Species Within the *Bemisia tabaci* (Hemiptera: Aleyrodidae) Complex in Soybean and Bean Crops in Argentina

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ABSTRACT The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a cryptic species complex that contains some of the most damaging pests in tropical and subtropical regions. Recent studies suggested that this complex is composed of at least 24 distinct species. We use the approach from these studies to consider the identity of *B. tabaci* in Argentina. Previous studies have suggested the presence of a *B. tabaci* presumably indigenous to the Americas and referred to as the BR biotype in Argentina. We placed the entity referred to as the BR biotype within the *B. tabaci* cryptic species complex using whiteflies collected in soybean and bean crops in northern and central Argentina. The whiteflies were assigned using the mitochondrial cytochrome oxidase (mtCOI) gene. Four unknown haplotypes plus two Argentina sequences from GenBank formed a cluster that was basal to the rest of the New World sequences. These sequences diverged from the consensus sequence across the range of 3.6 to 4.3%. Applying the species assignment rules of recent studies suggests that the individuals from Argentina form a separate species. A fifth unknown haplotype fell within the New World putative species and formed a distinct cluster with haplotypes from Panama. These results suggest that Argentina has two indigenous species belonging to the *B. tabaci* cryptic species complex. Rather than using mtCOI sequencing for all *B. tabaci* collected, a simple random amplified polymorphic DNA-polymerase chain reaction diagnostic was used and tested along with previously published primers designed to work specifically with the BR biotype from Brazil. These primers were either unable to distinguish between the two indigenous members of the complex in Argentina or indicated a difference when none was evident on the basis of mtCOI sequence comparison.

KEY WORDS whitefly, mitochondrial cytochrome oxidase I, phylogenetic analysis

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is a cryptic species complex (Dinsdale et al. 2010, De Barro et al. 2011) that contains some of the most damaging pests in tropical and subtropical regions (Byrne and Bellows 1991), reducing the yields of a wide range of agricultural and horticultural crops (Cahill et al. 1996). This pest causes losses through direct feeding (Berlinger 1986) and indirect damage through the excretion of honeydew and the subsequent growth of saprophytic sooty molds that reduce both quality and photosynthesis, and through the transmission of numerous plant viruses (Bedford et al. 1994, Brown et al. 1995, McGrath and Harrison 1995, Idris et al. 2001).

Since the 1950s, the existence of biotypes or host races of *B. tabaci* has been proposed to distinguish morphologically indistinguishable populations, which differed in biological traits with respect to host range, host plant adaptability, and plant virus transmission capabilities (Bird 1957, Bird and Maramorosch 1978, Brown et al. 1995). However, more recent work across a greater range of the known diversity in the complex has shown that these biological traits are unable to provide reliable delimitation (De Barro et al. 2011).

Since then numerous studies have used a range of protein and DNA molecular markers to study the underlying genetic structure and relationships within *B. tabaci* (De Barro et al. 2011). This has seen the proliferation in the number of biotypes identified (Perring 2001, De Barro et al. 2011). However, many of these studies used only small numbers of taxa that weakened the capacity to critically observe the genetic relationships between different members of the *B. Tabaci* complex. However, larger genetic studies encompassing hundreds of unique haplotypes (Boykin et al. 2007, Dinsdale et al. 2010) revealed a consistent structure and clearly identified the flaws in the biotype nomenclature (De Barro et al. 2011). Our most recent understanding of *B. tabaci* is that it is not composed of biotypes. Rather, Dinsdale et al. (2010) and De Barro et al. (2011) in conjunction with crossing studies (Xu et al. 2010; Elbaz et al. 2010; Wang et al. 2010, 2011; Sun et al. 2011) shows a strong agreement between the rules devised by Dinsdale et al. (2010) to assign individuals to putative species and

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either complete or incomplete mating isolation, the later leading to reduced fitness in the progeny. This approached suggested that *B. tabaci* was a cryptic species complex composed of at least 24 distinct putative species (hereon species) and the approach has since been used by Hu et al. (2011) to add a further four species to the complex. Therefore, we use this approach to consider the identity of *B. tabaci* in Argentina.

