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Research article

Cryptic Species Identification and Composition of *Bemisia tabaci* (Hemiptera: Aleyrodidae) Complex in Henan Province, China

Min Jiu,[1,2] Jian Hu,[3] Lun-Ji Wang,[1] Jun-Feng Dong,[4] Yue-Qin Song,[4] and Hui-Zhong Sun[5]

[1]College of Food and Bioengineering, Key Laboratory of Microbial Resources Exploitation and Utilization, Henan University of Science and Technology, 471023 Luoyang, China (jiumin0912@163.com; wanglunji7215@163.com), [2]Corresponding author, e-mail: jiumin0912@163.com, [3]Ministry of Agriculture Key Lab of Southwestern Crop Gene Resources and Germplasm Innovation, Yunnan Provincial Key Lab of Agricultural Biotechnology, Biotechnology and Germplasm Resources Institute, Yunnan Academy of Agricultural Sciences, 650223 Kunming, China hujian712@gmail.com, [4]College of Forestry, Henan University of Science and Technology, 471023 Luoyang, China (junfengdong@126.com; songyueqin6@163.com), and [5]College of Agricultural, Henan University of Science and Technology, 471023 Luoyang, China (huizhong66@163.com)

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Abstract

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is a cryptic species complex, causing significant crop losses in China during the last decade. Although knowledge of cryptic species composition and dynamics within *B. tabaci* complex is critical for developing sustainable pest management strategies, limited information is available on this pest in the Henan province of China. A systematic survey of the cryptic species composition and distribution of *B. tabaci* complex in different locations of Henan province was conducted in 2012. The results of RAPD-PCR and the gene for the mitochondrial cytochrome oxidase subunit-1 (*mtCOI*) based phylogenetic relationships established using Bayesian method indicated there were four known cryptic species MEAM1, MED, Asia II 3, Asia II 9 and a new cryptic species named China 6 in Henan province. In the survey, the invasive cryptic species MED and MEAM1 were found to be predominant with wide spread distribution across the surveyed regions. On the contrary, the indigenous *B. tabaci* cryptic species including Asia II 3, Asia II 9 and China 6 remained with low prevalence in some surveyed regions. Cryptic species MEAM1 and MED have not completely displaced the native *B. tabaci* in Henan province. This current study for the first time unifies our knowledge of the diversity and distribution of *B. tabaci* across Henan province of China.

Key words: *Bemisia tabaci*, mitochondrial cytochrome oxidase I, cryptic species China 6

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is one of the most devastating agricultural pests of tropical, subtropical and temperate regions of the world (Brown et al. 1995, De Barro et al. 2000) and has been listed as one of the world's 100 worst invasive alien species (Lowe et al. 2000). *Bemisia tabaci* is a highly polyphagous insect that causes severe damage to more than 600 plant species either through direct feeding and excreting honeydew, or through the indirect transmission of more than 120 plant viruses primarily belonging to the genus Begomovirus (family Geminiviridae) (Jones 2003, Navas-Castillo et al. 2011).

Different whitefly populations exhibit different features. By 2007, people used variable molecular features and biological behavior with respect to host plant range, plant virus transmission capabilities, the ability to cause disorders, attraction by natural enemies, and the degree of fecundity and/or insecticide resistance, to distinguish the so-called biotypes of *B. tabaci* (Bedford et al. 1994, Brown 2000, Jones 2003). De Barro et al. (2011) indicated that the gene sequence for the mitochondrial cytochrome oxidase subunit-1 could be utilized as barcode for *B. tabaci* population classification. *Bemisia tabaci* is now considered as a cryptic species complex (Boykin et al. 2007, Dinsdale et al. 2010, De Barro et al. 2011). Based on a Bayesian analysis of 366 mtCOI DNA sequences, *B. tabaci* is a complex of 11 well-defined high-level groups, all separated by a minimum of 3.5% *mtCOI* nucleotide divergence (Dinsdale et al. 2010, De Barro et al. 2011). Till now, at least 40 cryptic species have been found in the *B. tabaci* complex based on mtCOI sequence divergence (Hu et al. 2011, 2014, 2017; Barbosa et al. 2014; Alemandri et al. 2015; Karut et al. 2015; Prasanna et al. 2015; Li et al. 2016). The existence of distinct cryptic species based on genetic distance is also supported by mating incompatibility

(Elbaz et al. 2010, Wang et al. 2010a, Xu et al. 2010, Sun et al. 2011, Qin et al. 2016) and genome studies (Wang et al. 2011, 2013).

