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Molecular differentiation of the B biotype from other biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae), based on internally transcribed spacer 1 sequences

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Abstract. *Bemisia tabaci* (Gennadius) is a worldwide pest of vegetable, ornamental and field crops. Biotype B of *B. tabaci*, which is economically most important of the biotypes, is distinct from all other biotypes (non-B biotypes). Fourteen populations of *B. tabaci* were collected from different localities and host plants in the Chinese mainland and Taiwan, namely TWYDH (tassel flower, Taiwan), HNYC (tobacco, Hainan), GXNG (pumpkin, Guangxi), GDYPH (poinsettia, Guangdong), GDBSM (croton, Guangdong), GDFS (Chinese hibiscus, Guangdong), SHYPH (poinsettia, Shanghai), FJGS (sweet potato, Fujian), SDFQ (tomato, Shandong), BJXHL (squash, Beijing), XJQZ (eggplant, Xinjiang), XJYPH (poinsettia, Xinjiang), XJJM (abutilon, Xinjiang) and XJMH (cotton, Xinjiang). The internally transcribed spacer 1 sequences (ITS1) of ribosomal DNA of B biotype and other biotypes were sequenced and analyzed. The B biotype-specific primers were then designed for rapid identification of B biotype of *B. tabaci*. The results show that the diagnostic primer only gave a positive result with the B biotype. This is the first report of a rapid means of identifying *B. tabaci* B biotype using a diagnostic primer based on ribosomal DNA. This protocol is especially useful for identifying the B biotype in *Bemisia* populations consisting of several biotypes.

INTRODUCTION

Bemisia tabaci (Gennadius), composed of numerous populations, has long been known as a relatively unimportant agricultural pest, but it is now a worldwide pest of vegetable, ornamental and field crops. *B. tabaci* is morphologically indistinguishable (Gill, 1990; Rosell et al., 1997) but varies considerably in its ability to transmit geminiviruses (Bedford et al., 1994), rate of development (Wang & Tsai, 1996) and ability to utilize different host plants (Brown & Bird, 1995). Biotype B (also known as *B. argentifolii* Bellows & Perring) is an extremely damaging pest of cotton and horticultural crops (Costa & Brown, 1991; Costa et al., 1993; De Barro & Driver, 1997). It is especially difficult to control as it is resistant to most of the insecticides used against whiteflies. Furthermore it has a wide host range, rapid rate of development and produces up to 300 eggs / female, which enable populations to increase rapidly in size (De Barro, 1995). It is the only biotype able to induce silverleaf in squash, a hallmark for identifying the B biotype (Costa & Brown, 1991; Costa et al., 1993; Brown et al., 1995).

It is very important to differentiate biotype B from other biotypes of *B. tabaci*, because of the biological traits and economic importance of this biotype. In the early 1990's, the economic impact of the "B" biotype prompted the development of diagnostic techniques for its rapid identification. The first diagnostic method used PAGE to produce non-specific esterase banding patterns, which were used to analyze many populations collected worldwide (Costa & Brown, 1991; Byrne et al., 1995). There are 20 biotypes that have been identified on the basis of these patterns (Banks & Markham, 2000) and the most recent published work on the *B. tabaci* species complex includes this criterion for identifying populations (Perring, 2001). The first DNA marker, RAPD-PCR, corroborated the esterase studies and simplified the experimental process for identifying biotypes (Mullis et al., 1986; Gawell & Bartlett, 1993). The later use of ALFPs produced similar results to RAPDs, and allowed the use of larger sample size in population analyses (Cabezas et al., 2000). The application of other genetic markers, such as the sequence of cytochrome oxydase I (COI) of mitochondrial DNA and the internal transcribed spacer of ribosomal DNA, provided an entirely new perspective of *B. tabaci* phylogeny (Frohlich et al., 1999; De Barro et al., 2000). Recently, microsatellite markers have provided new insights into the genetic structure of *Bemisia* populations (Tsagkarakou & Roditakis, 2003). While all the above markers have contributed to the genetic analysis of *Bemisia* populations, they are not suitable for rapid identification as they involve methods that are either time-consuming or inconvenient. We have located a diagnostic sequence deletion in ITS1 that may be used to differentiate biotype B from other biotypes (Wu et al., 2003) (Fig. 1, Table 1), but it would be a huge task if the complete ITS1 sequences are needed to identify *Bemisia* biotypes, because every population would have to be sequenced before the biotypes could be identified.

