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ORIGINAL PAPER

Evidence for a new genetic variant in the *Bemisia tabaci* species complex and the prevalence of the biotype Q in southern Italy

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Abstract The genetic diversity of *Bemisia tabaci* was investigated in Southern Italy using the mitochondrial cytochrome oxidase I (*COI*) gene as molecular marker and sampling whiteflies on cultivated plants, weeds and bushes. Phylogenetic analysis of *COI* sequences and restriction analysis of *COI* amplicons were used to genotype whitefly populations. A PCR-RFLP method based on digestion with the endonuclease *Apo*I was set up to identify the *B. tabaci* genetic variants so far recorded from the Mediterranean region. In general, biotype Q populations were most frequently collected (76.0% of all sampled populations). In greenhouse districts, 87.0% sampled populations were identified as biotype Q, and 13.0% were assigned to biotype B. Outside the greenhouse districts, the biotype B was never collected, whilst biotype Q populations were found on weeds and on plants cultivated in family gardens in different environments, also located in interior plains or in mountain areas distant from intensive cultivations of whitefly host plants. A new genetic variant unrelated to B and Q biotypes, which was named Ru, was collected on *Rubus ulmifolius* and grapevine. Phylogenetic analysis of *COI* sequences shown that Ru haplotypes form a well-supported clade sister to the clade including the Asian/Australian major genetic groups and the Italy major genetic group. The closest relative of the Ru clade (10.7% pairwise genetic distance) was the Italy group, with this latter so far including only the haplotypes of the T biotype. These results were discussed in the light of the recent *B. tabaci* species concept.

Keywords Cytochrome oxidase I gene · Biotypes · Haplotypes · Phylogeny · Sweetpotato whitefly

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Introduction

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is one of the most invasive and injurious agricultural pests worldwide. A wide variety of horticultural, ornamental and field crops in tropical, sub-tropical and Mediterranean regions suffer heavy economic losses as a consequence of its feeding on phloem, deposition of honeydew and development of sooty mould, and mainly because this insect is a vector of plant viruses (Brown et al. 1995; Olivera et al. 2001; Jones 2003).

Bemisia tabaci represents one of the most striking examples of cryptic biological diversity. It includes populations which, although morphologically indistinguishable, are highly diverse to each other for their own biological and genetic characters (Rosel et al. 1997; Calvert et al. 2001). Numerous genetic variants and at least 36 biotypes have been assigned to *B. tabaci* (Perring 2001; De Barro et al. 2005; Zang et al. 2006; De Barro et al. 2011). Biological traits including host range, life-history traits, tolerance to climatic conditions, induction of plant physiological disorders, insecticide resistance, ability to transmit plant viruses, composition of the endosymbiotic bacteria community have been found to characterize specifically some of the various biotypes (Perring 2001; Delatte et al. 2005; Horowitz et al. 2005; Chiel et al. 2007; Delatte et al. 2009). Most biotypes, however, have only been designated based on difference of molecular markers, without any consistent biological

characterization, and should be regarded more properly as haplotypes (Brown 2010) or genetic variants (De Barro et al. 2011). Most genetic variants of *B. tabaci* are confined to specific geographic regions, but some of them, namely the well-characterized B and Q biotypes, are distributed worldwide raising the status of global pests (Brown 2007; Ueda et al. 2009). The strong biological and genetic differentiation and the occurrence of incompatible matings between populations with different genotype have led to consider *B. tabaci* a complex of cryptic species (Perring 2001; Boykin et al. 2007; Brown 2010).

