profile obtained by PCR-RFLP (61/1, 140/1, 288/1, 7/1, 8/1, 8/2, 14/1, 45/1), as well as of one individual of *Bemisia tuberculata*, were cloned in pGem T-Easy (Promega) and sequenced in an automatic sequencer ABI Prism 3700 DNA Analyser (Applied Biosystems Inc.). Both strands were sequenced and the sequences were deposited in GenBank (Table 1). The alignment of the sequences was performed using the Clustal W program (Higgins *et al.*, 1994). A phylogenetic tree was constructed using the MEGA program version 3.0 (Kumar *et al.*, 2004), with the p-distance and the Neighbor-joining method. Bootstrap analysis was performed with 250 replications. Besides the sequences determined in this study, sequences of the ITS1 rDNA region of some biotypes available in GenBank were also included for comparison (Table 1). The *B. tuberculata* sequence obtained in this study was used as the outgroup.

The analysis of the amplicons generated by the RAPD reactions revealed that the biotypes of *B. tabaci* produced different molecular patterns (Figure 1A). The banding patterns of the A and BR biotypes were very similar, with several common bands when the four RAPD primers were used. These results agree with previous studies reported by Lima *et al.* (2002). When using the primer OPA04, for example, it was observed that the A and BR biotypes had a common fragment of 950 pb. However, the presence of an additional 800 pb RAPD fragment allowed