Molecular identification of different biotypes of *B. tabaci* based on diagnostic primers would be efficient and reliable if the PCR-based technique is reproducible, because the method is comparatively simple compared with those using other molecular markers but is thought to be unreliable. This study aims to establish a reliable protocol for rapid and easy differentiation of B-biotype from other biotypes of *B. tabaci*, which is especially efficient for analyzing samples from mixed populations of *Bemisia*.

MATERIAL AND METHODS

Insects

The whiteflies were collected from nine different locations and 12 different host plants (Table 2). The whitefly samples were morphologically identified by examining the fourth instars under a microscope (Bellows et al., 1994). Live insects were stored at –20°C, and some of them were preserved in 100% ethanol. Populations were assigned to the B biotype on the basis of their ability to cause silverleaf in squash (Costa & Brown, 1991) and a unique sequence deletion in their ITS1 sequences as indicated in Fig. 1.

TABLE 1. Citation of ITS1 sequences of B biotypes and other biotypes of *B. tabaci* (De Barro et al., 2000).

Clone	Isolate	SSL[a]	Biotype	GenBank acc. nos
Costarica8	Catie, Costa Rica	No	C	AF216013
Usaa6	USA	No	A	AF216067
Benin10	Benin	No	E	AF216000
SpainIpom2	Spain	No	S	AF216050
AusNarr1	Narrabri, Australia	No	Austra	AF215987
IndiaSth3	Gujarat, South India	No	H	AF216023
Nepal9	Nepal	No	P	AF216041
Pakistan4	Multan, Pakistan	No	K	AF216047
Nauru10	Nauru	No	Nauru	AF216036
IndiaNth2	Kerala, North India	No	G	AF216020
Turkey1	Turkey	No	M	AF216062
AusTown3	Townsville, Australia	Yes	B	AF215992
Brazil5	Brazil	Yes	B	AF216010
Usab7	Florida, USA	Yes	B	AF216072
Usab5	Florida, USA	Yes	B	AF216070
IsraelRes4	Israel	Yes	B	AF216029
IsraelSus1	Israel	Yes	B	AF216031
Iran4	Iran	Yes	B	AF216025

[a] SSL, squash silverleafing.

Chemicals

Tris aminomethane (Tris), Ethylenediamine tetraacetic acid (EDTA) and potassium chloride (KCl) were all purchased from Amersham (Uppsala, Sweden). Proteinase K, *Taq* polymerase, 100 bp ladder DNA marker I, dNTPs, DNA fragment quick purification kit and *p*GEM-T Vector were obtained from TW-Biotech (Beijing, China).

DNA extraction, PCR amplification, cloning and sequencing

Genomic DNA was extracted from single whitefly from different populations as listed in Table 2 and as described in Wu et al. (2003). The primers for amplification of ITS1 regions were forward-TW81: 5'-GTTTCCGTAGGTGAACCTGC-3' (Tm 59.6, GC% 55) and reverse – *B. tabaci* 5.8 R: 5'-ATCCGCGAGCCGAGTGATCC-3' (Tm 69.2, GC% 65). All reactions (25 μl) contained 2 μl DNA lysate, 1.5 U of *Taq* polymerase, 2.5 mM $MgCl_2$, 0.5 μl dNTPs (10 mM each), 7% dimethyl sulfoxide, 0.5 μl 25 μM primer and 10×PCR buffer supplied by the manufacturer. PCR reactions were carried out on a PTC-150 MiniCycler™ (MJ Research, Waltham, MA, USA) thermocycler (initially 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 57°C for 1 min 15 s, 72°C for 1 min 30 s and finally 72°C for 10 min).