Different molecular (protein and DNA) markers have been used to identify the genetic complexity of *B. tabaci* (Costa and Brown 1991; Brown et al. 2000; Cervera et al. 2000; Moya et al. 2001; Abdullhai et al. 2004; Maruthi et al. 2004; Jones et al. 2007; Ko et al. 2007; Tsagkarakou et al. 2007; Dalmon et al. 2008; Ahmed et al. 2010). The knowledge of the genetic diversity of *B. tabaci* populations and their phylogenetic relatedness has greatly improved thanks to the analysis of nucleotide sequences obtained from the mitochondrial cytochrome oxidase I (*COI*) gene and the ribosomal intergenic transcribed spacer 1 region (Frohlich et al. 1999; De Barro et al. 2000; De Barro et al. 2005; De la Rúa et al. 2006; Ahmed et al. 2010). Recently, based on the *COI* gene, global *B. tabaci* phylogenetic relationships revealed the occurrence of 11 major, well resolved genetic groups at 11% genetic distance limit containing 24 higher phylogenetic clades. These clades, defined on the basis of a >3.5% sequence divergence criterion, have been proposed to represent individual cryptic species (De Barro et al. 2011). Absence of gene flow has been observed between clades at 3.5% divergence limit (Elbaz et al. 2010; Xu et al. 2010), further supporting the concept of *B. tabaci* as a complex of morphologically indistinguishable species. According to present knowledge, B and Q biotypes have been considered as two different cryptic species, named the Mediterranean species and the middle east-Asia minor 1 species respectively, within the Africa-middle east-Asia minor major genetic group (Dinsdale et al. 2010). B and Q biotypes have in common the origin in the old world, with biotype B probably having originated in arid regions of middle eastern Mediterranean/North Africa/Asia minor, and biotype Q probably originated in Saharan and sub-Saharan Africa (De Barro et al. 2005; Boykin et al. 2007). From their native regions, both biotypes have spread all over the world because of the trade of the host plants; they have colonized the invasion areas and have displaced less-injurious indigenous populations (Brown 2007) because of their superior adaptability to environments highly disturbed by agricultural practices (Delatte et al. 2009), asymmetric mating interactions (Liu et al. 2007; Crowder et al. 2010) and indirect vector–virus mutualism via host plants (Jiu et al. 2007). Recent studies have shown a considerable genetic variability amongst mitochondrial lineages of biotype Q. Phylogenetic analysis of *COI* haplotypes revealed the occurrence of two widespread subgroups, Q1 (western Mediterranean populations) and Q2 (eastern Mediterranean populations), both of which have spread to not native geographic areas (Tsagkarakou et al. 2007; Chu et al. 2008; Ahmed et al. 2009), and two more groups, namely, Q3, consisting of populations from Burkina Faso only, besides ASL (Africa silverleafing) consisting of Sub-Saharan populations only (Gueguen et al. 2010).

Biotype B has become a serious problem starting from the late-1980s when the worldwide expansion of its populations caused severe epidemics of the transmitted *Geminiviruses*, like those causing the tomato yellow leaf curl disease (Polston and Anderson 1997; Brown 2007). Biotype Q, recorded for the first time at the beginning of 1990s in Spain (Guirao et al. 1997), has become a major pest in European Mediterranean regions, North Africa and Israel (Horowitz et al. 2003; Simón et al. 2007; Tsagkarakou et al. 2007), and most recently, it has spread in the North and Central America, in East Asia and New Zealand (Chu et al. 2007; Martinez-Carrillo and Brown 2007; Ueda et al. 2009). Both biotypes, owing to their characteristics of strong polyphagy, high reproductive rate and high level of resistance to insecticides (Olivera et al. 2001; Horowitz et al. 2005; Roditakis et al. 2005; Bonato et al. 2007) represent a serious threat to cultivated plants in any area of invasion. Frequently, populations of the B- and Q-types coexist, but no gene flow has been observed in the field (Moya et al. 2001). In some regions, biotype Q colonization has been parallelled by displacement of the B-type populations, particularly where insecticides are applied frequently (Horowitz et al. 2005). It is thought that key factors in the prevalence of the biotype Q are the higher insecticide resistance and the higher fitness of resistant Q-type populations than biotype B (Pascual and Callejas 2004; Horowitz et al. 2005; Pascual 2006). Furthermore, biotype Q has resulted more tolerant to high temperatures (Bonato et al. 2007). However, different responses to host plants (species and cultivars) have emerged between biotypes B and Q relative to life-history traits and population dynamics (Nombela et al. 2001; Bonato et al. 2007; Iida et al. 2009), and reproductive interference has been shown to advantage biotype B colonization in the absence of chemical control (Pascual and Callejas 2004; Pascual 2006; Elbaz et al. 2010). Hence, each biotype can be advantaged over the other depending on the specific environmental and agricultural context. Finally, variabilities in the efficiency to transmit *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Begomoviruses) have been observed between B and Q biotypes, with biotype Q being more efficient in virus

transmission from tomato to tomato plants than the other one (Sánchez-Campos et al. 1999; Jiang et al. 2004).

