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Genetic Population Structure of *Bemisia tabaci* in Spain Associated with *Tomato Leaf Curl New Delhi Virus* – Short Communication

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Abstract

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Tomato leaf curl New Delhi virus (ToLCNDV) originates from Asia where it is persistently transmitted by indigenous cryptic species of the whitefly *Bemisia tabaci*. The virus has recently invaded Spain, Tunisia, and Italy, and to investigate whether whitefly species new to the Mediterranean are involved, 35 populations were collected during 2015 from different crops in different regions of southern Spain. Comparison of partial mitochondrial cytochrome oxidase I sequences from the collected whiteflies revealed the existence of 7 different haplotypes belonging to the Mediterranean-Q1 cryptic species. ToLCNDV was detected in 15 populations collected from tomato, zucchini, and melon crops and from 5 different localities. The results suggest that MED-Q1 is also responsible for the current spread of ToLCNDV in Spain.

Keywords: whitefly; begomovirus; zucchini squash

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is present in most tropical and subtropical regions in the world where it is one of the most serious threats to economically important crops (EFSA Panel on Plant Health 2013). It is considered as a complex of indistinguishable morphocryptic species which have been identified comparing mitochondrial cytochrome oxidase I (mtCOI) DNA sequences (De Barro *et al.* 2011), and this led to the identification of 24 distinct species: Mediterranean (MED), Middle East-Asia Minor (MEAM) 1 and 2, 2 Australian, 14 Asian, 4 Sub-Saharan Africa and Uganda species, and New World species. MEAM1 (formerly known as biotype B), and MED (or biotype Q) are of particular importance: they are found highly polyphagous, resistant to a large number of insecticides. MEAM1 is believed to have originally merged from northeastern Africa, the Middle East, and the Arabian Peninsula, and the MED species comes from the Saharan and Sub-Saharan Africa; yet, both species have been found to be very invasive and are now worldwide distributed (De Barro *et al.* 2000).

Apart from a direct damage to crops caused by feeding on the plants, *B. tabaci* acts as a vector for more than 120 plant viruses, and particularly begomoviruses (Jones 2003). *Tomato leaf curl New Delhi virus* (ToLCNDV) is a bipartite whitefly-transmitted begomovirus which was first described on tomatoes in India in 1995 (Padidam *et al.* 1995). Soon after, other Asian countries reported the occurrence of ToLCNDV on both solanaceous and cucurbitaceous crops (Mizutani *et al.* 2001). In September 2012, symptoms caused by ToLCNDV were first observed on zucchini squash (*Cucurbita pepo*) produced in the open field in the regional community of Murcia, in the south of Spain. In May 2013, the symptoms were found in the same crop, but produced in greenhouses from Almeria province, and by autumn 2013, the disease was widespread in both Spanish regions (Juárez *et al.* 2014; Ruiz *et al.* 2015). In January 2015, ToLCNDV

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was detected for the first time in Tunisia, causing a severe disease on melon, cucumber, and zucchini squash, grown in greenhouses (Mnari-Hattab *et al.* 2015). In October 2015, the virus was reported by growers of zucchini squash in open fields in the horticultural area of the Province of Trapani (Sicily, Italy) and the virus was subsequently identified (Panno *et al.* 2016). ToLCNDV is now considered an emerging virus in the Euro-Mediterranean region and is included in the EPPO Alert List.

Crops in the countries where ToLCNDV is established, such as India and Pakistan, are found infested with *B. tabaci* species Asia 1, Asia II 5, Asia II 1, Asia II 7, and MEAM1 (De Barro & Ahmed 2011). This latter species is also found in Taiwan (CABI 2015) but not in the Philippines (Sanchez & Caoili 2015). Although the invasive MEAM1 species was occasionally found to be a major vector for transmission of begomoviruses in India, ToLCNDV has been found transmitted mostly by indigenous *B. tabaci* populations (Maruthi *et al.* 2007). None of these indogenous Asian-type *B. tabaci* species has so far been detected in Spain. *B. tabaci* was reported in Spain for the first time in 1943 (Gómez-Menor 1943), but only in the late 1980s did it become a primary pest. Historically, both MEAM1 and MED species have been identified in greenhouse and outdoor crops in Spain (Guirao *et al.* 1997).