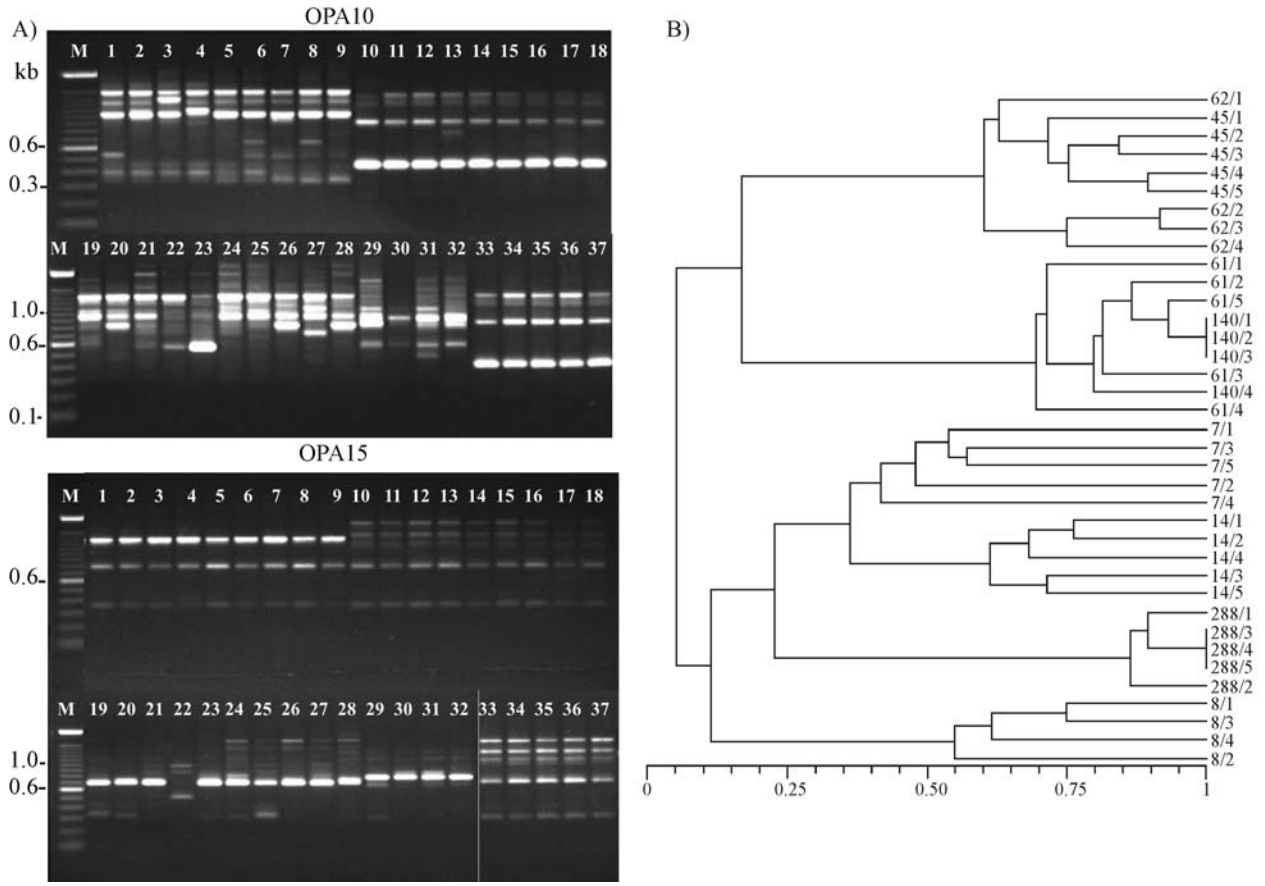

Figure 1 - A) RAPD amplicons produced by the OPA10 and OPA15 primers with DNA from different *Bemisia tabaci* biotypes. Numbers are: 1-4, A biotype (USA); 5-9, BR biotype (Brazil); 10-14, B biotype collected from melon (USA); 15-18, B biotype from soybean (Brazil); 19-23, Q biotype (Morocco); 24-28, Q biotype (Spain); 29-32, Cassava biotype (Nigeria); 33-37, B biotype collected from tomato (Brazil). M, ladder 100 bp. B) Dendrogram showing the similarity among the populations of the *B. tabaci* complex biotypes, constructed with RAPD data and obtained by a Jaccard's coefficient using UPGMA and NTSYS.

to discriminate the BR biotype. Moreover, the B and Q biotypes had a similar 700 pb fragment but two bands, one with 550 pb and another with 900 pb, were only present in the Q biotype. The amplified fragments can be further explored to develop specific primers which can be used as molecular markers for a rapid and accurate identification of *B. tabaci* biotypes.

Variation in the RAPD profiles was found among the B biotype from different hosts using the OPA04, OPA10 and OPA11 primers. A higher level of diversity was found within the populations of the Q and Cassava biotypes when the OPA10 and OPA11 primers were used. On the other hand, the OPA15 primer revealed a lower level of diversity among the *B. tabaci* populations.

The combined data sets obtained with the four primers were used to construct a dendrogram, which showed a high level of diversity among the *B. tabaci* biotypes and allowed the distinction of seven major clusters (Figure 1B). The first group (group I) was composed by the Brazilian biotype (BR) and the American biotype (A) populations. These populations showed 15% similarity in relation to group II, formed by the B biotype populations of soybean and melon. The third group (group III) was composed of Q biotype individuals from Morocco. Unexpectedly, one individual (7/4) formed a separate group (group IV) distantly related to the other Q biotype individuals from Morocco and presented a very distinct RAPD profile with the OPA15 primer. A high level of diversity has been previously reported in Q biotype populations (Moya *et al.*, 2001).

Group V was formed by Q biotype individuals from Spain. Interestingly, one B biotype population of *B. tabaci* (288) collected from tomato in Brazil (group VI) showed a higher similarity to Cassava and Q biotypes from Spain. Another intriguing result was that population 288 did not group with the other B biotype populations (61 and 140). It is possible that population 288 is older and had more time to differentiate from the originally introduced population from the USA. Some authors suggest that isolation is the key mechanism that leads to genetic diversity (De Barro *et al.*, 2005). Group VII was composed by the Cassava biotype of *B. tabaci*, which differs from the other biotypes in its host specificity (Abdullahi *et al.*, 2003, 2004).

Overall, the populations analyzed in this study were grouped according to their biotypes, showing that RAPD was useful to distinguish the biotypes that occur in Brazil as well as the exotic ones. This technique can be further explored in order to design specific primers to rapidly identify new biotypes, which can be very useful as an identification tool to be used for quarantine and phytosanitary purposes.