One member of the cryptic species complex, the putative species Middle East-Asia Minor one (commonly referred to in the literature as the B biotype and hereon MEAM1) has been spread globally through the trade in ornamental nursery plants (Cheek and MacDonald 1994). MEAM1 was first detected in Argentina on horticultural crops in the provinces of Jujuy, on weed in Buenos Aires and cotton samples collected in Chaco (Viscarret et al. 2003). Since then a *B. tabaci*, presumably indigenous to the Americas and referred to as the BR biotype was detected on weeds and soybeans in three Argentine provinces, Córdoba, Tucumán, and Salta (Truol et al. 2003). BR was first found in Brazil and has an electrophoretic profile similar to the A biotype from Arizona that is now known to belong to the New World putative species (Lima et al. 2000, Dinsdale et al. 2010). To date no mitochondrial cytochrome oxidase one (mtCOI) sequences of BR have been published so it has not been possible to consider this *B. tabaci* within a molecular phylogenetic context. Our study aims place the entity referred to as the BR biotype within the *B. tabaci* cryptic species complex using whiteflies collected in soybean and bean crops in northern and central Argentina.

Materials and Methods

Whitefly Collection. Whitefly sampling was conducted in February and March 2007. The study area was located between the latitudes 22° 42′ 24,12″ and 31° 28′ 5,52″ S and the longitudes 63° 36′ 7,92″ and 65° 36′ 56,16″ W. Individual *B. tabaci* were collected from fields (30 soybean and 11 bean fields) distributed in 26 localities across four Argentine provinces (Salta, Tucumán, Santiago del Estero, and Córdoba) (Fig. 1; Table 1). Adult whiteflies were collected from the underside of leaves selected at random across the field using a mouth aspirator. Up to thirty adults per field were collected. Adults were placed in 100% ethanol and maintained at 4°C until DNA extraction. When fourth instars were present these were also collected to enable morphological identification (Caballero 1996).

DNA Extraction. Adults were sexed and females were then used. DNA was extracted following the protocol of De Barro and Driver (1997) and Truol et al. (2003). In total, 80 females were analyzed. Two females were analyzed from each of the 41 fields where female whiteflies were collected, except for fields 16 and 19, where only one female was collected (Table 1). Females were individually homogenized in 60 μl filtered lysis buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.3% Triton X-100, 60 μg/ml proteinase K) using 1.5 ml microcentrifuge tubes. The homogenate was incubated at 65°C for 15 min and at 98°C for 6 min.

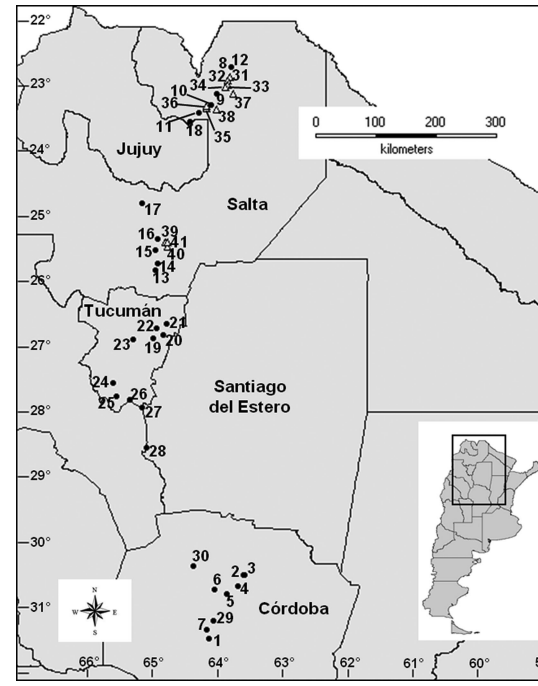

Fig. 1. Location of sites sampled for detection of species of *B. tabaci* complex in soybean and bean fields in Argentina (● = soybean field, △ = bean field).

Then it was centrifuged at 9,600 × *g* for 1 min and maintained at −20°C.