PCR products were run on a 2% TAE agarose gel. The target DNA bands were excised from gels and were then purified using DNA Fragment Quick Purification Kit, which were ligated into *pGEM*-T Vector and transformed into *E. coli* DH5α. Positive clones were screened according to the standard protocols (Sambrook et al., 1989). Several positive clones on each LB plate were picked for sequencing in Sangon (Shanghai, China).

Sequence analysis, primer design and molecular identification

All the ITS1 sequences that were obtained by molecular cloning were aligned using ClustalW1.8 and the particular region that is specific to biotype B of *B. tabaci* identified. Several potential primer candidates were designed based on the general primer-designing principles, and then tested for their specificity and reproducibility. Only those primer pairs that produced unique PCR bands on agarose gels from biotype B were selected. All PCR reactions were carried out on the PTC-150 MiniCycler™ (MJ Research, Waltham, MA, USA) thermocycler (initially 94°C for 5 min followed by 35 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 45 s and finally 72°C for 10 min). The PCR products were separated by electrophoresis on 2% agarose and the images auto-analyzed on a TM-26 MultiImage™ Gel

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AF216013        GATCCGGAAGCG-TTAACGCCGCTCC-GGCCCGGTCGCATAGTCGCCGGGCCGGCGCGAG 233
AF216067        GATCCGGAAGCG-TTTACGCCGCTCC-GGCCCGGTCGCATAGTCGCCGGGCCGGCGCGAG 232
AF216000        GATCCGGAAGCGTTTAACGTCGCTCC-GGCCCGGTCGCGTCGTCGCCGGGCCGGCGCGAG 232
AF216050        GATCCGGAAGCGTT-AACGTCGCTCCCGGCCCGGTCGCGTCGTCGCCGGGCCGGCGCGAG 232
AF215987        GATCCGGAAGCGGTTAACGTAGCCCCGGCCCCGGTCGCATCGTCGCCGGGCCGGCGCGAG 234
AF216023        GATCCGGAAGCGGTTAACGTCGCTCC-GGCCCGGTCGCATCGTCGCCGGGCCGGCGCGAG 234
AF216041        GATCCGGAAGCGGTTAACGTCGCTCC-GGCCCGGTCGCATCGTCGCCGGGCCGGCGCGAG 234
AF216047        GATCCGGAAGCGGTTAACGTCGCTCC-GGCCCGGTCGCATCGTCGCCGGGCCGGCGCGAG 234
AF216036        GATCCGGAAGCGGTTAACGTCGCTCC-GCCCCGGTCGCATCGTCGCCGGGCCGGCGCGAG 235
AF216020        GATCCGGAAGATGTTAACGTCGCTCC-GGCCCGGTCGCATCGTCGCCGGCCGGGCGCGAG 233
AF216062        GATCCGGAAGCGGTTAACGTCGCTCC-GGCCCGGCGGCATCGTCGCCGGGCCGGCGCGAG 236
AF215992        GATCCGGAAGCG-CTAACGTCGCTAC-GGCCCGGTAGAAT------CGGGTCGGAGCGAG 228
AF216010        GATCCGGAAGCG-CTAACGTCGCTAC-GGCCCGGTAGAAT------CGGGTCGGAGCGAG 227
AF216072        GATCCGGAAGCG-CTAACGTCGCTAC-GGCCCGGTAGAAT------CGGGTCGGAGCGAG 227
AF216070        GATCCGGAAGCG-CTAACGTCGCTAC-GGCCCGGTAGAAT------CGGGTCGGAGCGAG 227
AF216029        GACCCGGAAGCG-CTAACGTCGCTAC-GGCCCGGTAGAAT------CGGGTTGGAGCGAG 227
AF216031        GATCCGGAAGCG-CTAACGTCGCTAC-GGCCCGGTACAAT------CGGGTCGGAGCGAG 227
AF216025        GATCCGGAAGCG-CTAACGTCGCTAC-GGCCCGGTAGAAT------CGGGTCGGAGCGAG 227
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Fig. 1 Alignment of partial ITS1 sequences published previously (De Barro et al., 2000), showing the B biotype-specific sequence deletion as indicated by the quadrangle. GenBank accession numbers listed in the left column are in the same order as those listed in Table 1.

