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RESEARCH ARTICLE

Diversity and Genetic Differentiation of the Whitefly *Bemisia tabaci* Species Complex in China Based on mtCOI and cDNA-AFLP Analysis

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Abstract

The whitefly *Bemisia tabaci* are considered as a taxonomically complex that contained some destructive pests. Two of the most prevalent cryptic species are *B. tabaci* Middle East-Asia Minor 1 (MEAM1) and Mediterranean (MED). In an extensive field survey of the *B. tabaci* complex present throughout part of China from 2004 to 2007, we obtained 93 samples of *B. tabaci* from 22 provinces. We determined that these Chinese haplotypes included 2 invasive species (MEAM1 and MED), and 4 indigenous cryptic species (Asia II 1, Asia II 3, China 3 and Asia II 7) by sequencing mitochondrial cytochrome oxidose one gene (mtCOI). The diversity and genetic differentiation of a subset of 19 populations of *B. tabaci* were studied using cDNA amplified fragment length polymorphism (AFLP). Prior to 2007, MEAM1 was a dominant species in many provinces in China. By 2007, MED was dominant in 11 provinces. Both invasive and indigenous species were simultaneously found in some regions. Indigenous species of *B. tabaci* were found in six provinces in southern China. MED and MEAM1 have broad ranges of host plants, and indigenous species appeared to have much narrower host ranges. All Asia II 3 samples were found on cotton except one on aubergine. China 3 has more host plants than Asia II 3. Twelve samples of China 3 were collected from sweet potato, Japanese hop, squash and cotton. A total of 677 reproducible bands amplified with 5 AFLP primer combinations were obtained. The highest proportion of polymorphic bands was 98.7% and the lowest was 91.9%. Unweighted pair-group method analysis indicated that the clustering was independent of the different species. MED showed the lowest degree of similarity than the other species. The data indicate that both MEAM1and MED were rapidly established in China.

Key words: *Bemisia tabaci*, mtCOI, cDNA-AFLP, diversity, whitefly

INTRODUCTION

Bemisia tabaci is considered taxonomically as a species complex which contained some destructive pests worldwide (Brown *et al*. 1995; Boykin *et al*. 2007; De Barro *et al*. 2011). In addition to direct feeding, some *B. tabaci* cause serious crop losses through transmission of over 100 plant viruses (Byrne *et al*. 1990; Oliveira *et al*. 2001; Jones 2003; Rekha *et al*. 2005). *B. tabaci* also caused major agricultural losses in many parts of China, e.g., an estimate of over $600 million losses of crops by *B. tabaci* was reported in Hubei Province in 2007 (Luo *et al*. 2010). Recently, tomato production has been seriously threatened by *Tomato yellow leaf curl virus* in Zhejiang, Shandong, Hebei, and Beijing, which is transmitted by *B. tabaci*.

Significant differences occur in host range, geographical distribution, transmission of plant viruses in some geographical populations or host races of *B. tabaci*,

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which were indistinguishable by the traditional classification of morphology (Gill 1992; Perring 2001). However, with molecular technology, they can be identified by some molecular markers. Analysis of the amplification products generated from simple primers, different putative species shared less similarity (Perring *et al*. 1993). Abdullahi *et al*. (2004) used PCR and restriction fragment length polymorphism (PCR-RFLP) of the ITS rDNA to distinguish cassava-associated populations of *B. tabaci*. De Barro and Driver (1997) used random amplified polymorphic DNA (RAPD-PCR) to distinguish the Middle East-Asia Minor 1 (MEAM1, formerly referred to as 'biotype B') from other species of *B. tabaci*. Amplified fragment length polymorphism (AFLP) was carried out in the genetic study and putative species differentiation (Cervera *et al*. 2000; Zhang *et al*. 2005). ITS1 gene sequencing (De Barro *et al*. 2000) and mtCOI gene sequencing (Frohlich *et al*. 1999; Boykin *et al*. 2007) were used to identify species and analyze the genetic relations between different species. Jones *et al*. (2008) distinguished the *B. tabaci* MEAM1 and Mediterrenean (MED, formerly referred to as 'biotype Q') based on an allelic discrimination real-time PCR assay using fluorescent dye-labelled probes. Up to 2005, 41 geographic populations had been described, and 26 genetic groups were identified (Perring 2001; Simon *et al.* 2003; Delatte *et al.* 2005).