In Italy, *B. tabaci*, although recorded from a long time (Silvestri 1939), has raised the status of pest in the late 1980s following the spread of TYLCSV (Rapisarda and Tropea Garzia 2002). The first genetic characterization of the Italian populations revealed the presence of both B and Q biotypes (Demichelis et al. 2000). To date, B and Q biotypes have been recovered in the warmer areas, including the southern regions, main islands (Sardinia and Sicily) and the north-western coast (Liguria region). Coexistence of the two biotypes has been found in some areas although a general trend towards increasing frequencies of Q-type populations can be inferred from the literature (Simón et al. 2003b; Bosco et al. 2006; Cavalieri and Rapisarda 2008). Concurrently, with the invasions of B and Q biotypes, TYLCSV has spread in many horticultural districts causing severe economic losses to tomato crops, especially in greenhouse cultivations (Rapisarda and Tropea Garzia 2002; Fanigliulo et al. 2008). Recently, the spreadings of two more *B. tabaci*-transmitted viruses, the TYLCV (Begomovirus) (Davino et al. 2006; Parrella et al. 2006) and the *Tomato chlorosis virus* (ToCV, Crinivirus) (Accotto et al. 2001) have posed new concerns about the possible aggravation of the injuriousness of *B. tabaci* populations, on tomato as well as other vegetables. In addition to B and Q biotypes, a third one, the biotype T, has been recorded in Italy (Simón et al. 2003b). To date, biotype T has only been collected from restricted natural areas of Italy, where it is monophagous on *Euphorbia characias* L. (Demichelis et al. 2005; Bosco et al. 2006). In the laboratory, biotype T has been able to reproduce only on a single alternative host and to transmit TYLCSV on tomato inefficiently, and so it has been considered to be of no agricultural importance (Demichelis et al. 2005). Biotype T is genetically unrelated to B and Q biotypes and forms a distinct group sister to the Asian/Australian clade, possibly representing a distinct species (Dinsdale et al. 2010).

A precise knowledge of the genetic diversity of *B. tabaci* populations in the areas of invasion is fundamental for understanding the epidemiological behaviour of whitefly-transmitted viruses and for the management of viral diseases (Maruthi et al. 2002; Legg et al. 2002; Simón et al. 2003a; Brown 2007). Furthermore, identification of genetic diversity of *B. tabaci* is weighty for an integrated pest management, to develop effective strategies to delay the selection of insecticide-resistant populations (Ma et al. 2007; Feng et al. 2009; Tsagkarakou et al. 2009) as well as to improve the success of biological control programmes (Kirk et al. 2008). In the present study, by using *COI* gene sequencing and phylogenetic analysis, and PCR-RFLP of *COI* amplicons, we examined the genetic diversity of *B. tabaci* populations sampled from southern Italy and collected in greenhouse horticultural districts as well as in areas not subjected to intensive cultivations of host plants.

Materials and methods

Bemisia tabaci populations studied

Thirty-three whitefly populations were collected on cultivated and uncultivated (weeds and bushes) plants from 2004 to 2008 in five regions of southern Italy. Samples were taken mainly from coastal greenhouse districts characterized by intensive cultivations of vegetables and ornamentals. Furthermore, samplings were carried out in orchard and vineyard districts, located in interior plains or in mountain areas. The geographical origin and the host plant of each population are described in Table 1. *Bemisia tabaci* pupal cases were isolated from the infested leaves, and the emerging adults were used for molecular characterization. Adults were stored in 95% ethanol at −20°C until DNA template's preparation. Each sample consisted of 10–30 individuals, in general; 1–5 individuals per samples were collected from elmleaf blackberry (*Rubus ulmifolius* L.) and grapevine (*Vitis vinifera* L.) as *B. tabaci* colonies were very sparse on these latter host plants. In addition to our samples, individuals of reference biotypes recorded so far in the Mediterranean region were analysed. Adults of B and Q biotypes from Israel were supplied by Dr. Rami Horowitz (Department of Entomology, Gilat Research Center, Israel), the biotypes T from Italy by Dr. Domenico Bosco (Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Entomologia e Zoologia Applicata all'Ambiente, Università degli Studi di Torino, Italy) and the biotype S from Spain by Dr. Josè Luis Cenis (Centro de Investigación y Desarrollo Agroalimentario, La Alberca, Murcia, Spain). It was not possible to obtain specimens of the biotype M.

Template's preparation, COI amplification and sequencing

Templates for polymerase chain reactions (PCRs) were prepared from single insects, previously air-dried under vacuum for 5 min to eliminate ethanol, by using a modification of Rose et al. (1994). Each individual was homogenized with a plastic pestle in 1.5 ml microcentrifuge tube containing 25 µl TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.4). The homogenate was boiled for 5 min, chilled on ice, centrifuged at 14,000 rpm for 5 min to pellet insect debris, and the supernatant was used as template for the amplification. A 866-bp region of the *COI* gene was amplified in 50-µl reaction mixture using the forward primer C1-J-2195 in combination with TL2-N-

Table 1 Populations of *Bemisia tabaci* sampled in southern Italy and their genetic characterization based on *COI* gene sequencing or restriction analysis (B and Q are the biotype B and the biotype Q, respectively; Ru is the genetic variant discovered in this study)

