Genetic identification of members of the *Bemisia tabaci* cryptic species complex from South Africa reveals native and introduced haplotypes

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Abstract

The whitefly *Bemisia tabaci* cryptic species complex contains some important agricultural pest and virus vectors. Members of the complex have become serious pests in South Africa (SA) because of their feeding habit and their ability to transmit Begomovirus species. Despite their economic importance, studies on the biology and distribution of *B. tabaci* in SA are limited. To this end, a survey was made to investigate the diversity and distribution of *B. tabaci* cryptic species in eight geographical locations (provinces) in SA, between 2002 and 2009, using the mitochondrial cytochrome oxidase I (mtCOI) sequences. Phylogenetic analysis revealed the presence of members from two endemic sub-Saharan Africa (SSAF) subclades co-existing with two introduced putative species. The SSAF-1 subclade includes cassava host-adapted *B. tabaci* populations, whereas the whiteflies collected from cassava and non-cassava hosts

formed a distinct subclade, referred to as SSAF-5, and represent a new subclade among previously recognized southern Africa clades. Two introduced cryptic species, belonging to the Mediterranean and Middle East-Asia minor 1 clades were identified and include the B and Q types. The B type showed the widest distribution, being present in five of the eight provinces explored in SA, infesting several host plants and predominating over the indigenous haplotypes. This is the first report of the occurrence of the exotic Q type in SA alongside the more widely distributed B type. Furthermore, mtCOI PCR-RFLP was developed for the SA context to allow rapid discrimination between the B, Q and SSAF putative species. The capacity to manage pests and disease effectively relies on knowledge of the identity of the agents causing the damage. Therefore, this study contributes to the understanding the South African *B. tabaci* species diversity, information needed for the development of knowledge-based disease management practices.

Keywords: *Bemisia tabaci*, genetic diversity, indigenous, invasive, mtCOI PCR-RFLP

Introduction

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a cryptic species complex containing some of the most destructive and invasive pests of vegetable, ornamental and field crops worldwide. Some members of this species complex have a global distribution and cause damage directly through feeding and indirectly through the transmission of plant pathogenic viruses, primarily begomoviruses (Byrne and Bellows 1991; Moriones and Navas-Castillo 2000; De Barro et al. 2011).

Bemisia tabaci was formerly thought to be a sibling species group whose members are morphologically indistinguishable while exhibiting a range of genetic, biological and behavioural variation (Brown et al. 1995b). Approximately 33 'biotypes' have been classified based on the mtCOI gene sequences and a suite of concomitant phenotypic behaviours (Brown 2010; Gill and Brown 2010; Hadjistylli et al. 2010). Although evidence emphasises the existence of biological variants for the *B. tabaci* group, there is a lack of a definitive set of biological data that can be applied across the whole group

(De Barro et al. 2011). There has been considerable debate about whether this diversity indicates the existence of different species or diversity within a single species (Campbell 1993; Brown 2010; Perring 2001; Boykin et al. 2007). Recently Dinsdale et al. (2010) analysed *B. tabaci* globally and proposed subdividing *B. tabaci* in different cryptic species based on 3.5% pairwise genetic divergence. The 3.5% pairwise genetic divergence is further supported by complete or partial mating isolation between a number of the putative *B. tabaci* species (Dinsdale et al. 2010; Xu et al. 2010, Wang et al. 2011). *Bemisia tabaci* is now considered as composed of a complex of at least 24 cryptic species that barely interbreed and form different phylogenetic clades (Dinsdale et al. 2010; Xu et al. 2010; De Barro et al. 2011). The 24 putative species identified by Dinsdale et al. (2010) are (names of associated biotypes are placed in parentheses): Mediterranean (Q, J, L, Sub-Saharan Africa Silverleaf); Middle East-Asia minor 1 (B, B2); Middle East-Asia minor 2; Indian Ocean (MS); Asia I (H, M, NA); Australia/Indonesia; Australia (AN); China 1 (ZHJ3); China 2; Asia II 1 (K, P, ZHJ2); Asia II 2; Asia II 3 (ZHJ1); Asia II 4; Asia II 5 (G); Asia II 6; Asia II 7 (Cv); Asia II 8; Italy (T); Sub-Saharan Africa 1; Sub-Saharan Africa 2 (S); Sub-Saharan Africa 3; Sub-Saharan Africa 4; New World (A, C, D, F, Jatropha, N, R, Sida); and Uganda.

Two members of this cryptic species complex, referred to by Dinsdale et al. (2010) as Middle East - Asia minor 1 (also called *B. argentifolii*, herein referred to as B type) and Mediterranean (herein referred to as Q type), are known worldwide as invasive. The B and Q types invasion can largely be attributed to the trade in ornamental plant species (Frohlich et al. 1999; Bethke et al. 2009; Mckenzie et al, 2009; Dennehy et al. 2005, 2010), high fitness parameters, broad host ranges and propensity to develop insecticide resistance (Horowitz et al. 2003; Dennehy et al. 2005; Horowitz et al. 2005; Prabhaker et al. 2005; Chu et al. 2006; Dennehy et al. 2006, 2010). The introduction of these invasive *B. tabaci* types into non-native habitats has resulted in the displacement of some innocuous *B. tabaci* indigenous to Australia, China, Central and South America, Mexico, Turkey, and the USA (Costa and Brown 1991; Costa et al. 1993; Brown et al.1995a, b; Bird and Brown 1998; Lima et al. 2002; Bayhan et al. 2006; Zang et al. 2006; Liu et al. 2007; Chu et al. 2010a, b; Hu et al. 2011). The recent spread of Q type

in regions already invaded by B has, in some locations, resulted in the rapid displacement of B by Q (Chu et al. 2010a, b; Luo et al. 2010). In many regions of the world, epidemics of plant diseases caused by begomoviruses transmitted by *B. tabaci* occurred soon after the invasion of the B and Q types (Varma and Malathi 2003; Seal et al. 2006; Hogenhout et al. 2008). Where the Q type has shown resistance to pyriproxyfen and neonicotinoid insecticides, management of whitefly and associated viral diseases are more complicated (Dennehy et al. 2010). Therefore where the B and Q types have established sympatrically, proactive monitoring of *B. tabaci* diversity and spread contribute to management strategies based on whitefly biology, behaviour, and responses to agri-chemicals (Ellsworth and Martinez-Carrillo 2001; Nauen and Denholm 2005; Sequeira and Naranjo 2008).