The global phytogeographic genetic structure of *B. tabaci* has been analysed, and cross mating experiments among 14 genotypes of *B. tabaci* have been conducted in the past 20 years (Dinsdale *et al*. 2010; De Barro *et al*. 2011; Liu *et al*. 2012). The data so far suggested that *B. tabaci* is a species complex including >28 putative species, which are similar to each other in morphology but quite distinct in genetic structure (Xu *et al*. 2010; Wang *et al*. 2010, 2011; Sun *et al*. 2011). According to De Barro *et al*. (2011) and Hu *et al*. (2011), two invasive species (MEAM1, MED) and 13 indigenous species (Asia 1, Asia II 1, Asia II 2, Asia II 3, Asia II 4, Asia II 6, Asia II 7, Asia II 9, Asia II 10, Asia III, China 1, China 2, and China 3) of the *B. tabaci* species complex were found in China.

MEAM1 now occurs worldwide and has the capacity to induce a number of physiological changes in a range of host plants (Bedford *et al.* 1994; De Barro *et al.* 2011). Since MEAM1 was firstly reported in 2002 in China, it has colonized successfully and caused enormous losses to agriculture due to its wide host-range and transmission of plant viruses in this country (Luo *et al*. 2002; Wu *et al*. 2002; Zhang *et al*. 2005; Liu *et al*. 2007). In addition to MEAM1, the invasive MED has been reported extensively in China recently (Hu *et al*. 2011). MEAM 1 showed high levels of insecticide resistance (Ma *et al*. 2007). However, MED had attracted increasing attention owing to its even higher levels of resistance to pesticides than MEAM1 (Horowitz *et al*. 2005, 2008; Luo *et al*. 2010; Rao *et al*. 2012).

In this paper, we analyzed 93 *B. tabaci* samples collected from 2004 to 2007 by sequencing mtCOI gene and provided evidence of the species status of *B. tabaci* in 22 provinces in China. In addition, we conducted cDNA-AFLP polymorphic analysis on a subset of 19 samples to reveal the genetic diversity and differentiation of *B. tabaci* species complex in this country.

RESULTS

Distribution of different species of the *B. tabaci* complex

Two invasive crytpic species (MEAM1 and MED) and 4 indigenous cryptic species (Asia II1, Asia II 3, China 3, and Asia II 7) of the *B. tabaci* complex were found in different parts of China in this survey. All the mtCOI sequences of *B. tabaci* and *B. afer* were submitted to GenBank (accession nos.: EF566753, EF566756, EF566757, EF566760, EF566761, EF667473, EU000319, EU376986, EU376989, EU694112, GQ139492, GQ139494 to GQ139506, GQ139508 to GQ139510, and GQ139512 to GQ139515).

Fifty-five samples were identified as MEAM1 and 19 samples as MED. The data indicated that MEAM1 and MED were broadly distributed over much the mainland of China. MEAM1 was found in Anhui, Beijing, Fujian, Gansu, Guangdong, Hebei, Henan, Hubei, Inner Mongolia, Shaanxi, Shandong, Shanghai, Shanxi, Xinjiang, Yunnan and Zhejiang, whereas MED was found in Anhui, Hebei, Hubei, Jiangsu, Jiangxi, Qinghai, Shaanxi, Xinjiang, and Zhejiang (Fig. 1).

Four indigenous cryptic species were identified from 6 locations, including Chongqing, Jiangxi, Hunan, Anhui,

Fujian and Zhejiang (Fig. 1). One sample was detected as Asia II 1 and found only in Zhejiang Province. Asia II 3 was found in 5 samples from 3 provinces of the Yangtze River Valley (Hubei, Hunan, and Zhejiang). Asia II 7 was only found in Fujian Province. China 3 was found in Anhui, Chongqing, Hubei, Hunan, Jiangxi, and Zhejiang. China 3 was more broadly distributed than other indigenous species.