Whitefly Identification Using mtCOI. Two individuals from each of Argentine provinces: Tucumán (field 23, JF901841, JF901842) and Santiago del Estero (field 27, JF901843, JF901844); one individual from Córdoba (field 29, JF901838); and six individuals from Salta (field 10, JF901845, JF901846; 18, JF901839, JF901840; 35, JF901836; 36, JF901837) were used for the analysis of mtCOI.

DNA was extracted as described above. Amplifications used the primers C1-J-2195 and L2-N-3014 (Simon et al. 1994, Frohlich et al. 1999). Polymerase chain reaction (PCR) reactions were carried out in a final volume of 25 μl containing 1 U *Taq*DNA polymerase (Invitrogen, São Paulo, Brazil), 2.5 mM $MgCl_2$, 0.20 mM dNTP, 0.6 μM of each primer and 2 μl of DNA. The reactions were performed with a thermal cycler programmed for one cycle of 2 min at 94°C, followed by 30 cycles (1 min at 94°C, 1 min at 52°C, and 1 min at 72°C) and a final 5-min extension at 72°C. Amplification products were analyzed by 1.5% agarose gel electrophoresis. The amplified products were purified using illustra MicroSpin S-200 HR Columns (GE Healthcare, United Kingdom) and sequenced in both directions with the amplification primers using an ABI 3130XL (Applied Biosystems, Foster City, CA) automated sequencer. Those PCR products that could not be sequenced directly were cloned into pGEMT-easy (Promega, Madison, WI) and at least two clones were sequenced. The resultant product was 633 bp.

Table 1. Fields sampled for collection of *B. tabaci* adults in 26 localities across four Argentine provinces

Field no.	Crop	Province	Department	Locality	Biotype
1	Soybean	Córdoba	Capital	Córdoba	BR
2	Soybean	Córdoba	Tulumba	Las Arrias	BR
3	Soybean	Córdoba	Tulumba	Las Arrias	BR
4	Soybean	Córdoba	Totoral	Cañada de Luque	BR
5	Soybean	Córdoba	Totoral	Totoral	BR
6	Soybean	Córdoba	Totoral	Totoral	BR
7	Soybean	Córdoba	Capital	Córdoba	BR
8	Soybean	Salta	Gral. J. San Martín	Coronel Cornejo	BR
9	Soybean	Salta	Gral. J. San Martín	Embarcación	BR
10	Soybean	Salta	Gral. J. San Martín	Embarcación	BR
11	Soybean	Salta	Orán	Pichanal	BR
12	Soybean	Salta	Orán	Coronel Cornejo	BR
13	Soybean	Salta	R. de La Frontera	R. de La Frontera	BR
14	Soybean	Salta	R. de La Frontera	Horcones	BR
15	Soybean	Salta	Metán	Metán	BR
16	Soybean	Salta	Metán	Río Piedras	BR
17	Soybean	Salta	Capital	Salta	BR
18	Soybean	Salta	Oran	Urundel	BR
19	Soybean	Tucumán	Cruz Alta	Los Ralos	BR
20	Soybean	Tucumán	Cruz Alta	Las Cejas	BR
21	Soybean	Tucumán	Burruyacu	La Ramada	BR
22	Soybean	Tucumán	Burruyacu	La Ramada	BR
23	Soybean	Tucumán	Lules	Ing. San Pablo	BR
24	Soybean	Tucumán	Juan Bautista Alberdi	Villa Alberdi	BR
25	Soybean	Tucumán	La Cocha	La Cocha	BR
26	Soybean	Tucumán	Graneros	El Zapallar	BR
27	Soybean	Santiago del Estero	Guasayan	San Pedro	BR
28	Soybean	Santiago del Estero	Choya	Pozo de la Puerta	BR
29	Soybean	Córdoba	Tulumba	Tulumba	BR
30	Soybean	Córdoba	Colon	Dean Funes	BR
31	Bean	Salta	Gral. J San Martín	Ballivián	BR
32	Bean	Salta	Gral. J San Martín	Ballivián	BR
33	Bean	Salta	Gral. J San Martín	Ballivián	BR
34	Bean	Salta	Gral. J San Martín	Embarcación	BR
35	Bean	Salta	Orán	Pichanal	Not BR, not B
36	Bean	Salta	Orán	Pichanal	Not BR, not B
37	Bean	Salta	Gral. J San Martín	Misión Chaqueña	BR
38	Bean	Salta	Gral. J. San Martín	Embarcación	BR
39	Bean	Salta	Metán	Metán	BR
40	Bean	Salta	Metán	Metán	BR
41	Bean	Salta	Metán	Metán	BR