In addition to the invasive-like members of the Mediterranean and Middle East-Asia minor clades, the sub-Saharan African region harbours indigenous and possibly less invasive *B. tabaci* types that vector many begomoviruses (Legg et al. 2002; De La Rua et al. 2006). They cluster in the major sub-Saharan Africa non-silverleafing clade (SSAF), into four subclades (SSAF1-4) (Boykin et al. 2007, Dinsdale et al. 2010). The extent to which *B. tabaci* populations vary genetically and biologically throughout Africa have yet to be fully explored. *Bemisia tabaci* members within the SSAF major clade have been documented colonizing vegetable crops of which the majority associate with cassava (*Manihot esculenta*) (Legg et al. 2002; Abdullahi et al. 2003; Berry et al. 2004; Maruthi 2004; Brown and Idris 2005; Sseruwagi et al. 2005; De La Rua et al. 2006; Sseruwagi et al. 2006; Brown 2010; Carabali et al. 2010). The cassava associated types transmit at least 7 species of begomoviruses to cassava that is a major staple food in sub-Saharan Africa (Legg 1996; Legg et al. 2002; Abdullahi et al. 2003; Carabali et al. 2010). The SSAF-1 subclade represents *B. tabaci* collections from 8 African countries and currently represents the dominant haplotype associated with cassava in Africa (Berry et al. 2004; De La Rua et al. 2006). SSAF-2 correspond to *B. tabaci* individuals previously collected in Uganda on cassava and identified as an invasive Uganda II population that was associated with severe cassava mosaic disease epidemics (Legg et al. 2002, Sseruwagi et al. 2004; De La Rua et al. 2006) as well as

individuals collected from Spain on *Ipoema* species (De Barro et al. 2005). SSAF-3 and SSAF-4 contains 2 divergent populations collected from cassava in Cameroon (Berry et al. 2004). *Bemisia tabaci* collected from cassava in Cameroon, Ghana, Ivory Coast, Nigeria and Zimbabwe grouped within the Mediterranean silverleafing clade rather than with other cassava associated collections in the SSAF (Berry et al. 2004).

The presence of the *B. tabaci* in SA has been recorded since the 1960's when it was associated with Tobacco leaf curl disease in tobacco-producing areas in the Gauteng and North West provinces (Hill 1967; Thatcher 1976). Hill (1967) and Thatcher (1976) differentiated the whitefly species, *Trialeurodes vaporariorum* (Westwood) and *B. tabaci* using mainly morphological characteristics. The *B. tabaci* type or species was only determined several decades later by Bedford et al. (1994) and Berry et al. (2004). Cassava associated *B. tabaci* collections from Mozambique, SA (Kwazulu-Natal) and Swaziland examined by Berry et al. (2004) were closely related to other cassava colonizing types from sub-Saharan Africa and grouped within the SSAF-1 subclade. The exotic B type was first reported from SA from potato (*Solanum tuberosum*) in 1992 (Bedford et al. 1994). Subsequently it emerged as an important agricultural pest with the appearance of *Tomato curly stunt virus* (ToCSV-[ZA:Ond:98]) in SA in 1997 (Pietersen et al. 2002, 2008).

In South Africa, studies on the host range and distribution of *B. tabaci* types have been limited, despite their economic importance in vegetable crops, including cassava. This study aimed at updating the information regarding the different *B. tabaci* types present in the country. The objective of this study was to establish the identity, locality and host association of *B. tabaci* in selected vegetable- and cassava-producing regions in SA. Adult *B. tabaci*, collected between 2002 and 2009 in several locations within eight provinces, were identified using the mtCOI sequences. To facilitate rapid identification in follow-on field studies in SA, a PCR-RFLP method was developed to differentiate between the B type, Q type, and indigenous sub-Saharan subclades SSAF-1 and SSAF-5.

