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Distribution and dynamics of *Bemisia tabaci* invasive biotypes in central China

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Abstract

The tobacco whitefly, *Bemisia tabaci* (Gennadius), causes severe crop losses in many agricultural systems. The worst of these losses are often associated with the invasion and establishment of specific whitefly biotypes. In a comprehensive survey of biotypes present in central China between 2005 and 2007, we obtained 191 samples of *B. tabaci* from 19 districts in Hubei province and its surrounds. Biotypes were identified by RAPD-PCR and by sequencing the mitochondrial cytochrome oxidase I gene (mtCO1). We determined that these central Chinese haplotypes included the world's two most invasive *B. tabaci* biotypes (B and Q) and two indigenous biotypes (ZHJ1 and ZHJ3). The B biotype shared >99.7% identity with other Chinese B biotypes and the Q biotype shared >99.5% of its identity with Q samples from the Mediterranean, USA, Africa and East Asia. By 2007, the Q biotype was dominant over much of Hubei province and appeared to be supplanting all other biotypes, although both the invasive and indigenous biotypes existed in sympatry in some regions. The invasion and rapid establishment of the Q biotype in China mirrors events elsewhere in the world, and we suggest that this is a consequence of its reproductive isolation, its polyphagous nature and its broad-spectrum resistance to insecticides. Its dominance has severe implications for the sustainability of some insecticide groups and for the production of a number of crops.

Keywords: *Bemisia*, biotypes, diagnostics, invasions

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Introduction

The whitefly *Bemisia tabaci* (Gennadius) is one of the most devastating agricultural pests of tropical and subtropical areas. It is present in America, Europe, Africa, Asia and Oceania (Brown *et al*., 1995a; Oliveira *et al*., 2001; Boykin *et al*., 2007) and causes serious damage to vegetables, ornamental crops and cotton by direct feeding (Yokomi *et al*., 1990), the promotion of sooty moulds which exploit excreted honeydew and inhibit photosynthesis (e.g. Byrne

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et al., 1990) and the transmission of plant viruses (e.g. the begomoviruses: Feng *et al*., 2001; Oliveira *et al*., 2001; Rekha *et al*., 2005).

B. tabaci exists as a number of genetically distinct groups (Boykin *et al*., 2007) which are distinguishable through the analysis of biochemical markers, such as esterases (Perring *et al*., 1993; Brown *et al*., 1995b, 2000), and through molecular diagnostics, such as restriction fragment length polymorphism (RFLP: Abdullahi *et al*., 2004), random amplified polymorphic DNA (RAPD: Gawel & Bartlett, 1993; Bellows *et al*., 1994; De Barro & Driver, 1997; Qiu *et al*., 2003; Chu *et al*., 2007), amplified fragment length polymorphism (AFLP: Cervera *et al*., 2000; Zhang *et al*., 2005), mitochondrial CO1 gene sequencing (Frohlich *et al*., 1999; Boykin *et al*., 2007, Dinsdale *et al*., 2010) and ribosomal ITS1 gene sequencing (De Barro *et al*., 2000).

The phylogenetic relationships between these different biotypes remains under investigation, but one recent analysis of CO1 and ITS1 sequences supported the existence of 12 major forms (Boykin *et al*., 2007) whilst Dinsdale *et al*. (2010) identified 24 distinct forms by COI analysis alone. As there remains confusion over whether the groupings differentiated by these methods correspond to biological species, we refer to them in this paper as biotypes.

Among these, the B biotype is one associated with 'silver leaf' symptoms (Costa & Brown, 1991; Delatte *et al*., 2005; Sseruwagi *et al*., 2005). It has a very broad geographical distribution, widespread resistance to older insecticide groups and causes serious economic damage (Yokomi *et al*., 1990; Horowitz *et al*., 2005). Boykin *et al*. (2007) showed that it originated from the Middle East or Asia Minor. The Q biotype has also received widespread attention because of its recent and rapid global spread and its broad-spectrum resistance to both older insecticides and relatively novel insecticide groups, such as the juvenile hormone analogues and neonicotinoids (Horowitz *et al*., 2003, 2005, 2008; Luo *et al*., 2010). A number of indigenous Chinese *B. tabaci* biotypes have also been described (Zang *et al*., 2005a; Liu *et al*., 2007, Dinsdale *et al*., 2010). Some of these appear less competitive and less polyphagous than B or Q biotypes (Zang *et al*., 2005a; Liu *et al*., 2007).