TABLE 2. Host plants, SSL, where collected and date, and ITS1 sequences studied in this paper and registered in GenBank, of clones of *Bemisia*.

Clone	Host plants	SSL[a]	Collection Location	Collection time	No. of individuals[b]	GenBank acc. nos
FJGSa1	Sweet potato	non-B	Fuzhou, Fujian	Jul 2001	1	AF509595
FJGSa2	Sweet potato	non-B	Fuzhou, Fujian	Jul 2001	1	AF509596
GDBSMa3	croton	non-B	Guangzhou, Guangdong	Mar 2004	1	AY764369
GXNGa1	pumpkin	non-B	Nanning, Guangxi	Aug 2001	1	AF509593
XJQZb1	eggplant	B	Shihezi, Xinjiang	Oct 2001	2	AY764381
XJQZb3	eggplant	B	Shihezi, Xinjiang	Oct 2001	2	AY764382
XJYPHb1	poinsettia	B	Shihezi, Xinjiang	Oct 2001	3	AY764383
SDFQb1	tomato	B	Qingzhou, Shandong	Oct 2001	1	AF509594
GDFSb3	Chinese hibiscus	B	Guangzhou, Guangdong	Jan 2004	3	AY764371
GDFSb6	Chinese hibiscus	B	Guangzhou, Guangdong	Jan 2004	3	AY764372
GDFSb2	Chinese hibiscus	B	Guangzhou, Guangdong	Jan 2004	3	AY764370
XJMHb6	cotton	B	Shihezi, Xinjiang	Oct 2001	2	AY764380
XJYPHb2	poinsettia	B	Shihezi, Xinjiang	Oct 2001	3	AY764384
HNYCb2	tobacco	B	Sanya, Hainan	Feb 2002	1	AY764374
SHYPHb3	poinsettia	B	Shanghai	Oct 2001	1	AY764375
XJJMb1	abutilon	B	Shihezi, Xinjiang	Oct 2001	3	AY764376
XJJMb2	abutilon	B	Shihezi, Xinjiang	Oct 2001	3	AY764377
XJJMb3	abutilon	B	Shihezi, Xinjiang	Oct 2001	3	AY764378
XJYPHb3	poinsettia	B	Shihezi, Xinjiang	Oct 2001	3	AY764385
GDYPHb1	poinsettia	B	Guangzhou, Guangdong	Sept 2003	1	AY764373
XJMHb4	cotton	B	Shihezi, Xinjiang	Oct 2001	2	AY764379
BJXHLb1	squash	B	Beijing	Apr 2002	1	AF509592
TWYDHa2	tassel flower	non-B	Taibei, Taiwan	Oct 2001	1	AY764386

[a]B biotype was identified by its ability to cause silverleaf in squash;
[b]The number of individuals indicates the number of individuals that were sequenced. Each sequence originated from one individual.