Materials and methods

Whitefly collection

Field collections of South African *B. tabaci* were made during 2002-2009. Adult whiteflies were collected from field and greenhouse-grown crops, as well as, non-cultivated species that include *Ipomoea batatas*, *Solanum lycopersicum*, *Cucurbita species*, *Phaseolus vulgaris, Salvia tiliifolia,* and *Manihot esculenta* (Fig. 1 and Table 1). In each location, whiteflies were collected from several plants within the same field. Samples were collected directly into 70% ethanol and stored at -20˚C until analysis.
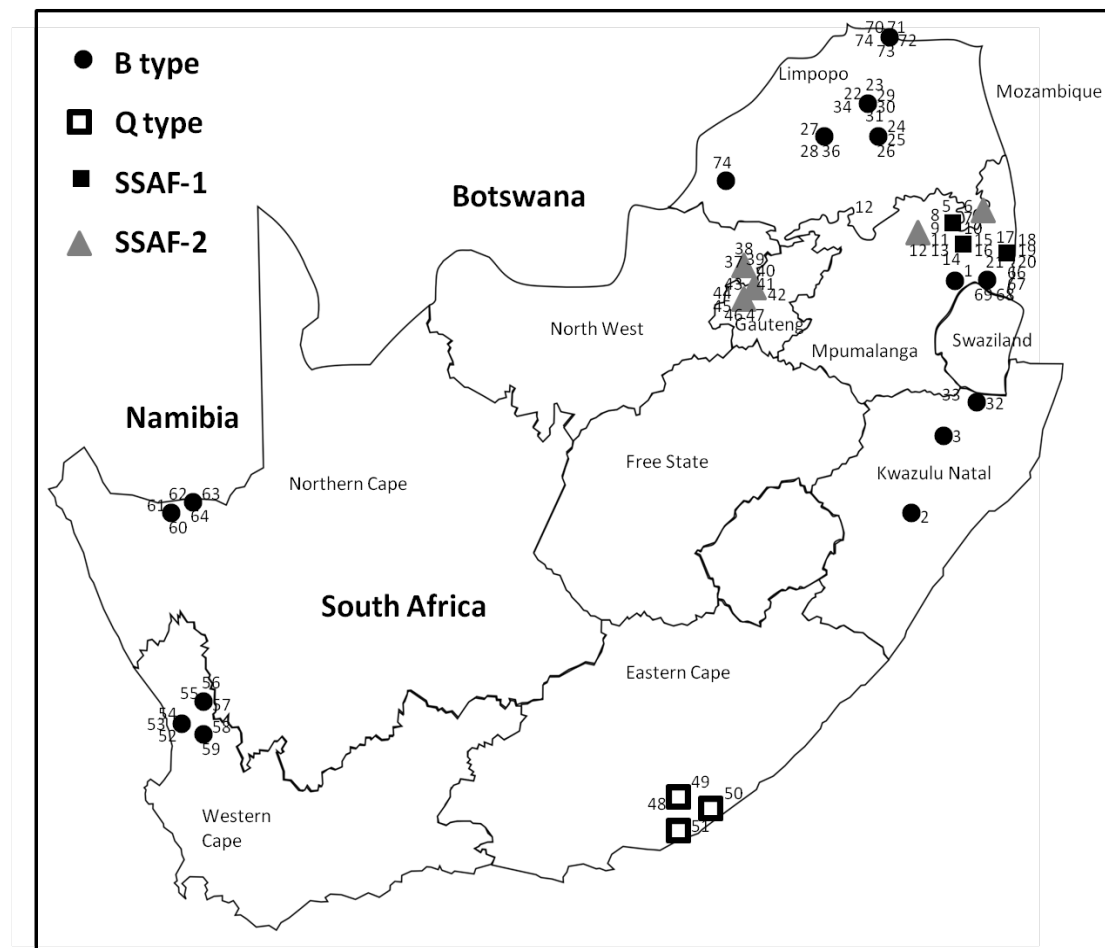

Fig. 1 South Africa map showing the areas where *B. tabaci* samples were collected. The numbers on the map correspond to sample numbers in Table 1. The whitefly mtCOI haplotypes are indicated as follows: ● Middle East/Asia minor 1 (B type), ◻ Mediterranean (Q type), ▲ SSAF-1 and ■ SSAF-2.

Table 1 Representative *Bemisia tabaci* collected in South Africa and identified by sequencing the mtCOI gene fragment.

Code [‡]	Area and province in South Africa	Host plant	Year	mtCOI haplotype[•]
1[†]	Onderberg, Mpumalanga	Unknown	2002	B
2[†]	Cedara, KwaZulu-Natal	Unknown	2002	B
3[†]	Kwalini, KwaZulu-Natal	Unknown	2002	B
4[†]	Tzaneen, Limpopo	Unknown	2002	B
5- 10 [#]	Bushbuckridge, Mpumalanga	*Manihot esculenta*	2004	SSAF-1/ SSAF -5
11-16[#]	Mariti, Mpumalanga	*M. esculenta*	2005	SSAF -1/ SSAF -5
17-21[#]	Tonga, Mpumalanga	*M. esculenta*	2006	SSAF -1
22-23	Mooketsi, Limpopo	*Solanum lycopersicum*	2006	B
24-26	Trichardsdal, Limpopo	*S. lycopersicum*	2006	B
27-28	Tom Burke, Limpopo	*S. lycopersicum*	2007	B
29-31	Mooketsi, Limpopo	*S. lycopersicum*	2007	B
32-33	Pongola, KwaZulu-Natal	*S. lycopersicum*	2008	B
34-35	Mooketsi, Limpopo	*S. lycopersicum*	2008	B
36	Tom Burke, Limpopo	*S. lycopersicum*	2008	B
37-38	Brits, North West	*S. lycopersicum*	2008	SSAF-5
39-40	Brits, North West	*Ipoema* spp.	2008	SSAF-5
41-42	Sterkfontein, Gauteng	*Cucurbita* spp.	2008	SSAF-5
43-45	Tarlton, Gauteng	*Cucurbita* spp.	2008	SSAF-5
46-47	Lanseria, Gauteng	*S. lycopersicum*, *Malva parviflora*, *Phaseolus vulgaris. Datura stramonium*	2009	SSAF-5
48-51	East London, Eastern Cape	*S. lycopersicum* *	2009	Q
52-54	Vredendal, Western Cape	*Ipomoea batatas*	2009	B
55-57	Van Rhynsdorp, Northern Cape	Unknown	2009	B
58-59	Klawer, Western Cape	Unknown	2009	B
60-64	Noordoewer, border of northern Cape	*S. lycopersicum*	2009	B

	and Namibia			
65-69	Komatipoort, Mpumalanga	*S. lycopersicum*	2009	B
70-74	Mussina, Limpopo	*S. lycopersicum*; *Cucurbita sp.*	2009	B

‡ Codes 1-74 correspond to *B. tabaci* individuals collected from field and identified by sequencing the mtCOI gene fragment as indicated in Fig. 1.

B. tabaci samples 22-74 collected by L. Esterhuizen (JN104702-JN104716).

† Samples collected by Kirsten Kruger, department of Entomology and Zoology, University of Pretoria, South Africa in 2002 from unknown host plants.

B. tabaci samples collected by K. Mabasa (JN104718-JN104726).

* Samples collected in greenhouse.

• mtCOI haplotype designation based on mtCOI sequenced samples, where B type refers to the Middle East-Asia minor 1 clade, Q type refer to the Mediterranean clade and SSAF-1 and SSAF-5 refer to two sub-Saharan subclades, using the *B. tabaci* mtCOI phylogenetic designation (Dinsdale et al. 2010).