The B biotype invaded China in the mid-1990s (Luo *et al*., 2002) and caused major agricultural losses in the eastern part of the country, where it appeared to displace the indigenous ZHJ1 biotype (Luo & Zhang, 2000; Liu *et al*., 2007). Another highly invasive form, the Q biotype, was recently identified from Beijing, Yunnan, Henan and Zhejiang provinces (Chu *et al*., 2007; Hsieh *et al*., 2007). These Q biotypes were separated into two subclades, both indigenous to the Mediterranean (Chu *et al*., 2008), which implies that they were introduced, possibly, through the importation of plant material. In recent years, *B. tabaci* of unidentified biotype have continued to have a serious impact upon vegetable and cotton production in Hubei province, central China (Zhou & Dong, 2006).

In this paper, we document the distribution of *B. tabaci* biotypes in Hubei and its surrounds over a three year period, and we provide evidence of rapid and ongoing changes in biotype composition. We characterised 191 *B. tabaci* samples by RAPDs. The mitochondrial CO1 genes of a subset of 56 samples were also examined. This allowed a phylogenetic analysis of the biotypes that we collected and the corroboration of our RAPD results with a more repeatable technique.

Materials and methods

From September 2005 to October 2007, we collected *B. tabaci* from 15 districts in Hubei and four surrounding provinces (Henan, Anhui, Chongqing, Jiangxi; fig. 1, table 1). In total, 191 samples were taken, each from a specific crop or weed host (table 2). These were stored in 75% ethanol at −20°C.

DNA extraction

Total DNA was extracted from each sample (Luo *et al*., 2002). Individuals were homogenized in 20 µl extraction buffer (50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, and 1% SDS) on parafilm. The extract was incubated at 60°C for 3 h, and then 2 µl of proteinase K was added. Distilled water (78 µl) was added and incubated at 100°C for 5 min. After incubation, 100 µl of the extract was mixed with 200 µl absolute alcohol. The sample was then placed at −20°C for 2 h and centrifuged at 12,000 rpm for 20 min. The suspensions were decanted and the DNA samples were re-suspended in 20 µl TE buffer (pH 8.0) and stored at −20°C.

RAPD-PCR reaction

The samples of *B. tabaci* were analysed by RAPD-PCR to detect B, Q and other biotypes according to the methods of De Barro & Driver (1997) and Chu *et al*. (2007). Known samples of B, Q and indigenous biotypes (ZHJ1, ZHJ2 and ZHJ3, respectively) were used as standards. And the mtDNA CO1 sequences analysis was employed to identify the ambiguous samples. The primer H16 (5′-TCTCAGCTGG-3′) was used as a marker to generate the RAPD profile. RAPD-PCR reactions were carried out in a final volume of 25 µl containing 2.5 µl of 10× PCR buffer, 0.25 mM of each dNTP, 0.25 mM of $MgCl_2$, 20 ng of primer, 1.5 U *Taq* DNA polymerase and 10–20 ng of DNA. The amplification was done according to the following parameters: 5 min at 94°C, 2 min at 40°C and 3 min at 72°C, followed by 39 cycles of 1 min at 94°C, 1.5 min at 40°C and 2 min at 72°C. The products of amplification were analyzed on 1.5% agarose gels.