The recent introduction and spread of ToLCNDV in Mediterranean countries of Spain, Tunisia, and Italy need to be studied in order to develop adequate control measures. In Spain, *B. tabaci* whiteflies have been collected from tomato, eggplant, pepper, pumpkin, cucumber, melon, and zucchini squash crops across different regions within southern Spain after ToLCNDV detection. These populations were partially sequenced to identify the cryptic species and extracts were also analysed for ToLCNDV.

MATERIAL AND METHODS

Insects. Samplings were carried out in 2015 in 28 locations from the regional communities of Andalusia and Murcia, and on 7 different cultivated host plant species: tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), pumpkin (*Cucurbita maxima*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*), and zucchini squash (*Cucurbita pepo*) (Figure 1 and Table 1). Five adults each from a total of 35 *B. tabaci* whitefly populations

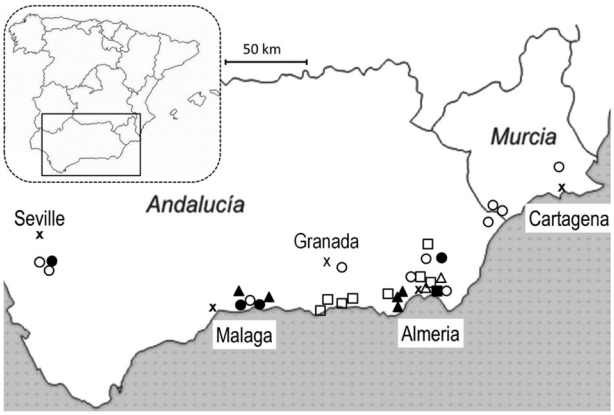

Figure 1. Location of the 35 cultivations where the *B. tabaci* adults where collected between June and September of 2015

The cultivations were pumpkins (Δ), zucchini squash (▲), cucumber (□), melon (■), tomato (○), pepper (●), and eggplant (◺). Mayor cities are shown (X). The rectangle represents the relative position of the surveyed are with respect to the map of Spain

were collected with a handheld aspirator, and were preserved immediately in 95% ethanol at –20°C for later processing. The insects were morphologically sexed under a binocular (40×) stereomicroscope, as described in Calvert *et al.* (2001), before DNA extraction.

DNA extraction. Total nucleic acid extractions from individual whiteflies were done according to Cenis *et al.* (1993) with minor modifications (Gil-Salas *et al.* 2007). After they were washed with double-distilled water to remove ethanol, individual whiteflies were placed, using wooden toothpicks, in a 0.5 ml microcentrifuge tube with 100 µl extraction buffer (200 mmol/l Tris-HCl, pH 8.5, 250 mmol/l NaCl, 25 mmol/l ethylenediaminetetraacetic acid, 0.5% w/v sodium dodecyl sulphate) and ground using a pellet grinder (Anachem Ltd., Luton, UK). Fifty µl of 3 mol/l sodium acetate (pH 5.2) was added, and tubes were incubated at –20°C for 10 min followed by centrifugation for 5 minutes. The pellet was discarded and one volume of iso-propanol (–20°C) was added. The tube contents were mixed well and incubated at room temperature for 30 minutes. Following centrifugation for 30 min the pellet was washed with 70% ethanol and vacuum dried. The resulting pellet was resuspended in 25 µl diethylpyrocarbonate-treated water and stored at –80°C.