Nucleic acid extraction

Genomic DNA was extracted from individual whiteflies as described by Frohlich et al. (1999) with modifications. Single whiteflies were placed on a Petri dish covered by a piece of parafilm and the round end of a 0.5 ml microfuge tube was used to grind them in 35 µl lysis buffer containing 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Triton-X 100 and 3 mg/ml proteinase K. The extracts were incubated at 65°C (15 min) and 95°C (10 min) prior to 5 min centrifugation (9600 *x g*) to pellet debris. Nucleic acid extracts were stored at -20°C until further use.

mtCOI PCR amplification and sequencing

The mtCOI gene (850 bp) (Frohlich et al. 1999) was amplified by PCR from 5 or more adult whiteflies per field collection, using the universal COI primers C1-J-2195 (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and TL2-N-3014 (5'-TCCAATGCACTAATCT GCCATATTA-3') (Simon et al. 1994). The 25 µl PCR reaction contained 1x buffer (JMR Holdings); 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1.2 mM of each primer, 1U DNA *Taq* polymerase (JMR Holdings) and 5µl DNA. The PCR conditions consisted of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 52°C for 1 min and 72 °C for 2 min, with a 1final 10 min at 72°C. The mtCOI amplicon from 2-3 samples per field collection was

sequenced bi-directionally using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, United States) and Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems). Sequence fragments were assembled using ChromasPro (www.technelysium.com.au/chromas.html) and edited manually to obtain a consensus sequence. The *B. tabaci* putative species for each sample was initially assigned by homology determination using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and then by phylogenetic analysis of the mtCOI sequences using ClustalW alignments of each sequence (Boykin et al. 2007; Brown 2010; Dinsdale et al. 2010).

Phylogenetic analysis

In addition to sequences obtained in the present study, mtCOI sequences of *B. tabaci* from Genbank were selected to represent major geographical regions (Dinsdale et al. 2010) and included in the analyses. Outgroups included in the alignment were *B. afer* (GU220055) and *B. subdecipiens* (GU220056). The mtCOI sequences were aligned using MUSCLE implemented in CLC Sequence Viewer 6.5 (Edgar 2004). The portion (634 bp) of the *B. tabaci* mtCOI gene sequence (AY521259) was used in this analysis is located between nucleotide 806 and 1439 (Thao et al. 2004). The optimal nucleotide substitution model determined by MrModeltest 2.3 (Nylander 2004) and PAUP 4.0b10 were used for phylogenetic reconstruction (GTR+I+G). Phylogenetic analysis was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The Markov chain Monte Carlo analyses were run in four independent analyses with one cold and three heated chains with 10 million generations each. Chains were sampled every 500 generations. To assess the mixing, convergence and a suitable burn-in for the chains we used Tracer 1.5 (Rambaut and Drummond 2007). Burn-in was set to 1 million and the trees represent 50% majority rule consensus trees. Node values represent posterior probabilities. The percentages of shared nucleotide identity within and among the major clades based on the mtCOI sequence (634 bp) were determined using Mega 4.1. Genetic distances based on the 634 bp mtCOI sequences were calculated based on the Kimura-2-paramater model using Mega 4.1 (Table 2). In this study, the term haplotype

was taken to mean a genetically distinct mtCOI sequence or group of genetically related sequences.

Table 2 Mean distance estimates calculated using Kimura-two parameter method. Along the diagonal is the genetic distance among the haplotype within each clade.

Population*	% shared nt id [#]	Middle East Asia Minor 1	Mediterranean	Sub-Saharan Africa				
				I	2	3	4	5
Middle East Asia Minor 1 (19)	96-99	-0.004						
Mediterranean (18)	97-99	0.053	-0.01					
SubSahAf 1 (n=12)	97-99	0.167	0.177	-0.014				
SubSahAf 2 (n=4)	97-99	0.173	0.181	0.082	-0.014			
SubSahAf 3 (n=1)	n/c	0.168	0.178	0.075	0.065	(n/c)		
SubSahAf 4 (n=4)	99	0.176	0.181	0.091	0.075	0.071	-0.01	
SubSahAf 5 (n=6)	99	0.174	0.179	0.061	0.081	0.067	0.081	-0.003

* The number of haplotype *B. tabaci* individuals included in each clade for analysis.

[#] The within-clade variation based on the percentage of shared nucleotide identity (nt.id) is also indicated.

n/c: Not computable

mtCOI PCR-RFLP marker

A mtCOI PCR-RFLP method was developed to differentiate between the observed mtCOI haplotypes found in SA. A computer simulation was performed to identify polymorphisms in restriction sites for the mtCOI sequence of representative members from the Mediterranean (Q type); Middle East-Asia minor 1 (B type) and the SSAF-1 and SSAF-5 subclade (this study). Selected sequences were aligned and trimmed to 732 bp using Bioedit and restriction fragment pattern determined using DistinctiEnz (www.bioinformatics.org/~docreza/cgi-bin/restriction/DistinctiEnz.pl). *Bfa*I (C/TAG) was selected for use as it generated a clear polymorphism between the B type, Q type and SSAF subclades (SSAF-1 and SSAF-5). Four or more samples per field collection were

mtCOI haplotyped using the PCR-RFLP method. The same PCR reaction and conditions as described above were used. The amplified mtCOI PCR product (10 µl) was digested with *BfaI* (Fermentas) (2.5 U) at 37°C for 1h. The fragments were visualized in a 2% agarose gel using Gelgreen (Anatech, Johannesburg, South Africa). Reference B and Q type provided by Prof H. Czosnek were included as controls.

Results

MtCOI sequences and analysis

A total number of 384 *Bemisia tabaci* samples were collected during 2002 to 2009 from various host plants throughout SA (Table 1). The 74 mtCOI sequences (Genbank JN104702 - JN104726), representative of samples per collection were blasted to determine the mtCOI haplotype of each collection and are represented in the phylogenetic trees depicted in this study. The Bayesian trees (Fig. 2) grouped the South African and reference mtCOI haplotypes into 3 of the 11 major phylogeographic clades reported by Boykin et al. (2007) and Dinsdale et al. (2010). The South African haplotypes, grouped either in the Middle East-Asia minor 1, Mediterranean or the SSAF clades (Fig. 2).