PCR and sequence analysis of mtDNA CO1 genes

One individual from each of a subset of 56 samples was used for the analysis of the mtCO1 gene sequence. DNA was extracted as above and PCR reactions (Luo *et al*., 2002) were carried out in a final volume of 20 µl containing 1 U Taq DNA polymerase, 2.5 mM $MgCl_2$, 0.25 mM dNTP, 2 mg ml^{-1} BSA, 2 µl of DNA and 2.5 ng μl^{-1} of each primer (Cl-J-2195: 5′-TTGATTTTTTGGTCATCCAGAAGT-3′ and L2-N-3014: 5′-TCCAATGCACTAATCTGCCATATTA-3′) (Simon *et al*., 1994; Frohlich *et al*., 1999). The amplification followed these steps: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and a final elongation during 5 min at 72°C. The products of amplification were analyzed on 1.6% agarose gels. The amplified products were gel-purified using DNA Clean/Extraction Kit (Promega, Madison, WI, USA) and sequenced in one direction on an ABI 3730XL DNA analysis system using TIAN gel Mini/Midi Purification Kit (Tiangen, Beijing, China).

Fig. 1. Distribution of *B. tabaci* samples from 15 districts of Hubei province and four districts of neighbouring Chinese provinces 2005–2007. Insert is a sketch map of China highlighting the area sampled.

Table 1. Biotype and location of 191 *B. tabaci* samples.

Year of collection	Place of collection (district, province)	Biotype and number of samples			
		Q	B	ZHJ1	ZHJ3
2005	Wuhan, Hubei	2	5	1	
2006	Huangshi, Hubei	5			
	Wuhan, Hubei	21	3	1	
	Xianning, Hubei	2			2
	Xiaogan, Hubei	2	1		1
	Xiantao, Hubei	6			
	Jingzhou, Hubei	1		2	2
2007	Huangshi, Hubei	4			
	Huanggang, Hubei	16		1	
	Ezhou, Hubei	3			
	Wuhan, Hubei	6			
	Xianning, Hubei	4			
	Xiaogan, Hubei	10	1		
	Xiantao, Hubei	5			
	Suizhou, Hubei		6		
	Tianmen, Hubei	3			
	Qianjiang, Hubei	9			
	Jingzhou, Hubei	16		2	1
	Jingmen, Hubei	6			
	Xiangfan, Hubei	3	3	1	2
	Yichang, Hubei	7	8		
	Shiyan, Hubei				4
	Hefei, Anhui	3			1
	Nanchang, Jiangxi	2			1
	Chongqing, Chongqing				3
	Xinyang, Henan		3		

Table 2. Host-plants and biotypes of 191 *B. tabaci* samples.

Host-plant	Common name	Biotype and number of samples			
		Q	B	ZHJ1	ZHJ3
Brassica oleracea	cabbage	4	1		
Brassica chinensis	pakchoi	1			
Capsicum frutescens	pepper	16			
Cucurbita moschata Duch	squash	6	5		4
Cucumis sativus	cucumber	18	4		3
Cucumis melo	melon	1			
Euphorbia pulcherrima Willd	poinsettia	1			
Gossypium hirsutum	cotton	31	9	8	2
Glycine max(L) Merr.	soybean	1			
Humulus scandens	Japanese hop	9			1
Ipomoea batatas	sweet potato	4			2
Lycopersicon esculentum Mill.	tomato	5	3		
Nicotiana tabacum	tobacco	1	2		
Raphams sativus	radish	4			
Solanum melongena	aubergine	29	5		5
Solanum tuberosum	potato		1		
Vigna sinensis	cowpea	5			

Data analysis

The sequence fragments that resulted from the mtDNA CO1 assay were aligned using ClustalX (Thompson *et al.*, 1997). Different locations and haplotypes were chosen for phylogenetic analysis. mtDNA CO1 sequences were obtained from several additional *B. tabaci* collections to serve as geographic references. One sequence of *Bemisia afer* (Priesner & Hosny) (AJ784260) was also used as an outgroup (see fig. 2). The evolutionary divergence of different samples of *B. tabaci* based on mtDNA CO1 used the Kimura 2-parameter model and an unweighted pair-group method analysis (UPGMA) to build a dendrogram using the software MEGA 3.0 (Kumar *et al.*, 2004). One thousand bootstrap replicates were performed for each analysis.

Nucleotide sequence accession numbers

Nucleotide sequence accession numbers for the sequences submitted to the GenBank nucleotide sequence database are shown in fig. 2.