Middle East-Asia Minor 1 (hereon MEAM1, formerly B biotype) and Mediterranean (hereon MED, formerly Q biotype) are the most invasive and widely distributed cryptic species of *B. tabaci* complex. MEAM1 was deduced to have invaded China in the mid to late 1990s (Luo et al. 2002), while MED was first detected in China in 2003 (Chu et al. 2006). Cryptic species MED is displacing the earlier invader MEAM1 and become the predominant cryptic species due to rapidly developing pesticide resistance and highly competitive on field crops in China during the past few years (Chu et al. 2010, Wang et al. 2010b, Yuan et al. 2012). The field survey of the cryptic species of *B. tabaci* complex is critical to biological research and effective management (Boykin and De Barro 2014). In China, large-scale field surveys on cryptic species composition and diversity of *B. tabaci* complex have been undertaken in some provinces such as Jiangsu (Zhou et al. 2003), Shandong (Chu et al. 2007), Zhejiang (Liu et al. 2007), Hubei (Rao et al. 2011), Guangdong and Yunnan (Ahmed et al. 2009). The presence and distribution of different *B. tabaci* cryptic species in Henan is poorly known and limited to five localities sampled in the former campaigns (Chu et al. 2006, Ji et al. 2010, Hu et al. 2011, Rao et al. 2011). No large-scale field surveys on *B. tabaci* complex are available for Henan until now.

The objectives of this study are to determine the cryptic species composition and distribution of *B. tabaci* complex in Henan, one of the most important agriculture and food provinces in China. This work provides updated information concerning the distribution of native *B. tabaci* populations in Henan province and the replacement caused by MEAM1 and MED, which will be important for pest control.

Materials and Methods

Whitefly Sampling

Bemisia tabaci individuals (males and females) were collected from representative locations and plant species (from vegetables, ornamental plants and weeds, and from urban as well as agricultural landscapes) in Henan province in 2012. Details of sampling information were summarized in Table 1. Alive adult individuals of *B. tabaci* were collected in tubes containing 95% ethanol and stored at −20 °C prior to molecular analysis. Voucher specimens were deposited in the collection of the College of Food and Bioengineering, Henan University of Science and Technology.

DNA Extraction and RAPD-PCR Analyses

Total DNA was extracted from adult individuals according to De Barro and Driver (1997) and Frohlich et al. (1999). Herein, we analyzed 24 individuals' DNA for every host plant. A total of 1,056 individuals from 44 host plants were identified to putative cryptic species. The DNA of *B. tabaci* was analyzed by RAPD-PCR firstly to determine whether the individual belongs to MEAM1 or MED according to the methods of De Barro and Driver (1997). The primer H16 (5′-TCTCAGCTGG-3′) was used as a marker to generate the RAPD profile. Each PCR reaction was performed in a volume of 20 µl containing of 2.0 µl 10 × PCR buffer, 2.0 µl 10 mM $MgCl_2$, 1.6 µl 2.5 mM dNTPs, 2.0 µl of template DNA, 1.0 U of *Taq* polymerase (Takara, Dalian, China) and 2.0 µl 10 µM primer. PCR procedure consisted of one cycle of 94 °C for 5 min, 40 °C for 2 min and 72 °C for 3 min, followed by 39 cycles of 94 °C for 1 min, 40 °C for 1.5 min and 72 °C for 2 min and a final extension of 72 °C for 10 min. Amplification products were analyzed with 1.5% agarose gel, and ethidium bromide-stained bands were recorded using a Gel-Doc 2000 system (Bio-Rad). Genotypes corresponding to putative cryptic species MEAM1 and MED were easily identified based on RAPD banding patterns. 16 individuals belonging to MEAM1 or MED and all individuals shown not to belong to MEAM1 or MED were subjected to *mtCOI* sequence analysis.