Population acronym	Geographic origin (Region/ Location)	Host plant species (family)	Type of plant	Agricultural district	Accession number	Haplotype
CamPo1	Campania/Portici, Napoli	*Raphanus sativus* L. (Brassicaceae)	IG	Greenhouse	FN557444	Q
CamPo2	Campania/Portici, Napoli	*Ipomoea purpurea* L. (Convolvulaceae)	W	Greenhouse	FN557454	Q
CamPo3	Campania/Portici, Napoli	*Ocimum basilicum* L. (Lamiaceae)	F	Greenhouse	FN557467	B
CamPo4	Campania/Portici, Napoli	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	FN557450	Q
CamNa1	Campania/Napoli	*Ipomoea purpurea* L. (Convolvulaceae)	W	Greenhouse	FN557455	Q
CamNa2	Campania/Napoli	*Passiflora caerulea* L. (Passifloraceae)	W	Greenhouse	FN557447	Q
CamSav1	Campania/Saviano, Napoli	*Rubus ulmifolius* Schott (Rosaceae)	Bu	Hazelnut orchard	FN557463	Ru
CamTr1	Campania/Trecase, Napoli	*Capsicum annuum* L. (Solanaceae)	IG	Greenhouse	FN557468	Q
CamTr2	Campania/Trecase, Napoli	*Capsicum annuum* L. (Solanaceae)	IG	Greenhouse	FN557469	Q
CamNo1	Campania/Nola, Napoli	*Solanum lycopersicum* (Solanaceae)	IG	Greenhouse	FN557446	Q
CamTG1	Campania/Torre del Greco, Napoli	*Solanum lycopersicum* L. (Solanaceae)	F	Greenhouse	FN557448	Q
CamTG2	Campania/Torre del Greco, Napoli	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	FN557449	Q
CamEr1	Campania/Ercolano, Napoli	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	FN557458	Q
CamBt1	Campania/Boscotrecase, Napoli	*Vitis vinifera* L. (Vitaceae)	F	Vineyard	FN557456	Ru
CamBt2	Campania/Boscotrecase, Napoli	*Vitis vinifera* L. (Vitaceae)	F	Vineyard	FN557457	Q
CamCa1	Campania/Caserta	*Hibiscus rosa-sinensis* L. (Malvaceae)	F	Orchard	FN557453	Q
CamCa2	Campania/Capua, Caserta	*Solanum lycopersicum* L. (Solanaceae)	F	Orchard	FN557451	Q
CamTN1	Campania/Torre le Nocelle, Avellino	*Rubus ulmifolius* Schott (Rosaceae)	Bu	Vineyard	FN557461	Ru
CamTN2	Campania/Torre le Nocelle, Avellino	*Conyza* spp. (Asteraceae)	W	Vineyard	FN557465	Q
CamTN3	Campania/Torre le Nocelle, Avellino	*Menta* spp. (Lamiaceae)	W	Vineyard	FN557464	Q
CamDo1	Campania/Domicella, Avellino	*Rubus ulmifolius* Schott (Rosaceae)	Bu	Hazelnut orchard	FN557462	Ru
CamSc1	Campania/Scafati, Salerno	*Capsicum annuum* L. (Solanaceae)	IG	Greenhouse	FN557445	Q
CamNc1	Campania/Nocera inferiore, Salerno	*Solanum nigrum* L. (Solanaceae)	W	Greenhouse	FN557452	Q
CamPa1	Campania/Pagani, Salerno	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	FN557459	B
BaRi1	Basilicata/Rivello, Potenza	*Rubus ulmifolius* Schott (Rosaceae)	Bu	Vineyard	FN557460	Ru
BaMe1	Basilicata/Metaponto, Matera	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	nd	Q

Table 1 continued

Population acronym	Geographic origin (Region/ Location)	Host plant species (family)	Type of plant	Agricultural district	Accession number	Haplotype
CalLT1	Calabria/Lametia Terme, Catanzaro	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	nd	Q
CalAm1	Calabria/Amantea, Cosenza	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	nd	Q
SiNo1	Sicily/Noto, Siracusa	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	nd	Q
SaCt1	Sardinia/Capoterra, Cagliari	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	nd	Q
SaCt2	Sardinia/Capoterra, Cagliari	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	nd	B
SaCt3	Sardinia/Capoterra, Cagliari	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	nd	Q
SaCt4	Sardinia/Capoterra, Cagliari	*Solanum lycopersicum* L. (Solanaceae)	IG	Greenhouse	nd	Q

Bu bush, *F* plant cultivated in a family garden, *IG* intensive greenhouse cultivation, *W* weed, *nd COI* sequence not determined and biotype assignment by restriction analysis