Results

Identification of biotypes

Using primer H16, four different biotypes were identified by RAPD-PCR. These were subsequently confirmed by mtCO1 sequencing as representing two exotic biotypes (B and Q) and two indigenous ones (ZHJ1 and ZHJ3). Consistently reproducible banding patterns of 250–1000 bp fragments differentiated the biotypes. Of the 191 samples analysed by RAPD-PCR, the ratio of Q : B : ZHJ1 : ZHJ3 was 136 : 30 : 8 : 17.

Sequence analysis of mtCO1 gene of B. tabaci

The sequences of the mitochondrial CO1 genes of a subset of 56 of the 191 Chinese samples were analysed (fig. 2). All samples of *B. tabaci* fell into one of four clusters representing the B, Q, ZHJ1 and ZHJ3 biotypes (fig. 2). A *B. afer* sample clustered separately as an out-group, sharing 77–74.8% of its identity with all *B. tabaci*. The B biotype cluster comprised samples from the USA, Beijing, Shaanxi and Guangzhou, and ten central Chinese samples. These shared 99.7–100% of their nucleotide sequence. The Q biotype cluster contained samples from USA, Morocco, Spain and Japan, and 42 central Chinese samples. These shared 99.5–100% of their nucleotide sequence. Individuals belonging to each indigenous cluster were almost identical (ZHJ1: 99.5–100%; ZHJ3: 99.5–100%). Overall, the sequence variation indicated four different biotypes among the samples, commensurate with the four groupings identified through RAPD analysis. There were no discrepancies. A phylogenetic tree was developed using a 602 bp portion of the CO1 sequence (fig. 2).

Trends in spatial distributions

The first samples of *B. tabaci* were collected from Wuhan, Hubei province in 2005 (fig. 1). Eight samples were analysed and separated into the categories Q, B, ZHJ1 and ZHJ3 in the ratio 2 : 5 : 1 : 0. In 2006, a further 49 samples from six districts of Hubei (including the sites sampled in 2005) were analysed. These samples had a Q : B : ZHJ1 : ZHJ3 ratio of 37 : 4 : 3 : 5. The Q biotype of *B. tabaci* was clearly predominant at this stage (fig. 3). In 2007, a still more extensive survey was undertaken to determine the spread of the Q biotype. The 134 samples sampled from 19 districts comprised Q : B : ZHJ1 : ZHJ3 in the ratio 97 : 21 : 5 : 11. The ratios differed between the southeast and the north and west (figs 1 and 3). All samples from the southeast were $\geqslant$90% Q biotype. In the northwest, non-Q biotype samples were more common than in the southeast: Q : B : ZHJ1 : ZHJ3; 7 : 8 : 0 : 0 (Yichang); 16 : 0 : 2 : 1 (Jingzhou); 3 : 3 : 1 : 2 (Xiangfan); 0 : 6 : 0 : 0 (Suizhou); and 0 : 3 : 0 : 0 (Xinyang) (fig. 3). Samples from Shiyan (0 : 0 : 0 : 4) and neighbouring Chongqing (0 : 0 : 0 : 3) province were exclusively of the ZHJ3 biotype.

Over the course of the study, the proportion of the B biotype in the southeast of Hubei steadily decreased as the Q biotype became dominant (fig. 3). This is most obvious in the Wuhan samples collected over all three years ($\chi^2 = 13.7$, df = 4, $P = 0.008$). The proportion of indigenous biotypes in Jingzhou, Xiaogan, Xianning and Wuhan also decreased