Insect mitochondrial and ToLCNDV genome DNA amplification. Whitefly mtCOI gene amplification by PCR and sequencing were carried out essentially as described by Frohlich *et al.* (1999): the two

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Table 1. Populations of *Bemisia tabaci* collected from different hosts and localities in Andalusia and Murcia in southern Spain during the year 2015 of the state of São Paulo. GenBank sequence numbers and PCR detection of ToLCNDV

Sample ID	Location	Province (Community)	Crop	GenBank No.	ToLCNDV
1	Viator	Almeria (A)	cucumber	KU840834	+
2	El Ejido		cucumber		+
3	La Mojonera		zucchini squash		+
4	Vícar		zucchini squash		+
5	Roquetas de Mar		zucchini squash		+
6	Vícar		eggplant		-
7	Santa María del Águila		eggplant		-
8	Vícar		pumpkin		-
9	El Alquiàn		pumpkin		-
10	La Cañada		pumpkin		-
11	La Cañada		cucumber		+
12	Viator		tomato		+
13	El Alquian		melon		+
14	San Isidro		tomato		+
15	Los Albaricoques		eggplant		-
16	Tahal		cucumber		+
17	Tabernas		tomato		+
18	Uleila del Campo		pepper		-
19	Castell de Ferro	Granada (A)	cucumber		+
20	Albuñol		cucumber		+
21	La Rábita		cucumber		+
22	Bérchules		tomato		-
23	Carchuna		cucumber		-
24	Torrox	Malaga (A))	pepper		-
25	Velez Málaga		zucchini squash		+
26	Velez Málaga		pepper		-
27	El Trapiche		zucchini squash		+
28	Pulpí	Almeria (A)	tomato		-
29	El Moche	Malaga (A)	tomato	KU840828	+
30	Águilas	Murcia (M))	tomato	KU840822	-
31	Águilas		tomato		-
32	El Charco		tomato	KU840827	-
33	Los Palacios	Seville (A)	tomato	KU840829	-
34	Los Palacios		pepper	KU840830	-
35	Villafranca		tomato	KU840835	+

primers used in the PCR reaction were C1-J-2195 (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and L2-N-3014 (5'-TCCAATGCACTAATCTGCCAT-ATTA-3'). The PCR reaction was performed in a 50-µl volume containing 2 µl template DNA, 2 units Taq polymerase, 5 µl 2.5 $MgCl_2$ (25 mmol/l), 2 µl dNTPs (10 mmol/l), 1 µl of 20 µmol/l of each primer, and 5 µl of 10 × PCR buffer (Ferments Inc., Glen Burnie, USA). The amplification procedure was: 95°C for 2 min for pre-denaturation; followed by 30 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and a post-cycle incubation at 72°C for 10 minutes. After that, 5 µl of PCR reaction was electrophoresed using 1.0% agarose gels. When bands of the expected size of approximately 850 bp were visible in the gels, the other 45 µl of PCR reac-

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tion was used for sequencing. For each population, at least three individuals were sequenced. Unique mtCOI sequences were submitted to GenBank, and their accession numbers are shown in Table 1. Partial ToLCNDV genome amplification was done essentially as described by Ruiz *et al.* (2015): the same total nucleic acid extractions from the whiteflies were used for PCR amplifications using primer pair 768 (5'-AGCACAGCCACGGTGAAGAAC-3') and 2028 (5'-TTTCATCCTTCGACAGAGTTC-3') designed to amplify a fragment of 1260 bp matching the sequence of the DNA-A component of ToLCNDV (GenBank KF891468). The PCR reactions were performed as described above in 50-µl volume containing 2 µl template DNA, but the amplification cycles were 35 times repeated, at 94°C for 35 s, 55°C for 35 s, and 72°C for 50 seconds.