3014 (Simon et al. 1994). The reaction mixture consisted of 1.25 U of GoTaq™ DNA polymerase (Promega), 10 µl (5×) Green GoTaq™ reaction buffer, corresponding to a final concentration of 1.5 mM $MgCl_2$, 1 µl dNTPs (2.5 mM each), 1 µl of forward and reverse primer (50 pmol/µl) and 4 µl of template prepared as described above. DNA fragments were amplified in a Perkin Elmer Cetus thermal cycler with 1 cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 45°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified DNA was analysed by electrophoresis in 1.2% agarose gel in 40 mM Tris–acetate, 1 mM EDTA, pH 8.2 and the band of expected size was cut from the gel with a sterile razor blade. The DNA fragment was eluted using the Montage Gel Extraction Kit (Millipore, USA), and 0.5–1 µg of the amplified product was ligated to the pGEM-T Easy vector (Promega, USA), according to the manufacturer's instructions, and cloned in competent *Escherichia coli* strain DH5α cells. DNA from recombinant plasmids was prepared as described by Hattori and Sakaki (1986), and the insert was sequenced at MWG Biotech (Ebersber, Germany). One adult per sampled population was sequenced, and for each adult, two clones were sequenced on both strands. In addition, one specimen for each of the Israeli B and Q reference biotypes was sequenced.

Phylogenetic analysis

COI sequences obtained from *B. tabaci* samples collected in southern Italy were assembled manually using EditSeq program available in the DNASTAR software package (Lasergene, Madison, WI, USA). As two clones per single adult whitefly were sequenced on both strands, a consensus sequence (866 bp) was produced for each adult representative of the populations sampled. The consensus sequences were aligned using the ClustalW method of MegAlign program in the same package, using default parameters, and compared with *COI* sequences of the reference B and Q biotypes that we produced (accession numbers FN557471 and FN557470, respectively) and with sequences available in GenBank. In particular, sequences of 657 bp from the alignment produced by Dinsdale et al. (2010) for global analysis of *B. tabaci* were adopted for phylogenetic analysis. Haplotypes representative of a different geographic origin for each major phylogeographic group were chosen. Furthermore, sequences of populations from Burkina Faso, representing the Q3 subgroup of the biotype Q, found in Gueguen et al. (2010), were included in the analysis. All the sequences used in the present study were unique haplotypes without nucleotide indels in the coding region or stop codons within the open reading frame. *Bemisia afer* (Priesner & Hosny) (GU220055) and *Bemisia subdecipiens* Martin (GU220056) sequences were chosen as outgroups. Sequences data were analysed with the program MODELTEST (version 3.06) (Posada and Crandall 1998), which uses hierarchical likelihood-ratio tests to determine the best-fit substitution model of evolution and the Bayesian phylogenetic analysis (BPA) was conducted with MrBayes 3.0 (Huelsenbeck et al. 2001) under the GTR + G model ($-\ln L = 6843.95$; $G = 0.2850$). BPA was run with six MCMC chains, each started from a random tree, and for 10 million generations, sampling the chain every 1000th cycle. All sample points

before reaching stationary (15,000 trees) were discarded as burn-in samples, and the remaining data were used to generate a majority-rule consensus tree, where the percentages of samples recovering any particular clade represent the clade's posterior probability. Convergence of parameters was assessed by calculating the effective sample size (ESS) using TRACERv1.4 (Rambaut and Drummond 2007). All the parameter estimates for each run showed ESS values >100. A final Bayesian majority-rule consensus tree was obtained. The tree was visualized using the FigTree v1.1.2 program available at http://tre.bio.ed.ac.uk/software/figtree.

The sequences of *B. tabaci* were deposited in the GenBank database under accession numbers FN557444–FN557465 and FN557467–FN557469 for the Italian populations, FN557471 for the Israeli reference B-type population and FN557470 for the Israeli reference Q-type population.

Pairwise genetic distance was analysed according to Dinsdale et al. (2010) in PAUP, using the HKY85 model to account for unequal base frequencies.

Restriction analysis

COI sequences obtained in the present study were analysed using MapDraw program (Lasergene software package-DNASTAR, Madison, WI) in combination with sequences available in GenBank, to identify restriction enzymes that would result in banding patterns specific for the Mediterranean genotypes of *B. tabaci*. We selected an endonuclease, *Apo*I, which appeared able to distinguish any biotype recorded so far in the Mediterranean region. Furthermore, *Apo*I produced in silico banding patterns specific for the Q1 (western Mediterranean) and Q2 (eastern Mediterranean) haplotypes of the biotype Q. Efficacy of the selected endonuclease was tested by in vivo electrophoresis experiments using specimens of Mediterranean reference biotypes (B, Q, T and S), and of the herein described Ru haplotypes. Digestion of the *COI* amplicons by using *Apo*I was performed on 6–7 µl of PCR products according to manufacturer's suggestions (New England BioLabs, Ipswich, MA, USA). The fragments were separated on 6% polyacrylamide gel (30% acrylamide:bisacrylamide 29:1) in TAE buffer. Finally, in order to extend the analysis to biotype M, the adult samples of which were not available, in silico restriction maps were produced. PCR-RFLP-analysis was then used to genotyping the Italian populations that in this study were not subjected to sequencing; 3–5 individuals of each population were screened. Similarly, as only one individual per population was sequenced, 3–5 individuals of each sequenced population were subjected to PCR-RFLP analysis.