The analysis of the ITS rDNA region was performed in an attempt to obtain another method for the identification of *B. tabaci* biotypes. The ITS rDNA is a conserved region and is useful in distinguishing recently divergent taxa (De Barro *et al.*, 2003). The amplification of the ITS1 rDNA region of the *B. tabaci* populations revealed one band of approximately 550 bp for all the individuals analyzed. The amplification product was digested with seven restriction enzymes. No digestion was observed when the enzymes *Xho*I, *Bam*HI, *Eco*RI and *Pst*I were used and different banding patterns were observed after digestion with *Dde*I, *Ava*I and *Sau*3A (Figure 2A).

The individuals of the B biotype populations from the USA and Brazil, as well as those of the Q biotype populations from Morocco and Spain showed identical banding patterns with the three enzymes (Figure 2A). An intrapopulation variation was observed in the Cassava biotype from Nigeria (Figure 2A). One of the five analyzed individuals from this population (8/1) presented a banding pattern identical to that of the B and Q biotypes from Spain and Morocco, while the other four individuals revealed a unique profile for the three enzymes used. The ITS rDNA region of these four individuals did not present a restriction site for the *DdeI* enzyme. The native Brazilian BR biotype also showed the same restriction pattern observed for the B and Q biotype populations with the three enzymes used (Figure 2A).

For a more detailed analysis of the divergence level of the ITS1 rDNA region between the populations and biotypes, the sequence of the ITS1 rDNA of one individual representing the populations and groups obtained by the PCR-RFLP analysis was determined. Sequences varying between 542 and 555 bp were obtained and aligned using the Clustal W program (Higgins *et al.*, 1994). The alignment of the sequences revealed a high similarity between the individuals of the different biotypes which ranged between 100% and 93,8%. The individuals of the B biotype presented almost identical sequences, differing only in positions 219 and 414. The BR biotype presented approximately 95% similarity with the other biotypes, including biotype B, which is dominant in Brazil. The individual 8/2 of the Cassava biotype from Nigeria presented the most divergent ITS1 rDNA sequence. Unexpectedly, the sequences of the BR and Cassava (8/2) biotypes presented a six-base insertion at position 239 of the ITS rDNA sequence. This region may be used to design specific primers that distinguish these biotypes. The ITS rDNA region of one *B. tuberculata* individual (58/1) (EF173286) was also determined and showed low similarity with the sequences of *B. tabaci* biotypes (66% to 64%). Interestingly, the *B. tuberculata* ITS rDNA sequence presented a 17-base deletion at position 89 and this region can also be used to design specific primers to rapidly identify *B. tuberculata*.

A phylogenetic tree was constructed using the ITS1 rDNA sequences herein obtained and sequences of individuals of the A, Q and B biotypes available in GenBank (Figure 2B; Table 1). The phylogenetic tree presents three major groups: one formed by the individuals of biotypes B from Brazil and the USA and Q from Spain, Morocco and Cassava (individual 8/1); a second group formed by biotypes A and BR, and a third group composed of individuals