Data Analysis. Sequences were compared against the consensus sequences developed for each of the cryptic species from Dinsdale et al. (2010) and those from Hu et al. (2011). Dinsdale et al. (2010) showed that members of the *B. tabaci* species complex could be readily assigned to species by comparison against consensus sequences. They determined that an unknown sequence was a match with a consensus sequence if it diverged by <3.5%; if an unknown sequence diverged by >3.5% from any of the 24 consensus sequences then this was likely to a new putative species. In this case several of the unknown sequences did diverge by >3.5% from the closest match, putative species New World. The divergence range for these sequences was 3.6 to 4.3%. As a result a Bayesian phylogenetic analysis was undertaken using the MrBayes (version 3.1; Huelsenbeck et al. 2001). We partitioned the data using a codon-partition model in which each codon position was allowed its own parameter estimates. The best-fit model of evolution was determined by the Likelihood Ratio Test using Modeltest 3.6 (Posada and Crandall 1998). All partitions were allowed a GTR + invariants + gamma model and analyses were run for 10 million generations by using eight chains and sampling every 1,000 generations. The burn-in period (n = 785) was determined by comparing graphical output from the SUMP command and checking that the harmonic means of the separate runs had converged to within two units. Dinsdale et al. (2010) showed that the members of the *B. tabaci* complex from the Americas formed an extremely well defined monophyletic group. As we were interested only in the relationships between New World members of the complex, we did not include non-New World members of the complex. The subsequent consensus tree was visualized using Treeview (Page RDM 2001) and putative species names are those from in Dinsdale et al. (2010) except where a new putative species has been identified.

The following sequences available in GenBank, AF340212, AF340213 (Argentina); DQ130053 (Belize); AJ550167, AJ550168, EU427728 (Colombia); AY057128 (El Salvador); AY057129 (Guatemala); AF342770, AY057133 (Honduras); AY057126, DQ130058, DQ130059, EU427729 (Mexico); DQ130060, DQ130061 (Panama); AY057134 (Puerto Rico) and AY521259 (USA). As outgroups *B. afer* (Priesner & Hosny) (GQ139515, GU220055), *B. atriplex* (Frog-

gatt) (GU086362, GU086363), *B. berbericola* (Cockerell) (HQ457046), *B. emiliae* Corbett (DQ989555), *B. subdecipiens* Martin (GU220056), and *B. tuberculata* (Bondar) (AY057220) were used in the analysis along with the five unique haplotypes from the Argentina dataset (Table 1). All sequences were checked for duplicates, gaps, and pseudogenes. Two of the unknown sequences each had a single ambiguous base. All sequences were aligned using ClustalX (version 1.81; Thompson et al. 1994).

Random Amplified Polymorphic DNA (RAPD)-PCR Diagnostic. Rather than use mtCOI sequencing for all *B. tabaci* collected, a simple RAPD-PCR diagnostic was developed using the method of Lima et al. (2002). The primers OPA 04 (AATCGGGCTG) and OPA 13 (CAGCACCCAC) Operon Technologies were used as these had enabled the indigenous individuals from Brazil (known as the BR biotype) to be distinguished from the invading MEAM1 (Queiroz da Silva 2006, Queiroz da Silva et al. 2007). Amplification reactions were performed in a 30 μl volume, containing 1× buffer (6 mM Tris-HCl pH 8.8, 50 mM KCl, 2 mM $MgCl_2$), 0.2 mM dNTPs, 0.4 μM of primer, 2 U of *Taq*DNA polymerase (Invitrogen), and 4 μl of whitefly DNA. Reactions were performed with a thermal cycler, under the following program: one cycle of 3 min at 94°C, followed by 35 cycles (1 min at 93°C, 1 min at 37°C, and 2 min at 72°C) and a 5 min-final extension at 72°C. Amplification products were analyzed by 1.5% agarose gel electrophoresis. Gels were submerged in 1X TBE buffer (90 mM Tris-borate, 1 mM EDTA), stained with ethydium bromide (0.5 μg/ml) and visualized under ultra violet light. A molecular weight marker (DNA Ladder 100 bp; Invitrogen) was used for amplified fragment size estimation. Adults of MEAM1 and another invasive member of the complex, Mediterranean (commonly known as Q biotype and hereon MED) from Israel and Spain were used as positive controls along with individuals previously identified as BR from Brazil. A fourth positive control, *Trialeurodes vaporariorum* (Westwood), was also used.