When the numbers of individuals from different *B. tabaci* species were compared, the ratio between MEAM1: MED:Asia II 3:Asia II 1:China 3:Asia II 7 was 55:19:5: 1:12:1. In 2004, only MEAM1 and one indigenous species were found (8 samples from 6 locations). In 2005, the 47 populations sampled from 26 locations in 12 provinces comprised 2 MED (2 locations), 35 MEAM1 (21 locations) and 10 indigenous (7 locations). In 2006, 11 samples from 4 provinces were analysed and separated into MED, MEAM1 and indigenous species in the ratio 3:7:1. In 2007, MED became dominant. The 26 samples from 11 provinces comprised with 14 MED (11 locations), 5 MEAM1 (5 locations) and 7 indigenous species (5 locations) (Appendix).

Host plant analysis

Samples of MEAM1 were collected from 17 plant species and those of MED from 9 plant species, which included vegetable, ornamental and weed host plant species (Table 1). For the four indigenous species, none was found on more than four host plants (Table 1). Thus the data indicated that the two alien whitefly species have much wide host ranges than those of the indigenous species. We only found one sample of Asia II 1 on cotton and one sample of Asia II 7 on an unidentified ornamental plant. All samples of Asia II 3 were found on cotton except one on aubergine. Other studies also suggested Asia II 3 has a narrow host range and is associated with *Gossypium hirsutum* (Zang *et al*. 2005a, b; Rao *et al*. 2011). China 3 has more host plants than Asia II 3. Twelve samples of China 3 were collected from sweet potato, Japanese hop, squash, and cotton.

Genetic diversity analysis

Amplification of the cDNA of 19 populations of whiteflies, using five AFLP primer combinations, produced a total of 677 AFLP bands ranging from 70 to 500 bp (Table 2). Of these, 653 fragments were polymorphic bands. The average number of polymorphic bands per AFLP primer combination was 135. The largest number of polymorphic bands was 158, produced with primer combination P_{GAA}/M_{CAG}. The least number of polymorphic bands was 108, produced with primer combination P_{GAA}/M_{CTC}. Different levels of polymorphisms were detected, and the proportion of polymorphic bands (PB) ranged from 91.9% for P_{GTG}/M_{GAA} to 98.7% for P_{GAA}/M_{CAG}.

The UPGMA dendrogram based on genetic distance and constructed with the program NTSYS-pc revealed the genetic relationships of 19 populations of *B. tabaci* and one population of *Trialeurodes vaporariorum* (Fig. 2). *T. vaporarionum* similarity coefficient was 0.563-0.615 with all *B. tabaci* populations and was clustered separately as an outgroup. The similarity coefficient was 0.783-0.866 among the 7 populations of MEAM1 (the average similarity coefficient (ASC) was 0.829), 0.730-0.826 among MED (ASC was 0.752), and 0.648-0.752 among the indigenous populations (ASC was 0.686).

DISCUSSION

This study presents an extensive field survey on the whitefly *B. tabaci* cryptic species throughout much of China from 2004 to 2007. Analysis of cDNA-AFLP revealed genetic differentiation of 19 populations of whiteflies. These results confirmed that two alien whitefly cryptic species MEAM1 and MED of the *B. tabaci* complex had invaded and established in the field in China, and confirmed the previous surveys as regards to the distribution of *B. tabaci* cryptic species in China and added more information for the indigenous *B. tabaci* species.