mtCOI Gene Amplification and Sequencing

The individuals from each host plant shown not to belong to MEAM1 or MED and some individuals belonging to MEAM1 and MED were selected for mtCOI sequencing. A fragment of the *mtCOI* gene (866 bp) was amplified via PCR using universal primers C1-J-2195 (5′-TTGATTTTTTGGTCATCCAGAAGT-3′) and TL2-N-3014 (5′-TCCAATGCACTAATCTGCCATATTA-3′). Reaction conditions were with a cycle consisting of an initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 1 min and 72 °C for 1.5 min, and a final extension of 72 °C for 10 min. PCR products were separated on 1% agarose gels, and bands were visualized by ethidium bromide staining under UV light source. PCR products were purified using the Agarose Gel DNA Purification Kit (Takara, Dalian, China). The PCR products were ligated into the pMD18-T vector (Takara, Dalian, China) and sequenced in GenScript Co., Ltd (Nanjing, China). A total of 32 new sequences of *mtCOI* were deposited in GenBank (Accession numbers KY100008 to KY100039).

Sequence Alignment and Phylogenetic Analysis

The sequences obtained here were aligned using ClustalX 1.81 of BioEdit. The 32 sequences obtained from the above analysis were supplemented with a further 56 sequences from GenBank. Aligned with consensus sequences proposed by Dinsdale et al. (2010), all the sequences were unified as 657 bp length for genetic distance and phylogenetics analyses. Genetic distances among 20 *B. tabaci* cryptic species (19 reported in previous studies and one new cryptic species detected in this study) were calculated as mean Kimura two-parameter sequence divergences as implemented in MEGA 6.06 (Tamura et al. 2013) and tabled as a distance matrix (Table 2). Sequence of *Bemisia afer* (Priesner and Hosny) (AJ784260) and sequence of *Trialeurodes vaporariorum* (Westwood) (AF418672) were selected as outgroups (see Fig. 2). The phylogenetic tree was constructed using Bayesian methods as implemented in MrBayes (ver 3.1; Huelsenbeck et al. 2001). The most appropriate model of sequence evolution (GTR + I+gamma) was selected using Modeltest (ver. 3.7; Posada and Crandall 1998). Four chains were run for 30 million generations, and sampling every 1000th generation. The 25% initial states were discarded as burn in of the analysis. The nomination of putative cryptic species was according to Dinsdale et al. (2010) and Hu et al. (2011).

Results

Distribution of the Invasive *B. tabaci* Cryptic Species

Extensive whitefly surveys were conducted in 2012 from multiple locations in Henan province, China (Table 1). The results obtained in this study showed that cryptic species MED was widely distributed across Henan province (Table 1 and Fig. 1). Cryptic species MEAM1 was found in Zhengzhou, Kaifeng, Shangqiu, Zhoukou, Zhumadian, Nanyang, Luoyang, Shanmenxia, Xuchang, Pingdingshan and Jiaozuo, but not found in Puyang, Anyang, Hebi, Xinxiang, Luohe and Xinyang. Compared to other cryptic species,