Results

Phylogenetic relationships of *B. tabaci* COI sequences

The Bayesian phylogenetic tree revealed that the 25 Italian populations here sequenced fall in three clades (Fig. 1). In particular, two Italian haplotypes and the Israeli reference B-type haplotype (Israel_FN557471) grouped within the Middle east-Asia Minor 1 genetic group (posterior probability, 1.00) including the other biotype B haplotypes. 17 Italian haplotypes and the Israeli reference Q-type haplotype (Israel_FN557470) grouped within the Mediterranean genetic group (posterior probability, 1.00) including the other biotype Q haplotypes. All the Italian Q-type populations were included in a group (posterior probability, 1.00) separated from the Israeli reference Q-type population (Israel_FN557470). Finally, five haplotypes obtained from different *B. tabaci* populations collected on elmleaf blackberry (four samples) and grapevine (one sample) formed a highly supported (posterior probability, 1.00) monophyletic group sister to the group including Asia 1, Asia 2, Asia 2 India, China, Australia, Australia/Indonesia and Italy major genetic groups. In conclusion, on the basis of phylogenetic analysis of *COI* sequences, two Italian populations were attributed to biotype B, 18 to biotype Q, and five represented a new genetic variant group that was named Ru (after *Rubus ulmifolius*) (Table 1; Fig. 1).

Based on *COI* sequence variation, the five Ru haplotypes exhibited high level of nucleotide similarity to one other (0.11–0.57% sequence divergence). Using BLAST searches, Ru haplotypes showed the highest similarity (89–90%) to *COI* sequences of the biotype T. Pairwise comparisons between the consensus sequence of Ru haplotypes and the consensus sequences produced by Dinsdale et al. (2010) for genetic groups of *B. tabaci* defined on the basis of a >3.5% sequence divergence criterion revealed that the consensus sequence of Ru haplotypes showed the lowest divergence (10.7%) with the consensus sequence of the Italy group, that is the biotype T.

Genetic discrimination of *B. tabaci* based on restriction analysis

Comparison of restriction sites present in the *COI* sequences of *B. tabaci* populations sampled in Southern Italy with restriction sites found in the sequences of the Israeli reference B and Q biotypes and in the sequences available in GenBank allowed the identification of an endonuclease, *Apo*I, useful to discriminate all biotypes recorded in the Mediterranean region (Table 2). Nevertheless, since the length of the sequences recovered from GenBank was in general shorter than the sequences produced in the present study, fidelity of in silico restriction