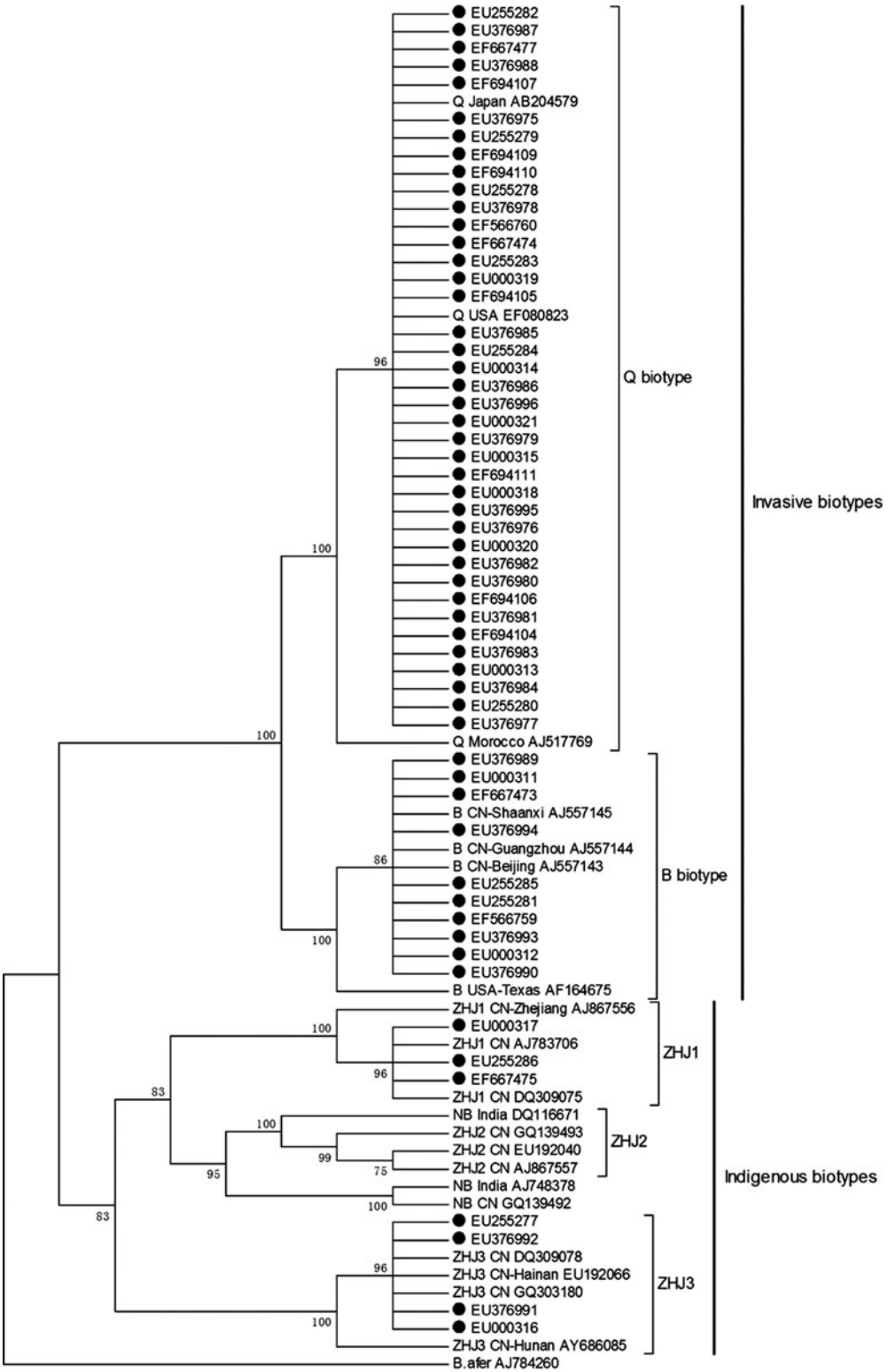

Fig. 2. Phylogenetic tree of mtDNA CO1 sequences for *B. tabaci* using UPGMA method with bootstrap test of phylogeny. The outgroup was *B. afer*. The code of sequences from GenBank includes the biotype, location and Genbank accession number. ●, sequences of mitochondrial cytochrome oxidase DNA from this study; CN, China; NB, no B/Q biotype.