Table 1. Summary of the field survey

City of collection	Location	Collection date	Host plants	Cryptic species identified
(1) Puyang	Suburb	July 2012	*Gossypium hirsutum*	MED (24)
			Lycopersicon esculentum	MED (24)
			Solanum melongena	MED (24)
(2) Anyang	Suburb	July 2012	*Solanum melongena*	MED (23), Asia II 9 (1)
			Gossypium hirsutum	MED (24)
(3) Hebi	Qixian	July 2012	*Gossypium hirsutum*	MED (24)
	Suburb	July 2012	*Gossypium hirsutum*	MED (24)
			Humulus scandens	MED (24)
(4) Jiaozuo	Mengzhou	July 2012	*Solanum melongena*	MEAM1 (20), MED (4)
			Gossypium hirsutum	MEAM1 (10), MED (14)
	Suburb	July 2012	*Sesamum indicum*	MEAM1 (24)
(5) Xinxiang	Yuanyang	July 2012	*Gossypium hirsutum*	MED (24)
	Suburb	July 2012	*Gossypium hirsutum*	MED (24)
			Lycopersicon esculentum	MED (24)
(6) Zhengzhou	Xinzheng	Aug. 2012	*Gossypium hirsutum*	MEAM1 (6), MED (18)
			Cucumis sativus	MEAM1 (2), MED (22)
			Solanum melongena	MED (24)
(7) Shangqiu	Suixian	Aug. 2012	*Cucurbita moschata*	MEAM1 (1), MED (23)
			Helianthus annuus	MEAM1 (1), MED (23)
	Minquan	Aug. 2012	*Gossypium hirsutum*	MED (24)
(8) Kaifeng	Lankao	Aug. 2012	*Gossypium hirsutum*	MED (24)
	Suburb	Aug. 2012	*Cucumis sativus*	MED (24)
(9) Sanmenxia	Yima	Sep. 2012	*Gossypium hirsutum*	MED (24)
	Jiaxian	Sep. 2012	*Glycine max*	MED (24)
	Lingbao	Sep. 2012	*Helianthus annuus*	MEAM1 (21), MED (3)
(10) Luoyang	Suburb	Aug. 2012	*Ipomcea batatas*	MEAM1 (22), MED (2)
			Sesamum indicum	Asia II 9 (1), MEAM1 (12), MED (11)
			Momordica Charantia	MEAM1 (16), MED (8)
(11) Xuchang	Yanling	Sep. 2012	*Solanum melongena*	MED (24)
			Cucumis sativus	MEAM1 (3), MED (21)
			Ipomcea batatas	MED (24)
			Nicotiana tabacum	MED (24)
(12) Luohe	Linying	Sep. 2012	*Solanum melongena*	MEAM1 (2), MED (22)
	Suburb	Sep. 2012	*Glycine max*	China 6 (5), MED (19)
(13) Zhoukou	Xiangcheng	Sep. 2012	*Solanum melongena*	MEAM1 (12), MED (12)
	Huaiyang	Sep. 2012	*Cucumis sativus*	Asia II 3 (1), MEAM1 (5), MED (18)
(14) Pingdingshan	Suburb	Sep. 2012	*Lycopersicon esculentum*	MEAM1 (10), MED (14)
(15) Zhumadian	Queshan	Sep. 2012	*Gossypium hirsutum*	Asia II 3 (2), China 6 (4), MEAM1 (9), MED (9)
(16) Nanyang	Xixia	Sep. 2012	*Lycopersicon esculentum*	China 6 (3), MED (21)
	Nanzhao	Sep. 2012	*Solanum melongena*	Asia II 3 (8), MED (16)
	Neixiang	Sep. 2012	*Solanum melongena*	China 6 (3), MEAM1 (4), MED (17)
			Gossypium hirsutum	Asia II 3 (8), MEAM1 (5), MED (11)
(17) Xinyang	Suburb	Sep. 2012	*Gossypium hirsutum*	China 6 (8), MED (16)
			Gossypium hirsutum	Asia II 3 (6), China 6 (4), MED (14)
Total		July 2012–Sep. 2012	44	Asia II 3, Asia II 9, China 6, MEAM 1, MED

MED has become the predominant cryptic species in Henan province (Table 1).