The alien MEAM1 was present in most parts of China before 2004. However, since 2007, the alien MED has become dominant in some regions (Hu *et al.* 2011; Rao *et al*. 2011). The spread of MED is correlated with the global trade in ornamental plants (Brown *et al*. 2000; Hsieh *et al*. 2007). Earlier reports of MED in China described its presence in Yunnan (Chu *et al*. 2005), Zhejiang (Xu *et al*. 2006), Beijing (Chu *et al*. 2006), and Liaoning (Fu *et al*. 2007). MED in Taiwan was

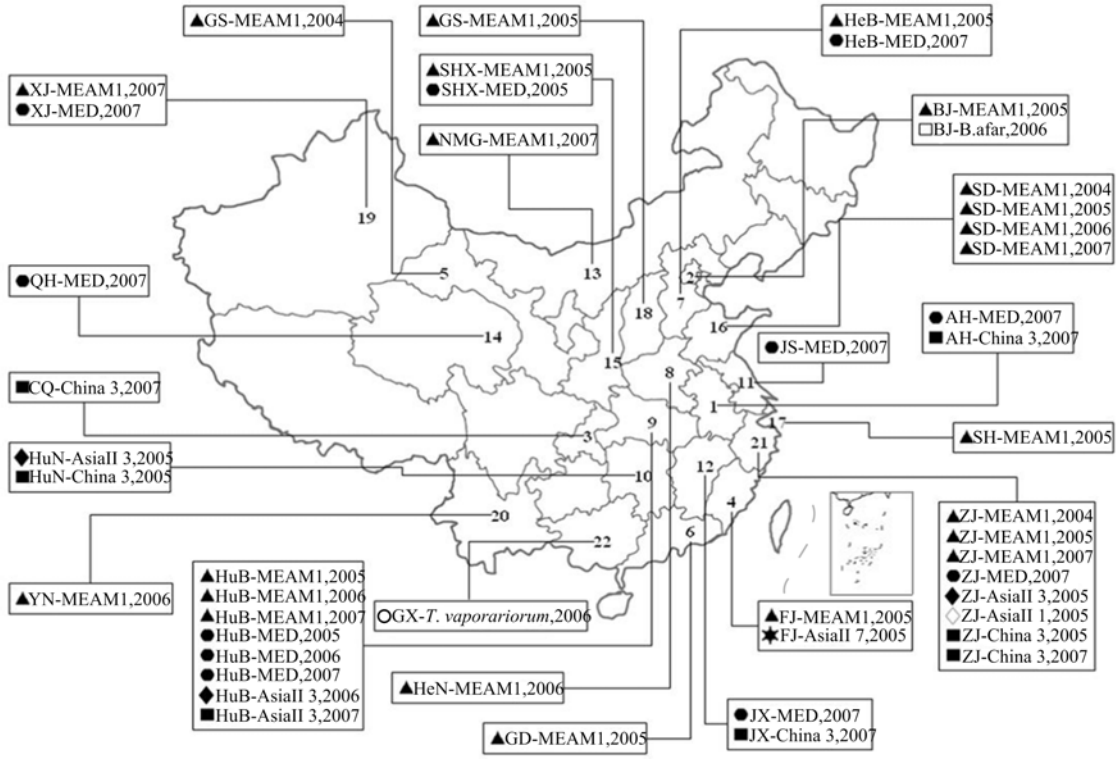

Fig. 1 Geographical distribution of the samples of various species of the *B. tabaci* complex. One sample of *B. afer* and one sample of *T. vaporarionum* were used in this study. 1, Anhui; 2, Beijing; 3, Chongqing; 4, Fujian; 5, Gansu; 6, Guangdong; 7, Hebei; 8, Henan; 9, Hubei; 10, Hunan; 11, Jiangsu; 12, Jiangxi; 13, Inner Mongolia; 14, Qinghai; 15, Shaanxi; 16, Shandong; 17, Shanghai; 18, Shanxi; 19, Xinjiang; 20, Yunnan; 21, Zhejiang; 22, Guangxi.▲ MEAM1;● MED;◇ Asia II 1;◆ Asia II 3;■ China 3;✱ Asia II 7;□ *B. afer*;○ *T. vaporarionum*