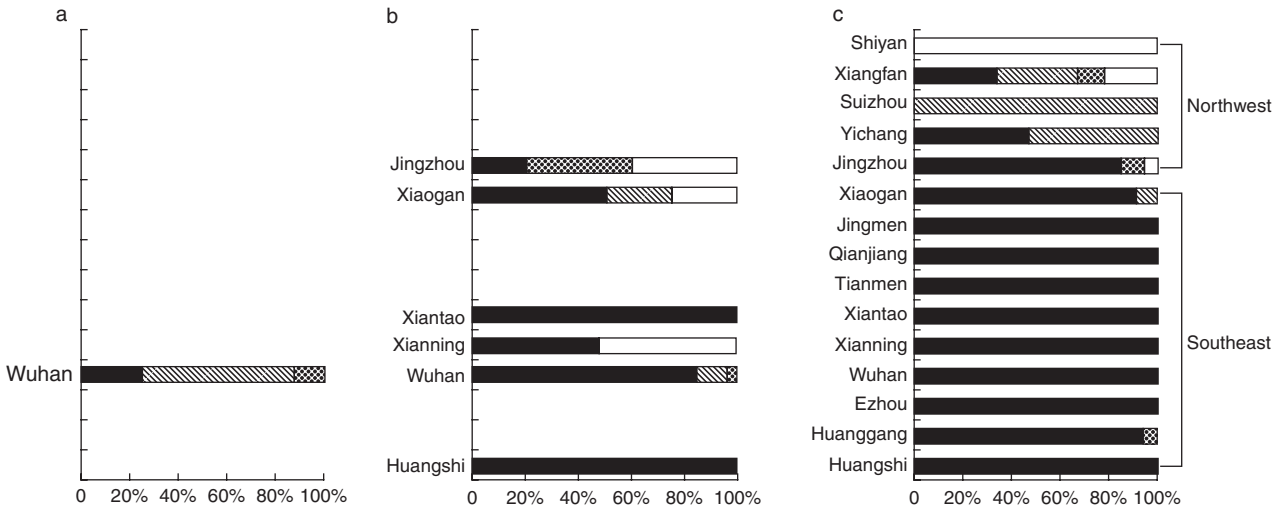

Fig. 3. The mean proportions of *B. tabaci* biotypes from samples sampled in Hubei province: (a) 2005, (b) 2006, (c) 2007 (■, Q; ▨, B; ▩, ZHJ1; □, ZHJ3).

steadily as the Q biotype increased (e.g. the Jingzhou samples, $\chi^2 = 8.19$, df = 2, $P = 0.017$) (fig. 3). Our sampling also revealed that, as expected, Q and B biotypes are broadly polyphagous. With a single exception, all biotypes collected from the weed *Humulus scandens* L. were of the Q form ($n = 9$). ZHJ1 was not found on any plant except *Gossypium hirsutum* L. ($n = 8$), while ZHJ3 was found on a wide range of hosts. Overall, the ratio of Q : B : ZHJ1 : ZHJ3 samples on different host plant groups was: cotton 31 : 9 : 8 : 2, weeds 9 : 0 : 0 : 1, vegetables 95 : 19 : 0 : 14 and tobacco 1 : 2 : 0 : 0 (see table 2).

Discussion

This study presents a detailed characterisation of the temporal and spatial dynamics of *B. tabaci* biotypes in central China. Analysis of RAPD banding patterns and cluster analyses of mtDNA CO1 sequences revealed the presence of four different groupings of *B. tabaci* in the region. These results confirm that the B biotype has a nationwide distribution in China (Luo *et al*., 2002; Qiu *et al*., 2003; Zhang *et al*., 2005; Ma *et al*., 2007). Sequencing of the CO1 gene showed that all B samples from around the world, including our own, share 99.7%–100% nucleotide identities. The B biotype insects in China have clearly resulted from one, or a number, of invasive events, beginning at least as far back as the 1990s (Luo *et al*., 2002; Zhang *et al*., 2005; Hsieh *et al*., 2007; Liu *et al*., 2007).

Dalmon *et al*. (2008) found that Q had spread very quickly and that the lack of spatial genetic structure among all Q populations again indicates recent colonization events and a massive competitive advantage. The Q biotype invaded more recently (Zhang *et al*., 2005; Hsieh *et al*., 2007) and, during the course of our study, has become increasingly dominant in Hubei province. It is now distributed widely over the Jianghan plain (Hubei), an agricultural area with highly developed transport links and intensive and extensive farming.