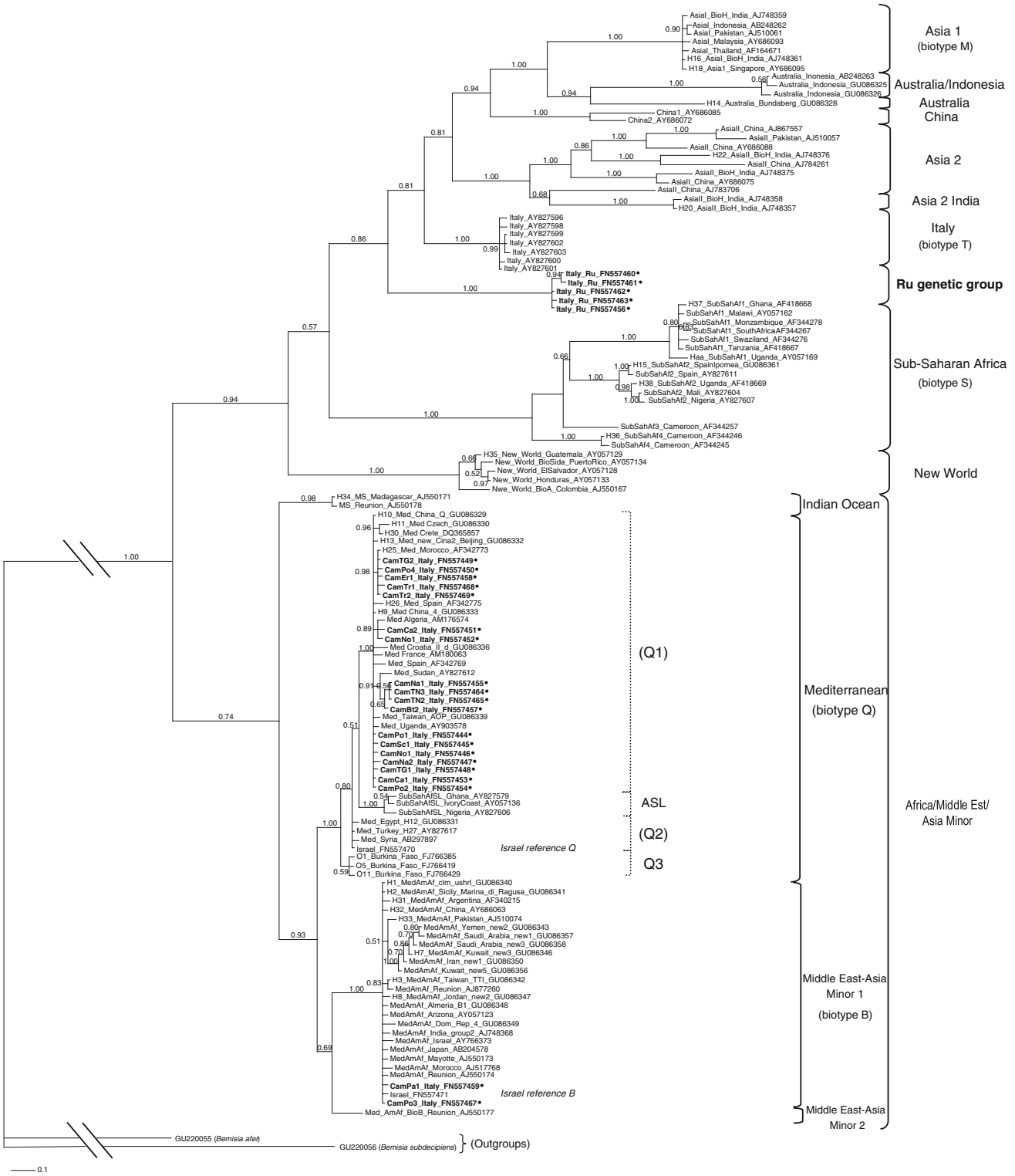

Fig. 1 Rooted phylogenetic tree based on Bayesian analysis of *COI* gene sequences. Bayesian posterior probabilities are given above branches of each node. *Bemisia afer* (GU220055) and *Bemisia subdecipiens* (GU220056) were used as outgroups. Acronyms and accession numbers of the Italian *Bemisia tabaci* populations sampled in this study are in *bold* and with a *dot*. The positions of reference Israelian B and Q populations are indicated. The 11 major phylogeographic groups (De Barro et al. 2011) are reported, and low level groups are highlighted only for the Africa/Middle East/Asia Minor major group. The phylogeographic positions of biotypes present in the Mediterranean are reported (*in brackets*). The *bar* represents the unit length of the number of nucleotide substitutions per site

Table 2 Predicted in silico restriction profiles of partial *COI* sequences of *Bemisia tabaci* obtained using the *Apo*I endonuclease

Genotype	Origin	Accession numbers	Sequence size (bp)	Number of cuts	Restriction fragment size
B	Italy	**FN557459**	866	3	310, **252**, *212*, 92
B	Israel	**FN557471**	866	3	310, **252**, *212*, 92
Q1	Italy	**FN557445**	866	3	266, **252**, *212*, 92, 44
Q2	Israel	**FN557470**	866	4	358, **252**, *212*, 44
T	Italy	AY827600	774	3	310, **197**, *175*, 92
Ru	Italy	**FN557456**	866	3	**562**, *194*, 92, 18
S	Spain	AY827608	750	2	**599**, 91, *60*
M	Turkey	AY827616	740	1	**599**, *141*

Accession numbers in bold are sequences produced in this study; accession numbers of the T, S and M biotypes are the longest sequences available in GenBank. Restriction fragments reported in bold and italic are the left and the right ends of the *COI* sequence, respectively

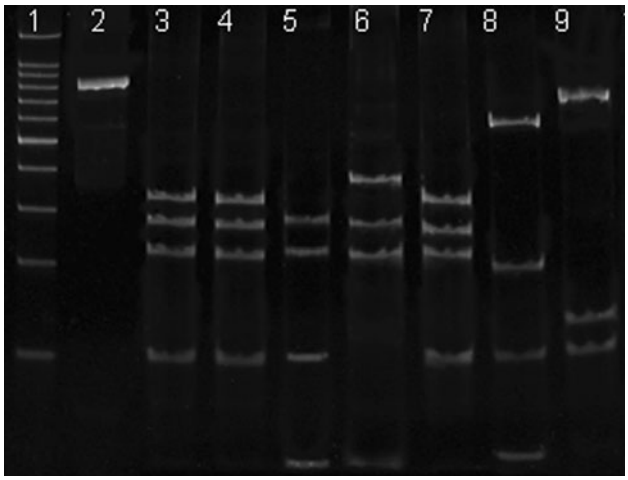

Fig. 2 Restriction profiles obtained after digestion with *Apo*I endonuclease of the 866 bp *COI* amplicon of *Bemisia tabaci* haplotypes. *Lane 1* 100 bp molecular weight ladder (Promega, USA), *lane 2* uncut amplicon, *lane 3* biotype B from southern Italy, *lane 4* biotype B from Israel, *lane 5* biotype Q from southern Italy (Q1), *lane 6* biotype Q from Israel (Q2), *lane 7* biotype T, *lane 8* haplotype Ru, *lane 9* biotype S