Table 1 Host-plants of 93 samples of various species of *B. tabaci* complex

Host-plant	Common name	No. of samples of different whitefly species					
		MEAM1	MED	Asia II 3	Asia II 1	China 3	Asia II 7
Brassica chinensis	Pakchoi	2					
Brassica oleracea	Cabbage	8					
Capsicum frutescens	Capsicum		3				
Cucumis melo	Melon		1				
Cucumis sativus	Cucumber	8	2				
Cucurbita moschata	Squash	9	1			2	
Euphorbia pulcherrima	Poinsettia	4	2				
Gossypium hirsutum	Cotton	5	6	4	1	1	
Hibiscus syriacus	Hibiscus	1					
Humulus scandens	Japanese Hop		1			3	
Ipomoea batatas	Sweet potato	2	1			6	
Lactuca sativa	Lettuce	1					
Lagenaria siceraria	Cucurbit	1					
Luffa cylindrica	Towel gourd	1					
Lycopersicon esculentum	Tomato	3					
Nicotiana tabacum	Tobacco	2					
Raphams sativus	Radish	1					
Ricinus communis	Castor-oil plant	1					
Solanum melongena	Aubergine	5	2	1			
Vigna sinensis	Cowpea	1					
Unknown ornamental	-						1

introduced with poinsettia plantlets imported from Italy in 2006 (Hsieh *et al*. 2007). Our field survey indicated that MED was first found in Hubei and Shaanxi provinces in 2005 and later in at least 8 provinces including

Table 2 Summary of different AFLP primer combination for different geographical populations of *B. tabaci*[1)]

PC[2)]	TNB	NPB	PB (%)	RP
P_{GAA}/M_{CAG}	158	156	98.7	61.5
P_{GAA}/M_{CTA}	154	149	96.8	57.9
P_{GAA}/M_{CTC}	108	103	95.4	49.4
P_{GAC}/M_{CTA}	133	131	98.5	59.7
P_{GTG}/M_{GAA}	124	114	91.9	44.9
Total	677	653	-	273.4
Average	135	131	96.3	54.7

[1)] PC, primer combination; TNB, total number of bands; NPB, number of polymorphic bands; PB, percentage of polymorphic bands; RP, resolving power.
[2)] *P*, *Pst* I; *M*, *Mse* I.

Zhejiang, Anhui, Jiangsu in East China, Xinjiang and Qinghai in Northwest China, and Hubei, Jiangxi, and Hebei in central and North China in 2007. MED had become dominant in many provinces by 2007. These data further confirm that MED had established populations in greenhouses and fields, and had become predominant in the Yangtze River Valley and eastern coastal areas (Hu *et al.* 2011; Rao *et al*. 2011).

Records of host plants indicated that both MEAM1 and MED were found mainly on cultivated *Solanaceous*