Distribution of the Indigenous *B. tabaci* Cryptic Species

Among the specimen, 16 specimen did not match the RAPD profile of MED or MEAM1. We sequenced the mtCOI gene and compared the sequences of those specimen to that of the known cryptic species. Finally, we found 7 specimen matched to the mtCOI of cryptic species Asia II 3 and 2 specimen matched to the mtCOI of cryptic species Asia II 9 based on the method of Dinsdale et al. (2010). In this analysis, *B. tabaci* Asia II 3 were distributed in Zhoukou, Nanyang, Zhumadian and Xinyang. *Bemisia tabaci* Asia II 9 was found in Anyang and Luoyang. However, the mtCOI sequences of the rest 7 specimen from our samples have pairwise divergences that exceed 3.5% either with the consensus sequences or between themselves. Thus, these specimen represent a new putative cryptic species, and it was clustered into a same clade with China 1, China 2, China 3, China 4 and China 5 with the posterior probability of 0.71 (Fig. 2). Furthermore, China 5 was newly reported and found in Yunnan province, China (Hu et al. 2017), so we named the new putative cryptic species in our study as China 6. China 6 is distributed in the south of Henan province including Nanyang, Luohe, Zhumadian and Xinyang. The distributions of the different indigenous cryptic species across Henan province are shown in Fig. 1. No indigenous cryptic species were found in Shanmenxia, Luoyang, Pingdingshan, Jiaozuo, Xinxiang, Hebi, Puyang, Kaifeng, Shangqiu and Zhengzhou.

Phylogenetic Analysis

The phylogenetic reconstruction based on 88 mtCOI sequences is shown in Fig. 2. The 866-bp fragments of the mtCOI sequence

Table 2. Mean Kimura two-parameter genetic distances among *B. tabaci* cryptic species based on *mtCOI*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. Asia I	0																			
2. Asia II 1	0.158	0																		
3. Asia II 2	0.140	0.056	0																	
4. Asia II 3	0.174	0.131	0.071	0																
5. Asia II 4	0.164	0.115	0.054	0.041	0															
6. Asia II 5	0.172	0.097	0.090	0.125	0.115	0														
7. Asia II 6	0.175	0.117	0.120	0.148	0.142	0.066	0													
8. Asia II 7	0.139	0.095	0.104	0.125	0.122	0.112	0.112	0												
9. Asia II 8	0.149	0.131	0.111	0.114	0.132	0.123	0.132	0.119	0											
10. Asia II 9	0.166	0.124	0.087	0.051	0.073	0.110	0.133	0.112	0.122	0										
11. Asia II 10	0.141	0.134	0.102	0.105	0.112	0.138	0.131	0.115	0.123	0.096	0									
12. Asia III	0.076	0.160	0.137	0.162	0.149	0.168	0.164	0.139	0.159	0.158	0.143	0								
13. China 1	0.132	0.148	0.124	0.150	0.145	0.136	0.145	0.145	0.147	0.144	0.132	0.125	0							
14. China 2	0.126	0.154	0.135	0.152	0.151	0.143	0.155	0.134	0.143	0.136	0.132	0.127	0.038	0						
15. China 3	0.140	0.152	0.124	0.150	0.159	0.153	0.153	0.137	0.128	0.137	0.131	0.132	0.114	0.103	0					
16. China 4	0.136	0.141	0.118	0.130	0.141	0.147	0.161	0.142	0.135	0.132	0.141	0.139	0.114	0.103	0.099	0				
17. China 5	0.149	0.156	0.137	0.154	0.161	0.163	0.161	0.149	0.140	0.140	0.128	0.122	0.125	0.115	0.048	0.110	0			
18. China 6	0.133	0.166	0.144	0.159	0.163	0.167	0.185	0.154	0.163	0.149	0.139	0.134	0.106	0.102	0.128	0.108	0.130	0		
19. MEAM1	0.165	0.170	0.115	0.172	0.151	0.173	0.183	0.176	0.158	0.166	0.174	0.175	0.159	0.157	0.150	0.161	0.158	0.170	0	
20. MED	0.163	0.174	0.132	0.174	0.167	0.181	0.177	0.172	0.162	0.158	0.172	0.183	0.166	0.162	0.164	0.168	0.176	0.177	0.053	0