The results of the RAPD-PCR were further confirmed using the primers that were designed by Queiroz da Silva (2006) and Queiroz da Silva et al. (2007) to work specifically with the indigenous *B. tabaci* from Brazil. Amplification reactions were performed in a 25 μl volume containing 1X buffer (6 mM Tris-HCl pH 8.8, 50 mM KCl), 1.5 mM $MgCl_2$, 0.6 μM dNTPs, 1 U of *Taq*DNA polymerase (Invitrogen), and 5 μl of DNA. The reactions were performed with a thermal cycler programmed for one cycle of 3 min at 94°C, followed by 30 cycles (1 min at 94°C, 1 min at 63.5°C, and 1 min at 72°C) and a final 5-min extension at 72°C. Those samples that did not amplify the expected band with specific primers were analyzed with specific primers to detect MEAM1 using conditions indicated by Ko et al. (2007). Amplification reactions were performed in a 30 μl volume containing 1X buffer (6 mM Tris-HCl pH 8.8, 50 mM KCL, 2 mM $MgCl_2$), 0,2 mM dNTPs, 0.4 μM of each primer (F: CCACTATAATTATTGCTGTTCCCACA and R: TCCAATGCACTAATCTGCCATA TTA), 2 U of *Taq*DNA polymerase (Invitrogen), and 4 μl of DNA. Reactions were performed using a thermal cycler, programmed for one cycle of 5 min at 94°C, followed by 35 cycles (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C) and a final 5-min extension at 72°C. Amplification products were analyzed by 1.5% agarose gel electrophoresis as described above.

Results

Whitefly Identification Using mtCOI. Four of the unknown haplotypes (JF901836, JF901837, JF901838, JF901844) plus the two Argentina sequences (AF340212, AF340213) formed a cluster that was basal to the rest of the New World haplotypes (Fig. 2). These sequences diverged from the consensus sequence across the range of 3.6 to 4.3%. Applying the assignment rules of Dinsdale et al. (2010) suggests that the individuals from Argentina formed a separate species that we refer to as New World2. The fifth unknown haplotype (JF901839) fell within the New World putative species and formed a distinct cluster with haplotypes from Panama. These results suggest that Argentina has two indigenous species belonging to the *B. tabaci* cryptic species complex.

RAPD-PCR Diagnostic. All individuals analyzed using RAPD and specific PCR primers to detect BR biotypes were identified as biotype BR except the individuals from fields 35 and 36 that were not considered to belong to BR or to biotype B (Table 1). The individuals identified as biotype BR using the RAPD technique, as well as the positive control, exhibited the characteristic band of 950 bp for this biotype using the primer OPA 04. With the primer OPA 13, all the samples exhibited the characteristic 800 bp band for biotype BR. All the individuals analyzed with specific primers to detect biotype BR exhibited the expected 500 bp band that is considered diagnostic for BR. This means that the RAPD and specific PCR primers are unable to distinguish between the two indigenous species found in Argentina. Furthermore, in the case of individuals from fields 35 and 36, these primers indicated that these individuals were distinct from those considered to be BR and on the basis of the pervading practice to assign new biotypes to different PCR profiles would have been considered new biotypes.