In 2005, our own farmer surveys in Hubei suggested that *B. tabaci* was strongly associated with severe effects on vegetable and cotton production. This realisation was the trigger for our initial brief survey of the Wuhan area. Between 2005 and 2007, the Q biotype established and became dominant over a large area of central China. In the north and west of our study area, fragmented agricultural landscapes and mountainous regions appeared to hinder the spread of the Q biotype and indigenous *B. tabaci* biotypes continued to persist in this more isolated region. The presence of ZHJ1, ZHJ2 and ZHJ3 biotypes in Hubei, Shandong and Zhejiang has been noted previously (Zhang *et al*., 2005; Zang *et al*., 2006; Liu *et al*., 2007). More recently, Dinsdale *et al*. (2010) described the COI sequences of a number of other indigenous biotypes from China. Most of these sequences originated from individuals collected from south and east China. Only the ZHJ3 group was described from central China. The non B or Q COI sequences described in the current paper clustered only with the ZHJ1 and ZHJ3 sequences described by Dinsdale *et al*. (2010).

The spread of the Q biotype has been associated with the global trade in ornamental plants (Brown *et al*., 2000; Horowitz *et al*., 2005). Hsieh *et al*. (2007) suggested that the Q biotype in Taiwan had entered on poinsettia plants originating from Italy in 2006. Chu *et al*. (2008) showed that Chinese Q biotype from the northeast Pacific region clustered with Q biotype from the Mediterranean. We suggest that the Q biotype from central China also originated from the Mediterranean, having spread throughout the Chinese mainland via the transport of vegetables, seedlings and ornamental crops.

All eight samples that were identified as ZHJ1 were taken from cotton. This suggests a narrow host range, and a strong association with *G. hirsutum*. This was also reported by Zang *et al*. (2005a). All other biotypes (B, Q and ZHJ3) were more polyphagous, again in agreement with previously published data (Bellows *et al*., 1994; Perring, 2001; Zhang *et al*., 2005). It is notable that, in our survey, the Q biotype had a stronger association with weed hosts than the other biotypes. In Spain, the Q biotype was also better adapted to weed hosts and highly competitive with the B biotype on some commercial crops (Nombela *et al*., 2001; Muniz & Gloria,

2001). The increasing rarity of indigenous biotypes in central China, driven by alien invasions, is mirrored in eastern China, where the B biotype may outcompete the indigenous ZHJ1 on a variety of commercially grown plants (Liu *et al*., 2007; Zang *et al*., 2005a,b). The B biotype has been shown to be more fecund than Chinese and Australian biotypes and neither is there any introgression between them (Liu *et al*., 2007). A combination of these competitive advantages and mating interactions appears to have led to the effective extinction of indigenous genotypes in some regions.

One factor that we suspect is of key relevance to the distribution of biotypes in central China relates to insecticide use patterns and the differential presence of resistance mechanisms in the various biotypes. B and Q biotypes have both evolved resistance to neonicotinoids through the overexpression of a P450 gene, CYP6CM1, but resistance is particularly potent and widespread in the Q biotype (Karunker *et al*., 2008). In a recent comparison of B and Q biotypes from China (Luo *et al*., 2010), the Q biotype exhibited far higher resistance to neonicotinoids than B biotype. When resistance to a key insecticide is associated with a single biotype, insecticide use will be a powerful determinant of biotype composition. We would, therefore, expect the Q biotype to predominate in regions where its common hosts are regularly treated with neonicotinoids. The manufacture of imidacloprid in China began in 1992 and was first used in Hubei to control the plant hopper *Nilaparvata lugens*. It has since become a standard treatment for many sucking pests, including whitefly, on a variety of crops. Approximately 650,000 kg of neonicotinoids were used annually between 2005 and 2008 and the area under neonicotinoid treatment in Hubei is 6500 km^2 per year (Peng, personal communication). This reflects multiple applications to the same areas.

The overall ascendancy of the Q biotype on the agricultural plains of central China probably results from its biotype-specific ability to withstand neonicotinoid treatment, its polyphagous and competitive nature and perhaps its reproductive isolation. Its presence has profound implications for the sustainability of insecticide use and for the agricultural economy of the region.

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