The majority of the South African *B. tabaci* populations from *S. lycopersicum, I. batatas* and *Cucurbita sp.* were identified as the B type based on 99% shared nucleotide identity and therefore clustered within the Middle East-Asia minor 1 subclade (Table 2 and Fig. 2). Whitefly collections from greenhouses in the Eastern Cape province (Fig. 1) grouped with the Spanish Q and other Q-like reference sequences in the Mediterranean subclade, with which they shared 99% nucleotide identity. Whitefly samples collected from cassava plants in Mpumalanga province (Fig. 1) were most closely related at 97-99% sequence identity (Table 2) to subgroup SSAF-1 members, which represent haplotypes previously collected in the southern Africa. Finally, whitefly collections from various vegetable and weed species in North West and Gauteng provinces and from cassava plants in Mpumalanga province (Fig. 1), formed a distinct subclade, herein identified as SSAF-5 (Fig. 2 and 3), and represented a new subclade among southern Africa clades. The latter subgroup haplotypes shared 99% nucleotide identity with each
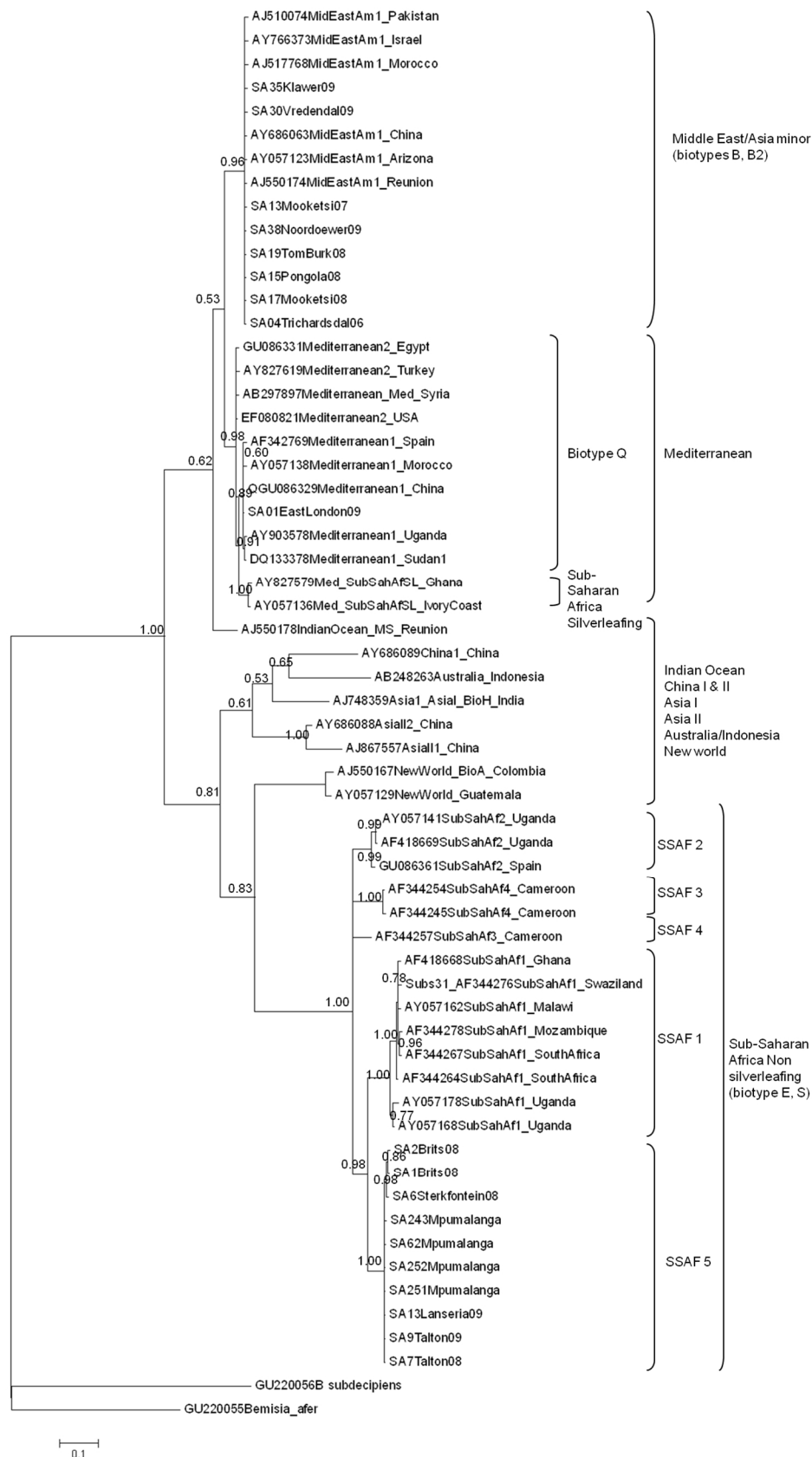
Fig. 2 Phylogenetic tree estimated using Bayesian inference for *Bemisia tabaci* mtCOI haplotypes collected in South Africa (JN104702-JN104726) showing their relationship to each other and to reference *B. tabaci* from representative locations worldwide. Bootstrap results after 1000 replicates are noted at each branch node.

other, and 92-94.4% with haplotypes in the other southern Africa subclades (SSAF 1, 2, 3 and 4) (Table 2). In addition, the new haplotype was found to colonize tomato, *Malva parviflora* and *Datura stramonium* plants in the Gauteng province that were infected with a newly discovered begomovirus that is closely related to ToCSV-[ZA:Ond:98] (at 83% nucleotide identity) (Pietersen et al. 2000, 2008). The new begomovirus show similar symptoms to ToCSV-[ZA:Ond:98] (Esterhuizen et al. 2010).