Discussion

Analysis of mtCOI supports the existence of two indigenous species in Argentina, one that belongs to the New World putative species and a second that we now refer to as New World2. This latter species appears ancestral to the New World putative species. The results also highlight the problem with the use of biotype nomenclature and the lack of any rule set that enables biotypes to be delimited. The analysis of the mtCOI clearly shows that the individuals from Argentina identified as BR using the PCR based diagnostics belong to two distinct species that diverge by at least 3.6%. Furthermore, the results suggest that individuals deemed not to be BR on the basis of different PCR

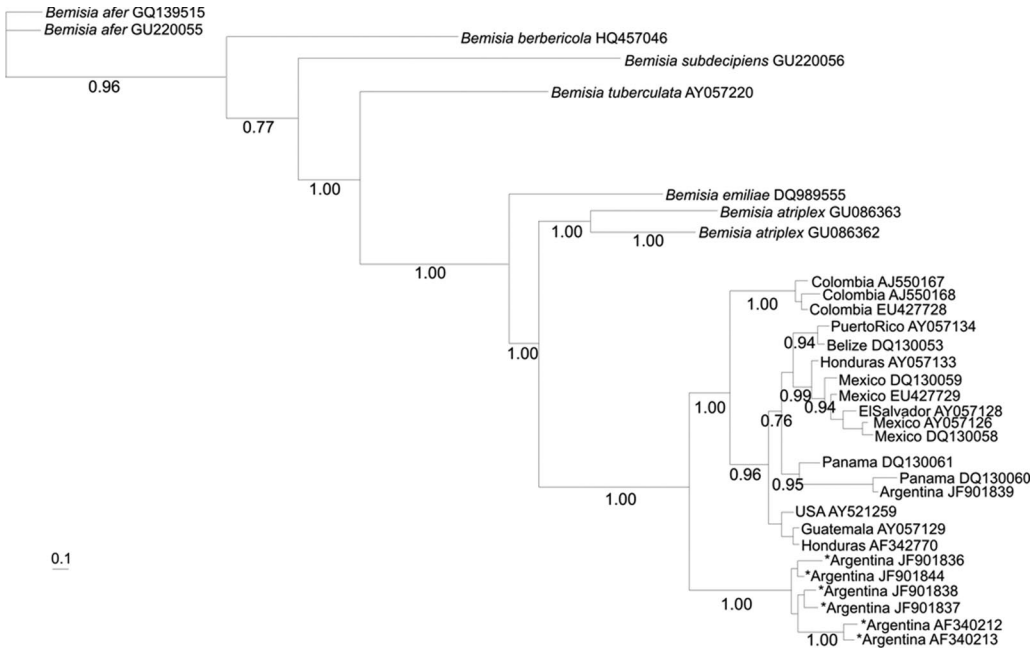

Fig. 2. Phylogenetic tree based on the Bayesian analysis of mtCOI sequences. Posterior probabilities are indicated at nodes. The haplotypes belonging to the proposed new putative species are indicated by an asterisk (*).

profiles are likely to belong to the same putative species, New World2. Therefore, the assumption that BR in Brazil is the same as BR in Argentina is not valid and needs to be reconsidered in the light of our findings.

The results obtained also confirm that the invasive MEAM1 was not detected. In Argentina, Viscarret et al. (2003) identified MEAM1 infesting horticultural crops in areas adjacent to the soybean and bean crops from which our samples were collected. MEAM1 is known to be able to readily displace the New World putative species (Perring and Symmes 2006), but the persistence of New World2 in areas being invaded by MEAM1 suggest that this species may retain some capacity to resist displacement. This needs to be explored more thoroughly.

In Brazil, MEAM1 is known to be invading areas that once were occupied by the indigenous biotype BR (Oliveira et al. 1998, Lima et al. 2001) and appears to be displacing the indigenous member of the complex (Lima et al. 2000, 2002). The guiding assumption here has been that BR in Brazil was the same as BR in Argentina and that as a consequence MEAM1 was expected to displace BR in Argentina. This has clearly not happened. The reason was unclear, but as we have now shown the BR RAPD-PCR diagnostic is unable to separate New World from New World2, it is quite possible that BR in Brazil is not that same as BR in Argentina. It would therefore be useful to obtain mtCOI from BR in Brazil to enable it to be identified more accurately.

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