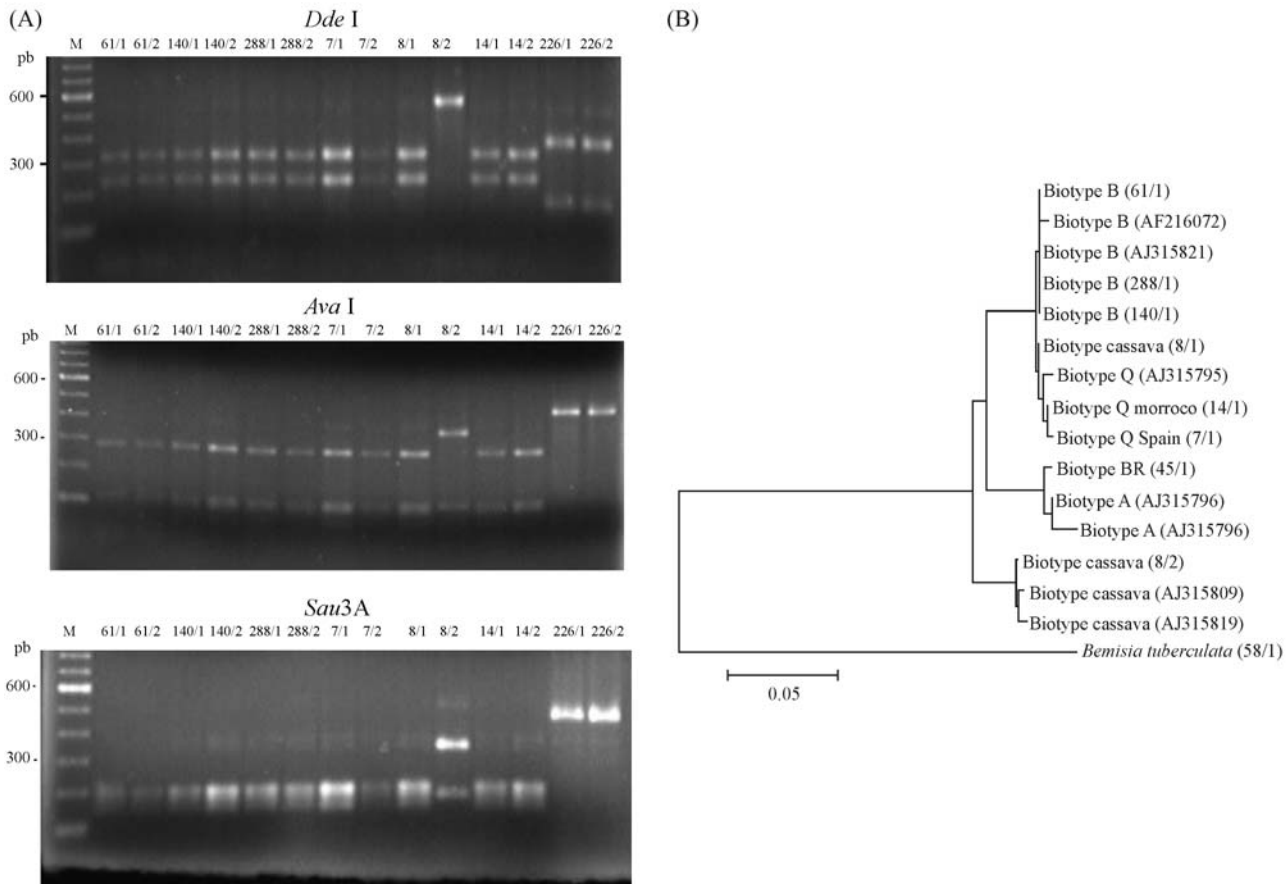

Figure 2 - A) Digestion of the *Bemisia tabaci* ITS1 rDNA region with the restriction enzymes *Dde* I (A), *Ava* I (B) and *Sau*3A (C). 61, B biotype collect from melon (USA); 140, B biotype from soybean (Brazil); 288, B biotype collected from tomato (Brazil); 7, Q biotype (Morocco); 8, Cassava biotype (Nigeria); 14, Q biotype (Spain). B) Phylogenetic tree of the different *Bemisia tabaci* biotypes and populations listed in Table 1, with the bootstrap values over the branches. The tree was constructed using the Neighbor-joining method with MEGA version 3.0. The bar indicates the distance value.

of the Cassava biotype from Nigeria (individual 8/2) (Figure 2B). Interestingly, the individual 8/1 of the Cassava biotype grouped with the Q biotype indicating that these biotypes possibly coexist and were therefore collected together from the same host plant. In general, the *bootstrap* values were high, mostly above 98%, indicating a high reliability in the obtained grouping. Similar results were observed by De Barro *et al.* (2000) when analyzing the ITS1 rDNA region of different biotypes. These authors reported that the populations of America (biotype A) formed a group closely related to the B biotype and to the biotypes from Northern Africa.

The results obtained herein showed that the analysis of the ITS1 rDNA region constitutes a valuable method, complementary to RAPD-PCR, that allows the inference of the relationships between species and biotypes of *B. tabaci*. Moreover, several differential bands detected in the RAPD-PCR and specific sequences of the ITS rDNA can be further explored to develop specific primers to rapidly and efficiently distinguish the *B. tabaci* biotypes. The use of specific primers represents an important tool as it may help to avoid the introduction of exotic biotypes of *B. tabaci* in Brazil. This is of interest because invasive alien species or biotypes may cause a severe economic, environmental and social impact in agricultural crops.

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