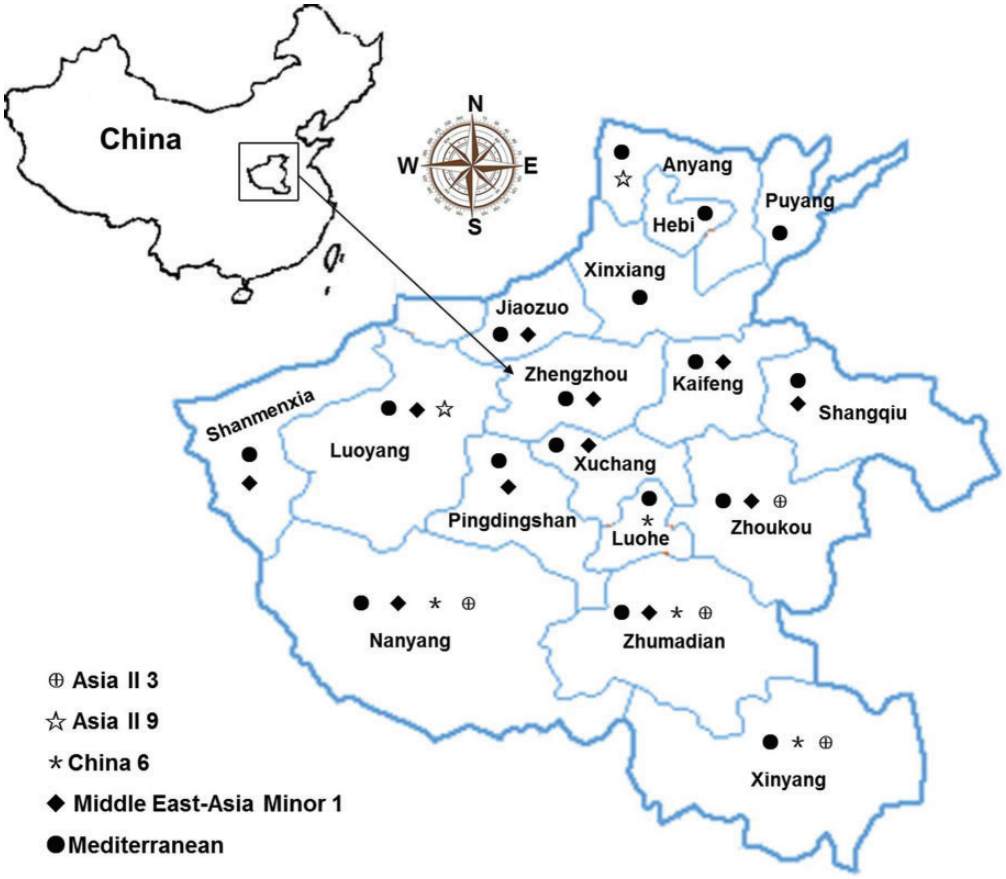

Fig. 1. Distributions of the different cryptic species belonging to the *B. tabaci* complex in Henan province, China. Insert is a sketch map of China highlighting the eara sampled.

was obtained from 32 adult *B. tabaci* collected in Henan province in 2012 followed by DNA sequencing. The sequence data generated for 32 specimen have been submitted to NCBI database. In the phylogenetic analysis, all Asian indigenous species were clustered into a larger clade (Fig. 2). A total of 11 whitefly sequences collected from Henan province clustered with the MED, 5 with the MEAM1, 7 with the Asia II 3, 2 with the Asia II 9 and 7 with the China 6. Genetic distances among the Asian members of *B. tabaci* complex ranged from 0.038 to 0.185 (Table 2).