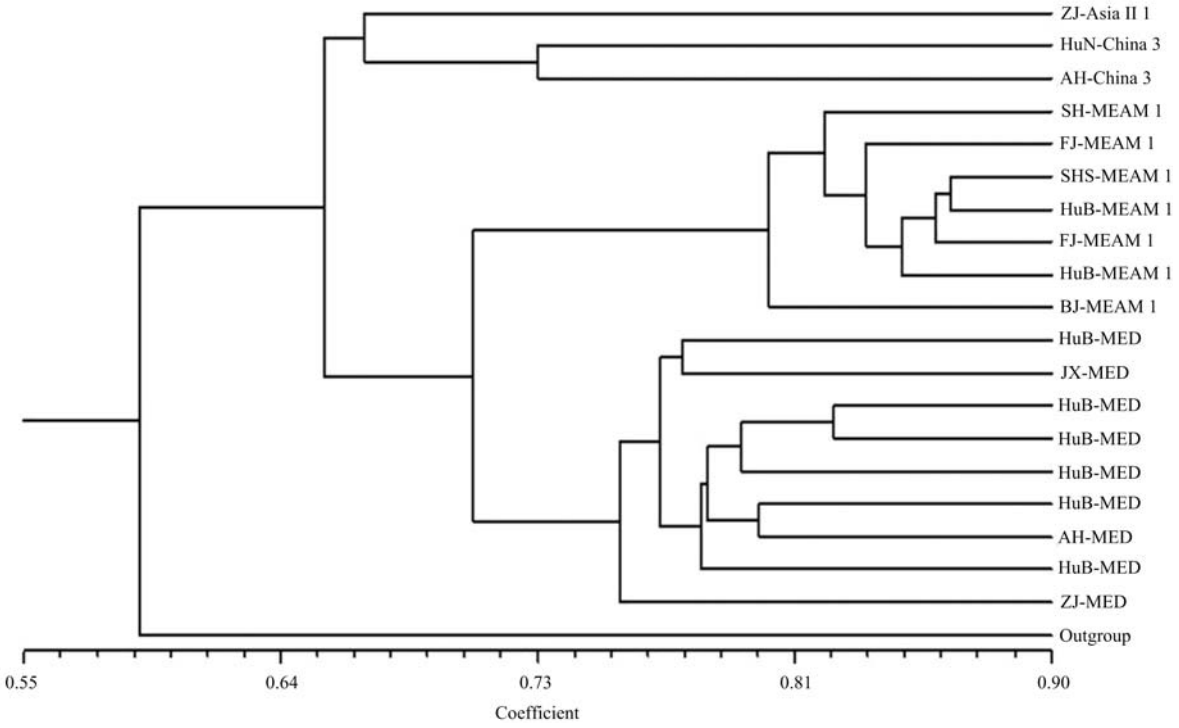

Fig. 2 Dendrogram of 19 *B. tabaci* based on UPGMA analysis using AFLP marker by NYSYS2.0.

and *Cucurbitaceous* vegetables. The indigenous *B. tabaci* Asia II 3, Asia II 1, China 3, and Asia II 7 were collected from few species of plants and showed a much narrower host range than that of MEAM1 or MED. A broad range of hosts including vegetable, ornamental and weed plants could have helped the alien MEAM1 and MED to distribute widely in the regions of invasion. The four indigenous *B. tabaci* species were found only in southern China in this survey, indicating a diversity of indigenous species of *B. tabaci* in this part of the country (Hu *et al.* 2011).

The high percentage of polymorphic cDNA-AFLP fragments in our study was similar to the percentage of polymorphic AFLP and RAPD markers found in other studies (Rekha *et al*. 2005; Zhang *et al*. 2005). MEAM1 showed a higher degree of similarity than MED. A study based on RAPD showed that the genetic diversity of MED was higher than that of MEAM1, and the high level of genetic diversity has been suggested to be beneficial to invasion (Chu *et al*. 2007).

MED is more resistant to neonicotinoids than MEAM1 (Horowitz *et al*. 2003, 2008; Karunker *et al*. 2008; Rao *et al*. 2012). The distribution of large numbers of MED could have been favoured by the extensive use of pesticides such as neonicotinoids or insect growth regulators in China (Rao *et al*. 2011). In addition, the high

level of genetic diversity of MED probably has played an important role in its establishment and spread. Long-term monitoring of the spread of MED should be conducted and further studies on the insecticide resistance of MED are also warranted.

CONCLUSION

In conclusion, our study confirmed that the two alien whitefly cryptic species MEAM1 and MED of the *B. tabaci* complex had invaded and established in the field in China. MED is now present in many provinces and has become dominant in the Yangtze River Valley and eastern coastal areas. Four indigenous *B. tabaci* species Asia II 3, Asia II 1, China 3, and Asia II 7 were found from six locations and they showed a much narrower host range than that of MEAM1 or MED.

MATERIALS AND METHODS

Insects

From 2004 to 2007, 93 samples of *B. tabaci*, one sample of *B. afer* (Priesner and Hosny) and one sample of *T. vaporariorum* (Westwood) were collected from 50 locations of 1 autonomousregion, 3 municipalities and 18 provinces in China. The year of collection, origin and whitefly species are presented in Fig. 1 and Appendix. For DNA analysis, the adults were collected from each location, and stored in 75% ethanol at -20°C. For RNA analysis, at least 100 adults (mixed-sex) were collected from each of 19 locations in 10 provinces and stored at -80°C in RNA later RNA stabilization reagent (Qiagen, German).

mtCOI assay

DNA extraction Total DNA was extracted from individual adult whiteflies (Luo *et al*. 2002). The supernatant was discarded and the DNA was suspended in 20 μL of TE buffer (pH 8.0) and stored at -20°C.