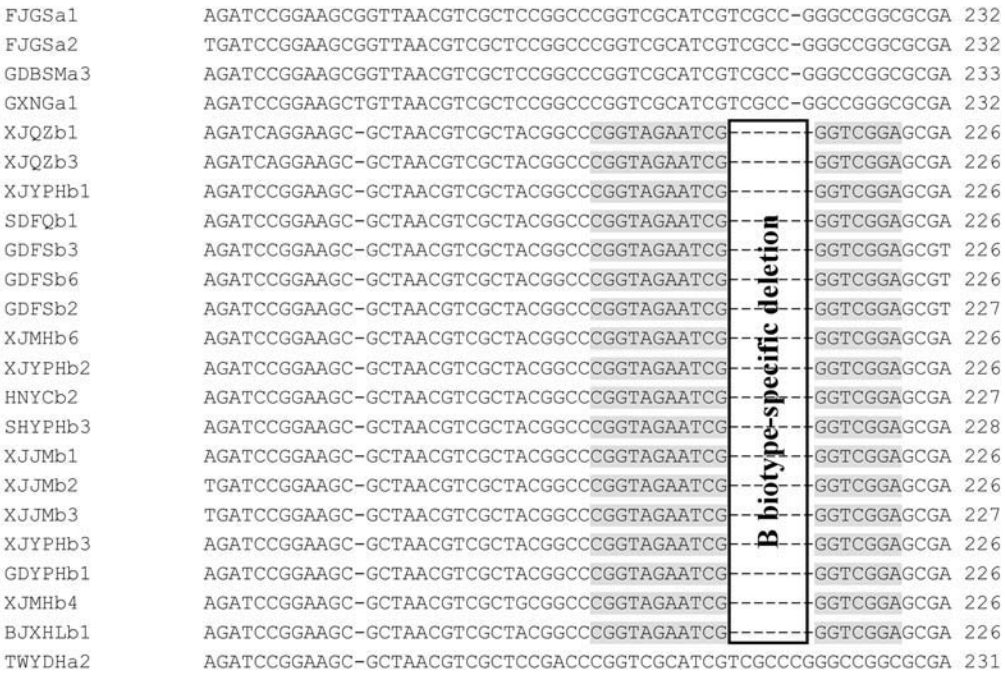

Fig. 2. Alignment of partial ITS1 sequences of *Bemisia* populations studied in this paper showing the B biotype-specific sequence deletion as indicated by the quadrangle. The shaded sequence covers the reverse primer, SSLr. The codes listed in the left column are in the same order as those listed in Table 2 and the ITS1 sequences are all retrievable from GenBank using the accession numbers shown in Table 2.

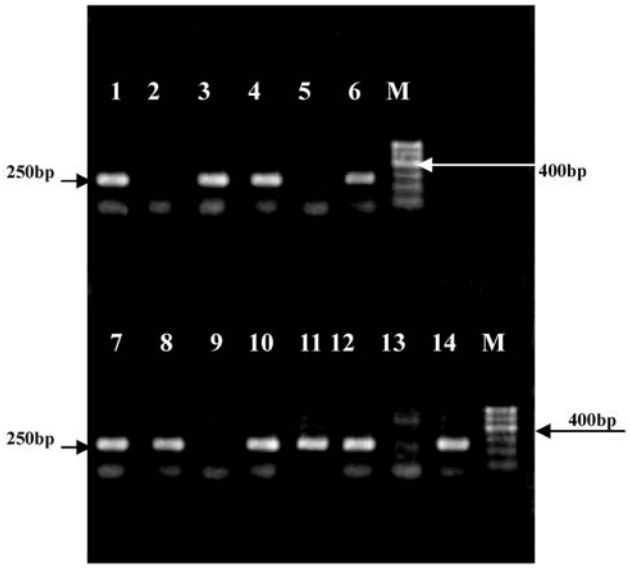

Fig. 3. Molecular differentiation of B biotype from other biotypes of *B. tabaci*. M: DNA molecular weight standard-DNA Marker I (100 bp Ladder); 1: HNYC (tobacco, Hainan); 2: GXNG (pumpkin, Guangxi); 3: GDYPH (poinsettia, Guangdong); 4: GDFS (Chinese hibiscus, Guangdong); 5: GDBSM (croton, Guangdong); 6: SHYPH (poinsettia, Shanghai); 7: SDFQ (tomato, Shandong); 8: BJXHL (squash, Beijing); 9: FJGS (sweet potato, Fujian); 10: XJQZ (eggplant, Xinjiang); 11: XJYPH (poinsettia, Xinjiang); 12: XJMH (cotton, Xinjiang); 13: TWYDH (tassel flower, Taiwan); 14: XJJM (abutilon, Xinjiang).

Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA).

RESULTS

ITS1 sequences, the region in ITS1 sequences specific to biotype B and diagnostic primers

All the ITS1s sequenced in this study were registered in GenBank (Table 2). Alignment of partial ITS1 sequences studied showed that the B-biotype has a specific sequence deletion (Fig. 2) and a reverse primer specific to B biotype was designed based on the unique sequence region shown as the shaded area in Fig. 2.