Data analysis. Besides the sequences of the whitefly populations sampled in this study, the mtCOI gene sequences of 19 *B. tabaci* populations were downloaded from GenBank as reference sequences: MEAM1 populations were from Spain, China, Arizona, Israel, and the United Arab Kingdom, and sequences from reference MED species were from Burkina Faso, Syria, Cyprus, USA, Israel, France, Spain, and Algeria. One representative mtCOI gene sequence of each haplotype in the whiteflies was selected and aligned with the reference sequences. The mtCOI sequences were aligned using the MUSCLE algorithm, and the neighbour-joining trees were constructed with 1000 bootstrap replications to assess the robustness of the trees (Geneious 7.0.6; Biomatters Ltd., Auckland, New Zealand). The mtCOI sequence of *B. atriplex* (HQ457047) was used as outgroup. To ascertain the homology and genetic distance between the *B. tabaci* populations associated with ToLCNDV and the *B. tabaci* population present in Spain since 1995, mtCOI gene sequences of 17 *B. tabaci* populations were selected from GenBank: 14 MED from Spain and 3 MEAM1 populations that were used as reference (2 from Spain and 1 from Argentina). Pairwise percentage of nucleotide sequence identity was calculated between *B. tabaci* GenBank sequence No. DQ 365875, and the haplotypes identified during 2015. The analysed mtCOI gene sequences were separated into three subclades: MED sequences since 1995 to 2014, mtCOI sequences corresponding to the 7 haplotypes identified in this article, and MEAM1 sequences using Mega 6.0 (Tamura *et al.* 2013) in order to calculate the genetic distances within and between the groups.

RESULTS AND DISCUSSION

Following the sudden introduction and spread of the first bipartite begomovirus in Europe across southern Spain, we collected and characterised populations of *B. tabaci* from different crops and agricultural regions where ToLCNDV has been reported. DNA extractions from all of 35 whitefly populations collected from tomato, eggplant, pepper, pumpkin, cucumber, melon, and zucchini squash crops yielded amplicons of the expected size (approximately 850 bp) when amplified with mtCOI-specific primers. The sequences from the amplicons revealed the existence of 7 different haplotypes. One single haplotype was

Table 2. Percentages of nucleotide identity of mtCOI sequences from the *B. tabaci* haplotypes identified during 2015, haplotypes collected in Spain between 1995 and 2014, and three MEAM1 haplotypes (GenBank Nos. GU968888, AF340215, and GU086348) with MED-Q1 collected in Spain during 2000 (DQ 365875)

GenBank No.	Identity (%)	Collection date
GU968888	94.2	2003
AF340215	94.4	NA
GU086348	94.5	NA
KU840835	98.9	2015
JX437455	99.1	2010
KJ411787	99.1	2007
KU840829	99.1	2015
KF870574	99.2	2008
KU840830	99.2	2015
AF342775	99.2	2000
AY057139	99.2	1995
KU840822	99.5	2015
DQ174539	98.7	2002
KU840828	99.3	2015
AF342769	99.3	1999
DQ302946	99.6	2005
KU840827	99.6	2015
EF398126	99.7	2006
KU840834	99.7	2015
LN614545	99.7	2014
DQ365874	99.9	1998
GU472435	99.9	2011
HG421088	99.6	2013

NA – not available

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found in 28 samples from zucchini squash, cucumber, melon, tomato, pepper, eggplant, and pumpkin, from the Andalusia provinces of Almeria, Granada, and Malaga. One additional and unique haplotype was found in tomato from Malaga. Tomatoes from Murcia were infested with *B. tabaci* populations consisting of two unique haplotypes, and whiteflies collected from the crops in the province of Seville belonged to three novel and unique haplotypes (Table 1). Phylogenetic analysis of partial mtCOI sequences from these and reference populations from *B. tabaci* separated 4 different groups, representing MEAM1, MED-Q1, MED-Q2, and MED-Q3 (Figure 2). The latter three *COI*-differentiated groups are believed to have different geographical origins: the subgroup Q1 in Europe, Q2 in the Middle East, and Q3 in Africa (Gueguen *et al.* 2010). All of the 35 populations collected during the year 2015 grouped together with MED-Q1 (Figure 2). MED-Q1 of *B. tabaci* from Spain constitutes a well characterised lineage (De Barro *et al.* 2011). The comparison of nucleotide sequences from *B. tabaci* MED-Q1 (DQ 365875) with the 17 selected haplotypes from GenBank plus the haplotypes identified during 2015 showed 98.7–99.9% nucleotide identity with all the MED-Q1 Spanish haplotypes, independent of the date of collection. Also, the analysis showed less than 94.5% of nucleotide identity with the three MEAM1 haplotypes used as references (Table 2). The genetic distance within and between MED-Q1 subclades of

Table 3. Genetic distance within and between subclades average calculations based on mtCOI sequences

	MEAM1	MED from 1995	Haplotypes 2015
MEAM1	0.005		
MED from 1995	0.057	0.008	
Haplotypes 2015	0.058	0.008	0.008

1995–2014 and of 2015 was 0.008, which suggested that the *B. tabaci* population associated with ToLCNDV in Spain was exactly the same as that described previously (MED-Q1) (Table 3). Differences found between COI sequences of MED-Q1 *B. tabaci* populations were expected, because this cryptic species presents values of intrapopulational diversity which are higher than values from other species such as MEAM1 (Moya *et al.* 2001).