The nomenclature system used to refer to the SSAF haplotypes by Berry et al. (2004) and De La Rua et al. (2006) and later by Dinsdale et al. (2010) are indicated in the Bayesian phylogenetic tree (Fig. 3). The African *B. tabaci* populations grouped into subgroups referred to as SSAF I-V by Berry et al. (2004), and as SSAF I-VI by De La Rua et al. (2006), with the addition of a subclade (VI) containing haplotypes from West Africa (Cameroon) and Spain. The system employed by Boykin et al. (2007) and Dinsdale et al. (2010) grouped the sub-Saharan Africa *B. tabaci* populations as SSAF 1-4 and Uganda sweet potato, with the exclusion of the haplotypes from Benin (ABA, AF1106930, Frohlich et al. 1999) and Ivory Coast (AY057135, Brown unpublished) and the addition of the distinct sweet potato clade from Uganda (Legg et al. 2002; Maruthi et al. 2004). Herein, the Dinsdale et al. (2010) nomenclature system is followed (SSAF 1-4) (Fig. 3), with the addition of the new SSAF-5 subclade, that represents a new subclade among previously recognized southern Africa clades. The whitefly samples examined and considered to be indigenous to sub–Saharan Africa, grouped either with the SSAF-1 (n=5) or SSAF-5 (n=11) subclade.

Genetic distance analysis

The genetic distance estimates within and among the South African members of *B. tabaci* based on mtCOI sequence characterization are shown in Table 2. The genetic distance among the haplotypes within the Middle East-Asia minor 1 clade (n=19) and Mediterranean clade (n=18) is 0.004 and 0.01 respectively, whereas the genetic distance between these two closely related invasive types is 0.053. The distance among the haplotypes within the 5 different sub-Saharan Africa subclades are 0.014, 0.014, 0.005 and 0.003 respectively for subclades 1 (n=12), 2 (n=4), 4 (n=4) and 5 (n=6).
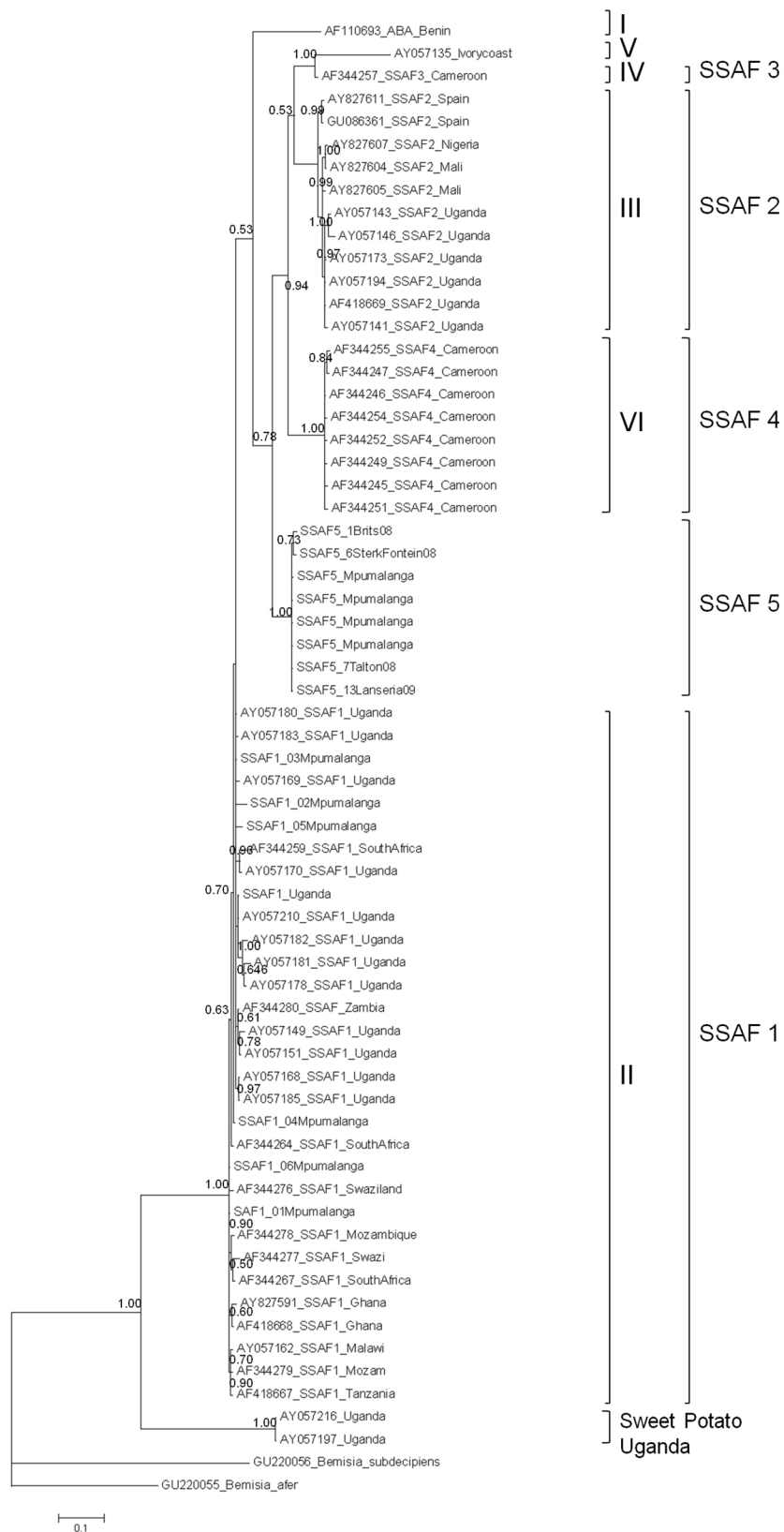
Fig. 3 Phylogenetic tree estimated using Bayesian inference of sub-Saharan Africa *Bemisia tabaci* mtCOI haplotypes indicating the different classification of subclades as named firstly by Berry et al. (2004) and De La Rua et al. (2006) (subclades I – VI) and later by Dinsdale et al. (2010) (subclades 1-4) with the addition of the newly reported subclade SSAF-5 described in this study. Bootstrap results after 1000 replicates are noted at each branch node.