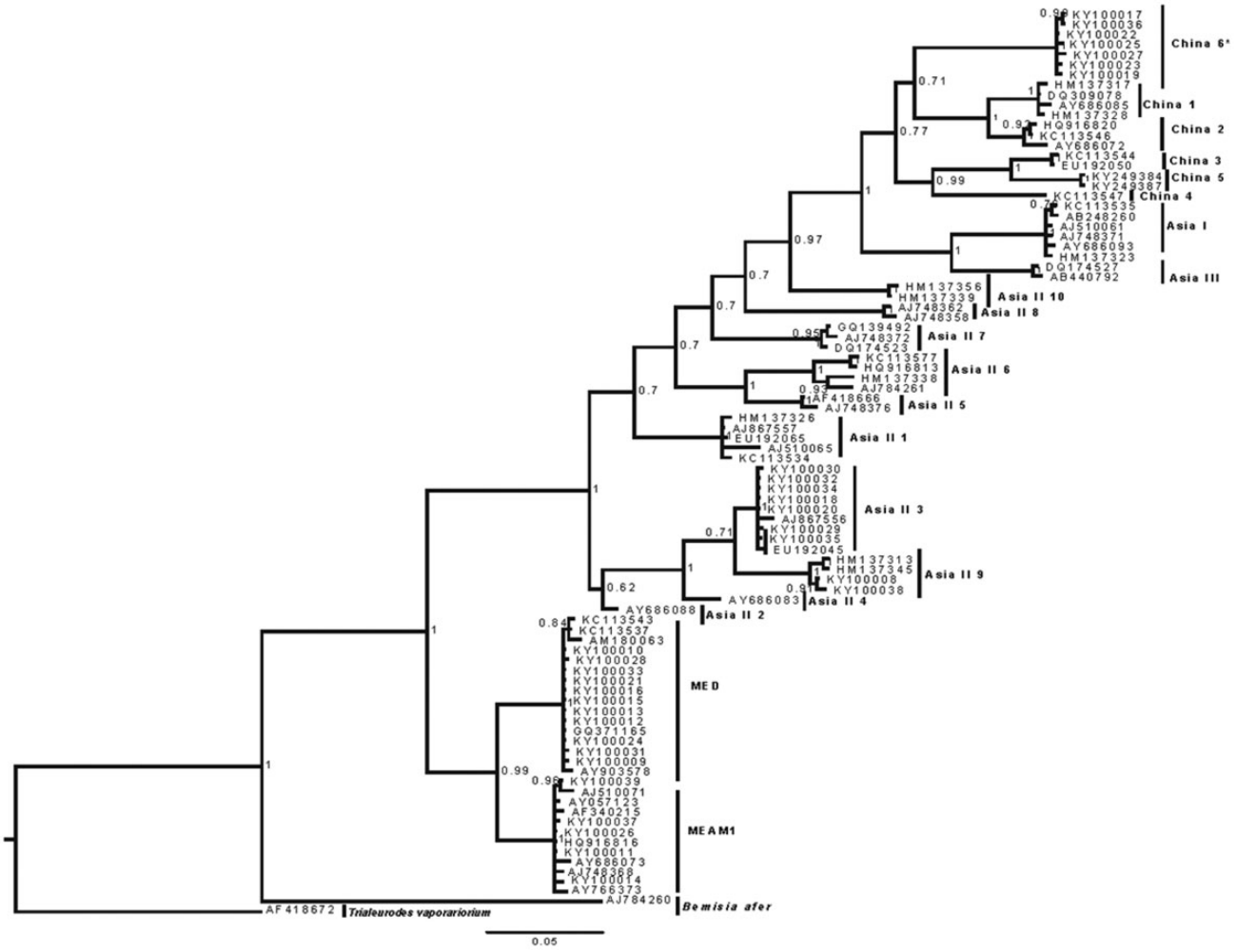

Fig. 2. Phylogenetic tree based on the Bayesian analysis of mtCOI sequences. Posterior probabilities are indicated at nodes. Species indicated by * was identified as being new species when pairwise sequence divergence exceeded 3.5%.