To date, ToLCNDV has been found associated with Asian indigenous cryptic *B. tabaci* species (Maruthi *et al.* 2007). However, among the collected 35 whitefly populations sampled, 15 reacted positive after PCR analyses with ToLCNDV-DNA-A-specific primers. All samples that contained ToLCNDV were restricted to the provinces of Andalusia (Almeria, Granada, Seville, and Malaga) and were collected from cucumber, zucchini squash, tomato, and melon (Table 1). This suggests that, apart from Asian endogenous cryptic

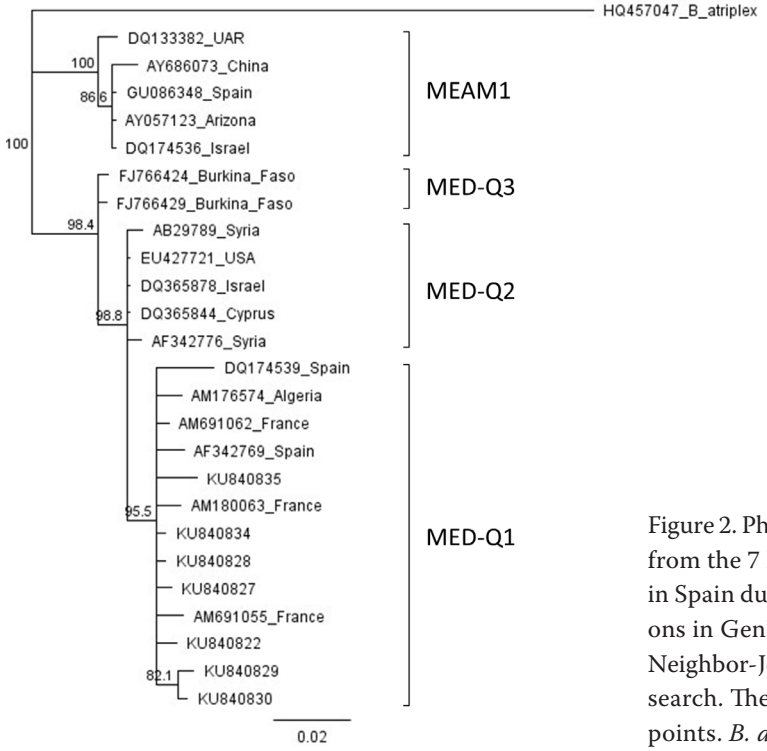

Figure 2. Phylogenetic analysis of partial mtCOI sequences from the 7 haplotypes of *B. tabaci* populations collected in Spain during 2015 (see Table 1) and selected populations in GenBank. The tree was constructed based on the Neighbor-Joining method with a 1000-replicate bootstrap search. The bootstrap values are indicated at the branch points. *B. atriplex* (HQ457047) was used as outgroup

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species, the virus would also spread by MED-Q1 for the first time, although the possibility exists that the virus entered through Asian *B. tabaci* species introduced into Europe. The probability of entry into the European Union through the importation of plants for planting is likely, because of the frequent association of insects with this pathway at origin and based on the number of interception records despite strict phytosanitary requirements for these particular commodities. Also entry of viruses that are transmitted in a circulative transmission mode (like ToLCNDV) can occur with viruliferous *B. tabaci* and with infected plants (EFSA Panel on Plant Health 2013). Despite the fact that we did not find any Asian species in the collections from 2015, it cannot be ruled out that such populations could have arrived from a region or country where ToLCNDV is endemic.

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