Molecular differentiation of B biotype from other biotypes

The primers used for checking whitefly samples were: sense-TW81: 5'-GTTTCCGTAGGTGAACCTGC-3' (Tm59.6, GC%55) and antisense-SSLr: 5'-TCCGACCCGATTCTAC CG-3' (Tm59.6, GC%61, product = 250 bp). Fourteen *Bemisia* samples collected from different locations and host plants were tested (Fig. 3). As shown in the figure, 10 samples belonging to B biotype tested positive using the B biotype-specific primer, while 4 samples tested negative, namely no specific PCR products were created by using this protocol.

DISCUSSION

The reliability of a molecular identification technique depends on the reproducibility of PCR reactions, which relies on the optimized reaction conditions, such as the suitability of the primers and thermocycling programs, especially the former. We have tried several different primer pairs, but only TW81 / SSLr produced specific PCR results. This is the first report of the rapid identification of *B. tabaci* biotype B using a diagnostic primer designed using ribosomal DNA.

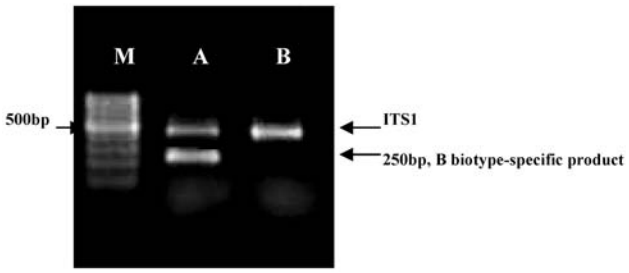

Fig. 4. Nested PCR checking of whitefly samples with two pairs of primers. M: DNA Marker I (100 bp Ladder); A: HNYC (tobacco, Hainan); B: GXNG (pumpkin, Guangxi).

The within-biotype sequence divergence in populations was very limited. The mean calculated ITS1 sequence divergence within B biotype is only 0.011 ± 0.002 (N = 25; Bootstrap = 500 replicates) based on the distance model Kimura 2-parameter (Kimura, 1980) of MEGA2.1, while the mean sequence difference for other biotypes is 0.041 ± 0.004 (N = 16; Bootstrap = 500 replicates). The larger ITS1 sequence difference for other biotypes may be due to their more complicated composition (Table 1). The narrow within-B biotype sequence difference and a B biotype-specific sequence deletion justify the use of a PCR-based protocol for the molecular identification of B biotype whiteflies. It must be noted that this method cannot differentiate subtypes in the non-B biotype group. However, the B biotype is the more important economically worldwide.

To increase the reliability of this PCR-based diagnostic technique, a so-called nested PCR method could be used. In this method, the primers for amplification of the complete sequence of ITS1 are used as a control and the B biotype-specific primers are then applied at the second step with ITS1 produced from the first primer pairs as a DNA template. The first primer sets test the integrity of the original DNA template. The gel image (Fig. 4) shows that the DNA template of B biotype (HNYC) and non-B biotype (GXNG) are both pure, which confirms that HNYC is diagnostic of B biotype in this case.

Molecular identification method is a convenient tool for the rapid and preliminary examination of whitefly populations, but does not replace the conventional approach to population ecology. This technique is especially useful for studying and monitoring mixed populations where biotype B and other biotypes co-exist. We did not test the diagnostic primers by using populations from the Mediterranean / North Africa region, but the primers were designed based on sequences that originated from populations from all over the world (Table 1). In fact, all biotypes other than the B biotype collected in China tested negatively when the B-specific primers were used. This technique is being used to monitor the dispersion and invasion of the B biotype in China mainland. A close relative, biotype Q is known to be present in China. Unfortunately, it was not found in the samples tested in this and our previous studies. So more *Bemisia* populations will be studied and compared in the future and the biology of other biotypes of the species complex will be studied. Our method may fail to distinguish the B biotype from some of its closest non-B relatives. If this is the case, other methods such as RAPD-PCR and even rDNA sequencing can be used to increase the reliability of the molecular identification.

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