Discussion

Understanding cryptic species composition and diversity within the *B. tabaci* complex is critical for developing sustainable and effective approaches for *B. tabaci* and whitefly-transmitted diseases control. Our survey shows that both MED and MEAM1 are widely distributed across Henan province (Table 1 and Fig. 1). Though the overall distribution of the two invaders largely overlapped, we observed a considerable disparity in relative abundance in open field crops (Table 1). Furthermore, the survey data suggest that the existence of MEAM1 was not found in some regions including Puyang, Anyang, Hebi, Xinxiang, Luohe and Xinyang (Table 1). Chu et al. (2006) reported that *B. tabaci* MEAM1 and MED cryptic species were found in Zhengzhou. Ji et al. (2010) reported that *B. tabaci* MEAM1 and MED cryptic species were found in Zhengzhou. Hu et al. (2011) reported that *B. tabaci* MEAM1 and MED cryptic species were found in Henan. Rao et al. (2011) reported that *B. tabaci* MEAM1 cryptic species was found in Xinyang. In this study, we only found MED cryptic species in Xinyang and the ratio of MED identified was much higher than MEAM1 in Zhengzhou. From these results, we can know that MED is displacing the earlier invader MEAM1 in Henan province. The same displacement also has been reported in other provinces of China such as Shandong (Chu et al. 2010), Hubei (Rao et al. 2011) and Zhejiang (Hu et al. 2011). The effects of differential resistance to various insecticides of MED and MEAM1 has been shown by Crowder et al. (2010a,b) to be a key factor in MED's capacity to displace MEAM1. As far as we know, the control of harmful insects in Henan province is mainly depended on insecticides (Zhang et al. 2016), which may lead MED to spread and displace MEAM1 continuously.

Aside from MED and MEAM1, the data also show that the presence of three indigenous *B. tabaci* cryptic species including Asia II 3, Asia II 9 and China 6 in Henan province (Fig. 2). This is the first time to report the existence of the indigenous *B. tabaci* cryptic species in Henan province. The indigenous *B. tabaci* cryptic species were successively found in other provinces of China from 2006 to 2016 (Zang et al. 2006; Qiu et al. 2007; Hu et al. 2011, 2014; Rao et al. 2011; Guo et al. 2012; Xu et al. 2014; Li et al. 2016). By 2016, the number of the indigenous *B. tabaci* cryptic species has reached 13 and the indigenous cryptic species in different provinces are not the same. It can be seen that the indigenous *B. tabaci* cryptic species are not only distributed in the south and the southeast coastal areas of China, but also can be found in the central regions of China. However, all of the researches demonstrated that the indigenous *B. tabaci* cryptic species remained with low prevalence in agricultural areas of China.

MED and MEAM1 have invaded many parts of the world and in many regions has displaced the indigenous population of *B. tabaci* (Brown et al. 1995, Boykin et al. 2007, Liu et al. 2007). Our data showed that the complete displacement of the indigenous population of *B. tabaci* by invading MEAM1 and MED has not yet occurred in Henan province. This study speculated that the indigenous population of *B. tabaci* still exist and may be due to the complex terrain in southern region of Henan province, the mild climate conditions and the small amount of insecticide application, and which may also affect the competing between the indigenous and invasive cryptic species. Hu et al. (2011) reported that indigenous cryptic species occurred more frequently in areas of less intense, small plot farming activity where they were observed to feed on hosts such as *Ipomcea batatas*, *Glycine max*, and *Humulus japonicas*. Our results also showed that indigenous cryptic species occurred more frequently in mountain area on hosts such as *Ipomcea batatas*, *Glycine max*, and *Sesamum indicum*. Indigenous cryptic species may have a specific preference to these host plants, therefore, this may be a key factor that indigenous cryptic species has not been replaced by the invasive cryptic species. The mechanisms of competitive replacement between the indigenous whitefly cryptic species and the invasive ones are worthy of further study.

This current study for the first time unifies our knowledge of the diversity and distribution of *B. tabaci* across Henan province of China. The data from RAPD-PCR and the mtCOI based phylogeny and pairwise sequence divergence analysis supported the existence of five cryptic species namely MED, MEAM1, Asia II 3, Asia II 9 and China 6 in Henan province. The information generated here could be useful for monitoring future patterns of whitefly population diversity, abundance and displacement. It would be interesting to conduct more intensive surveys in Henan province to evaluate whether the diversity of *B. tabaci* cryptic species is higher than that described so far.

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