When considering the genetic distance among the 5 subclades, the lowest values were observed between those haplotypes corresponding to subgroup 1 and 5 (0.061) and subgroup 2 and 3 (0.065) and the highest value between subgroup 1 and 4 (0.091).
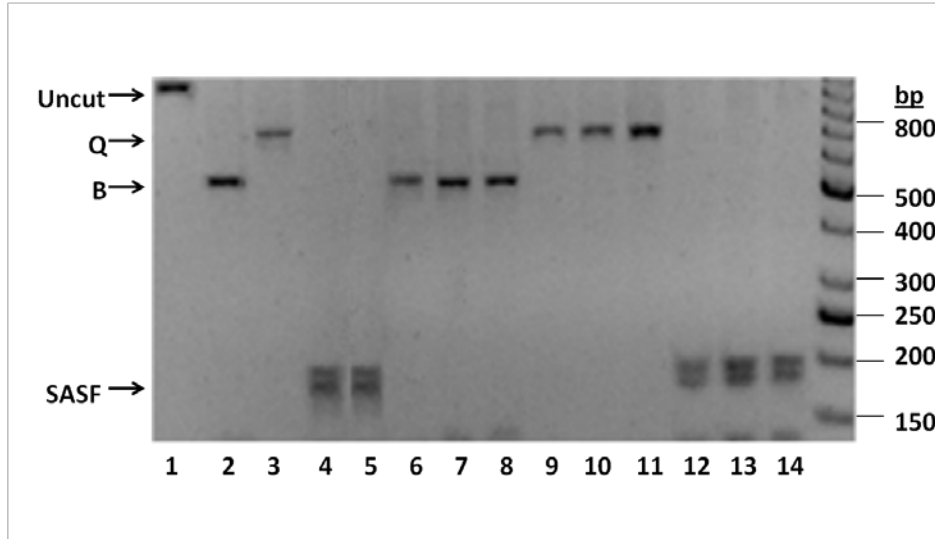

Fig. 4 PCR-RFLP profile of mtCOI amplicon (879bp) (uncut, Lane 1) using the universal COI primers sequence C1-J-2195 and TL2-N-3014 and digested with *Bfu*I (digested, Lane 2-13). Lane 2: B type control; lane 3, Q type control; lane 4, native SSAF-1 from South Africa; lane 5, native SSAF-5 from South Africa; Lane 6-14, samples of *B. tabaci* populations from various locations in South Africa (lane 6-8 samples from Limpopo province (B type); lane 9-11 samples from East-London (Q type), Eastern Cape and; lane 12-14 samples from Gauteng and Mpumalanga (SSAF 1 and 5) and lane 14, 50bp DNA Ladder (Fermentas).

MtCOI PCR-RFLP analysis

A PCR-RFLP method was developed based on the mtCOI gene sequence, to distinguish the genotypic clusters present in South Africa. The PCR-RFLP employed the mtCOI C1-J-2195 and TL2-N-3014 primers to amplify the 850 bp mtCOI fragment, followed by digestion with *Bfa*I that enables differentiation of the B, Q, SSAF-1 and SSAF-5 haplotypes by yielding different sized products (Fig. 4). The PCR-RFLP analysis of B and Q types yielded a distinctive restriction fragment of ~500 bp and ~700 bp respectively, and a unique RFLP pattern for both the SSAF-1 and SSAF-5 with distinctive restriction fragments of ~200, ~190 and ~100 bp (Fig. 4). Identification of 384 whitefly samples from different South African locations using the PCR-RFLP method,

grouped individuals into the Middle East-Asia minor 1 clade (B type), Mediterranean (Q type), SSAF-1 and SSAF-5 subclades, yielding results that were consistent with mtCOI sequence-based identification.

DISCUSSION

In a survey between 2002 - 2009, we elucided *B. tabaci* whitefly haplotype diversity in eight provinces in SA on several host plant species, using mtCOI sequences and a PCR-RFLP method which distinguished between the B type, Q type and SSAF subclades (SSAF-1 and SSAF-5) (Fig. 4). The survey consisted of two specific detailed studies on cassava and tomato hosts, respectively, that were undertaken from 2004-2009. Whitefly samples collected in 2002 from unknown hosts in several locales (Table 1) were also included for identification to broaden the sample base. The *B. tabaci* collected in SA clustered into the Middle East-Asia minor 1 comprising of *B. tabaci* endemic to the Middle Eastern / North African region consisting of the B type. The B type (Middle East-Asia minor 1 clade) was first reported in 1992 from SA on potato (Bedford et al. 1994), and was later associated with the outbreak of a newly described begomovirus (*Geminiviridae*), ToCSV, that emerged in 1997 (Pietersen et al. 2002, 2008). The most widely prevalent haplotype was the B type that shared 99% identify with the mtCOI sequences for other Middle East-Asia minor 1 members, irrespective of the extant geographical sites of collection. The results of this study demonstrated that the B type, is currently widely established in 5 of the 8 provinces explored in this study. Interestingly, the results indicated that the B type has not yet become established in Gauteng and North West province, even though vegetables are produced in that region. The exotic Q type within the Mediterranean clade endemic to the Mediterranean and the Arabian Peninsula was also identified (Frohlich et al. 1999; Guirao et al. 1997). This is the first report of the Q type in SA. Indigenous *B. tabaci* clustered in the sub-Saharan Africa (SSAF) clade in two subgroups namely SSAF 1 and a new haplotype (SSAF-5) presumed to be endemic to SA.