PCR and sequencing of mtCOI genes The PCR reactions (Luo *et al*. 2002) were done in a final volume of 20 μL containing 1 U of *Taq* DNA polymerase, 2.5 mmol L^{-1} $MgCl_2$, 0.25 mmol L^{-1} dNTP, 2 mg mL^{-1} of BSA, 2 μL of DNA and 2.5 ng μL^{-1} of each primer: Cl-J-2195 (5′-TTGATTTTTGGTCATCCAGAAGT-3′); L2-N-3014 (5′-TCCAATGCACTAATCTGCCATATTA-3′) (Simon *et al*. 1994; Frohlich *et al*. 1999). The amplification was done as Luo *et al*. (2002) described. The amplified products were gel-purified using a DNA Clean/Extraction Kit (Promega, USA) and sequenced in one direction by an ABI 3730XL DNA analysis system using a TIANgel Mini/Midi Purification Kit (Tiangen, Beijing, China).

RNA extraction and cDNA synthesis Total RNA was isolated from approximately 100 individuals from each sample using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. Total RNA was treated with 1 U of DNase I at room temperature for 15 min. All RNA samples were quantified using a NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the integrity of RNA structure was checked by formamide-containing denaturing agarose gel electrophoresis. First and second strand cDNAs were synthesized from approximately 1 μL of total RNA using an M-MLV RTase cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Double-stranded cDNA was used in the AFLP analysis.

Pre-amplification and selective amplification A 200 ng sample of purified cDNA was digested with 8 U of *Mse* I/*Pst* I and ligated to adapters with sequences as follows: *Mse* I adapter, 5′-GACGATGAGTCCTGAG-3′, 5′-TACTCAGGACTCAT-3′; and *Pst* I adapter, 5′-CTCGTAGACTGCGTACATGCA-3′, 5′- TGTACGCAGTCTAC-3′. After ligation of the adapters, pre-amplification of purified cDNA templates was done with the corresponding primers: *Mse* I, 5′-GATGAGTCCTGAGTAAC-3′; and *Pst* I, 5′-GACTGCGTACATGCAG-3′. After 20-fold dilution of the resulting PCR product, 2 μL samples of pre-amplified products were amplified with primers having selective nucleotides at the 3′ end: *Mse* I, CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT; and *Pst* I, GAA, GAC, GAG, GAT, GTA, GTC, GTG, GTT. We used 64 different primer combinations. Following selective amplification, the 26 samples were heat-denatured and resolved by electrophoresis in a denaturing 4% (w/v) polyacrylamide sequencing gel at 1 200 V for 2.5 h.

Data analysis

mtCOI assay Three to five individuals from each sample were selected randomly for mtCOI gene sequencing. mtCOI sequences from 93 *B. tabaci* samples were deposited to GenBank. To identify species identity, the sequences were aligned using ClustalX 2.1 (Thompson *et al*. 1997), and checked for duplicates, gaps and ambiguous bases. Then, all sequences were shortened as required for comparison against 28 *B. tabaci* putative species consensus sequences from GenBank. If a sequence diverged by <3.5% from others of the 28 consensus sequences, this was a known *B. tabaci* putative species (Dinsdale *et al*. 2010; Hu *et al*. 2011). If a sequence diverged by >3.5% from all the 28 putative species, this was likely to be a new putative species. Using this protocol, all the 93 *B. tabaci* samples were identified to putative species. mtCOI sequences were obtained for several additional *B. tabaci* collections to

serve as geographic references. One sequence of *B. afer* was obtained as an outgroup (Fig. 1).

cDNA-AFLP assay For the combination of five primers, each gel was analysed by Genescan 3.1, assigning a score of "1" for the presence or "0" for the absence of bands in individual lanes from 70 to 500 bp. The total bands and the polymorphic bands were determined to estimate the percentage of polymorphic bands (%):

PB (%)=$K/N\times100$

Where K is the number of polymorphic bands and N is the total number of bands tested (Table 2). The dendrogram was constructed on the basis of the similarity matrix determined by unweighted pair-group method analysis (UPGMA) (Rohlf 1997) (Fig. 2).

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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