Given the global occurrence of the Q type mainly through ornamental trade, the identification of the Q type in SA is perhaps not unexpected. The mtCOI sequences for

the Q type from this study were highly invariant, at 99-100% shared nucleotide identity (Table 2), indicating high homogeneity and therefore could have originated from a single introduction. The identification of the Q type in East London in 2009 is the first report of the exotic Q type in SA and poses a new threat to agriculture production throughout SA. The Q, like the B type, transmits ToCSV to tomatoes (Esterhuizen et al. 2010) and since the first detection of the Q type in the Eastern Cape in 2009, it has also been detected in vegetable production regions in Mozambique (Rey and Nuaila, personal communication). We therefore expect that the Q type, similar to the B type, has already spread to other regions, possibly through the movement of ornamental hosts and vegetable seedlings infested with *B. tabaci*. Wider geographical sampling, and inclusion of more host plants in future, will provide useful information on the spread and distribution of this Q type. Given the current wide scale use of neonicotinoids and pyrethroids to manage *B. tabaci* in tomato-producing regions in SA, the differential susceptibility of the invasive B and Q types to these compounds is expected to ensure the survival and spread of the latter (Dennehy et al. 2006; Horowitz et al. 2003, 2005; Nauen and Denholm 2005; Prabhaker et al. 2005; Chu et al. 2010a, b; Luo et al. 2010). Further spread and establishment of the Q type in the major tomato producing regions in South Africa could lead to difficulties controlling the Q type, and therefore also to the increased incidence and damage caused by ToCSV infection of tomato crops.

Whitefly samples collected from cassava plants in Mpumalanga province (Fig. 1) were most closely related at 97-99% sequence identity to subclade SSAF-1 members, which were previously collected in Africa. The SSAF-1 subclade, with *B. tabaci* collections from 8 African countries, is the largest and most widely established haplotype associated with cassava in Africa. *Bemisia tabaci* collections from vegetable- and weed species from the Gauteng and North Western province, as well as from cassava in Mpumalanga, clustered in a separate subclade, herein identified as SSAF-5. The assignment of this new putative cryptic species is supported by the 5.6-6.5% divergence between subclade SSAF-5 and its closest sister clade, SSAF-1, in accordance with the 3.5% pairwise genetic divergence identified by Dinsdale et al. (2010) as being the boundary separating different species (Table 2). This is the first report of this subclade

in the sub-Saharan Africa, which is robustly supported at a bootstrap value of 100% (Fig. 2).

Although *B. tabaci* is primarily a polyphagous 'species', evidence exists for host specialization in certain *B. tabaci* populations including the monophagous JAT-PR type on *Jatropa gossypifolia* (Brown and Bird, 1992), the T type colonizing *Euphorbia characias* (Simon et al. 2003) and the cassava colonizing *B. tabaci* from sub-Saharan Africa (Burban et al. 1992; Legg 1996; Legg et al. 2002; Abdullahi et al. 2003; Sseruwagi et al. 2005, 2006). Despite reports that some members of the SSAF 1-5 subclades prefer cassava (indicated as 'cassava-associated'), recent studies have suggested that haplotypes within the SSAF have a broader host distribution (Legg 1994; Thompson 2003; Sseruwagi et al. 2005, 2006). Sseruwagi et al. (2006) found that cassava-associated SSAF-1 whiteflies also colonize *M. glaziovii* (tree cassava), *J. gossypifolia* (jatropa), *Abelmoschus esculentus* (okra) and *E. heterophylla* (Mexican fireplant). In our study, the SSAF-1 haplotype was only collected in Mpumalanga province where cassava is grown. The SSAF-5 haplotype occurred with SSAF-1 in Mpumulanga and individually in the bordering provinces of Gauteng and North West (Fig. 1, Table 1) The subclade SSAF-5 consists of broadly polyphagous individuals collected on cassava in Mpumalanga and non-cassava species in Gauteng and North West province (since cassava is not cultivated in Gauteng and North West provinces). SSAF-5 has a broader host distribution that includes cassava and other host plants suggesting that adaptation is necessary for local haplotypes to survive in some regions, apparently reflecting dependency, in part, on host plant availability.

Bosco et al. (2006) and Sartor et al. (2008) described an mtCOI PCR-RFLP diagnostic tool using *Tru*9I restriction enzyme to differentiate 5 haplotypes belonging to different clades. The same PCR product was employed in this study to develop a PCR-RFLP using *Bfa*I restriction enzyme to rapidly distinguish between the native and introduced haplotypes present in SA. The PCR-RFLP results were in agreement with mtCOI sequence results, and facilitated differentiation between members of the Middle East-Asia minor 1 (B type), Mediterranean clade (Q type), SSAF-1 and SSAF-5 subclade

(Fig. 4). This method was developed to allow rapid differentiation between the invasive B and Q types from the indigenous sub-Saharan Africa clade, as members of SSAF-1 and SSAF-5 subclade produce a similar restriction fragments. The advantage of this approach is that it allows rapid identification of a large number of specimens without the added cost and time required to sequence the mtCOI fragment and will aid in studies to monitor haplotype distribution, insecticide resistance and begomovirus outbreaks.

The results of this study have provided new evidence regarding the *B. tabaci* types present in the country during the study period. Several distinct endemic and introduced haplotypes of *B. tabaci* co-existing in SA were identified. The identification of the cassava-associated SSAF-1 and the more polyphagous SSAF-5 haplotypes provide evidence for the occurrence of two phenotypes among haplotypes that group in the SSAF clade. In addition, two other haplotypes belonging to the major Middle East-Asia minor 1 and Mediterranean clades were identified, and include the B type and Q type. The B type has been identified previously in SA, however, our results show that it has become established there and is more widespread than previously known. The exotic Q type, which is endemic to Spain and known to be distributed nearly worldwide, was not previously identified in SA. The continued monitoring of *B. tabaci* haplotype distribution in southern Africa, together with studies to elucidate key phenotypic characteristics of these two endemic *B. tabaci* haplotypes, particularly in relation to host range, inseciticide resistance and gene flow between one another and the non-native B and Q types, will be crucial to designing sustainable approaches for the management of *B. tabaci* as a pest and vector of plant viruses in South African agriculture.

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