profiles was confirmed by in vitro restriction of the 866-bp amplicons. Electrophoresis experiments revealed that *Apo*I is able to produce banding patterns specific for the B, Q, T and S biotypes and the herein described Ru haplotypes (Fig. 2). Also *Apo*I was able to discriminate between Q1 (western Mediterranean) and Q2 (eastern Mediterranean) haplotypes of biotype Q. By using in silico digestion with *Apo*I, restriction analysis was extended also to biotype M, which was effectively discriminated from the other genotypes tested (Table 2). *Apo*I was then used for the identification of *B. tabaci* populations found infesting greenhouse tomato crops in Calabria, Basilcata, Sicily and Sardinia regions. which were not subjected to sequencing. The digestion profiles revealed that seven out of eight populations were of the biotype Q and one of the biotype B (Table 1). Finally, all the Italian biotype Q populations showed a unique restriction pattern, namely that of the Q1 subgroup.

B. tabaci distribution and association with the host plants

Twenty-three populations of *B. tabaci* were sampled from greenhouse districts of southern Italy, on cultivated plants as well as on weeds and bushes (Table 1). On the basis of the *COI* sequence and digestion profile, 20 out of 23 populations (87%) were assigned to biotype Q, and only three populations (13%) were assigned to biotype B. In addition, five biotype Q populations (CamBt2, CamCa1, CamCa2, CamTN2 and CamTN3) were found in orchard and vineyard districts located in interior plains or in mountain areas distant from intensive cultivations of whitefly host plants (Table 1).

Ru-haplotype individuals were collected on elmleaf blackberry (four samples from different locations, namely, CamSav1, CamTN1 and CamDo1 from Campania region, and BaRi1 from Basilicata region) and grapevine (the sample CamBt1 from Campania region) (Table 1). Ru-type populations were collected only outside greenhouse districts (although elmleaf blackberry bushes were sampled also from greenhouse districts). Furthermore, in two locations, Ru-type populations were found to be sympatric with the biotype Q populations: at Boscotrecase location, Ru individuals (CamBt1) shared the same host plant (grapevine) with Q individuals (CamBt2); at Torre le Nocelle location, Ru individuals were collected on elmleaf blackberry (population CamTN1), while Q individuals on two weed species (populations CamTN2 and CamTN3). Overall, the Ru haplotypes were found widely distributed as they were collected from mild coastal localities (population CamBt1), interior plains (population CamSav1) and cool inner mountain areas (populations CamDo1, CamTN1 and BaRi1).

Discussion

In the present study, by means of phylogenetic analysis of *COI* sequences and restriction analysis of *COI* amplicons, three genetic variants of *B. tabaci* were identified in southern Italy: the B and Q biotypes, and a new genetic variant including the haplotypes not described so far, collected on elmleaf blackberry and grapevine. In general, biotype Q populations were most frequently collected (76.0% of all sampled populations). In greenhouse districts, 87.0% of the sampled populations (20 out of 23) were identified as biotype Q, and only three populations (13.0%) were assigned to biotype B. Outside the greenhouse districts, the biotype B was never collected, whilst populations of the biotype Q were found on weeds and plants cultivated in family gardens grown in environments distant from intensive cultivations of whitefly host plants, like orchard and vineyard districts located in interior plains or in mountain areas. These results are congruent with the increasing frequencies of biotype Q populations and displacement of the biotype B observed during the last decade in Italy (Simón et al. 2003b; Bosco et al. 2006; Cavalieri and Rapisarda 2008) and in other Mediterranean countries (Moya et al. 2001; Khasdan et al. 2005; Tsagkarakou et al. 2007; Dalmon et al. 2008).

The large distribution of Q-type populations in different agro-ecosystems from southern Italy suggests that biotype Q is better adapted to Mediterranean environmental conditions than biotype B. Biotype Q is characterized by higher insecticide resistance and higher fitness of resistant populations (Horowitz et al. 2005; Pascual 2006), and by higher tolerance to temperatures (Bonato et al. 2007) than biotype B. It is possible that, although asymmetric reproductive interference favours biotype B when coexisting with the biotype Q (Crowder et al. 2010; Elbaz et al. 2010), a combination of climatic factors (e.g. outdoor conditions, and extreme temperatures reached under plastic greenhouses) and agricultural practices (e.g. insecticide sprayings, and genotypes of cultivated plants) could have hindered the biotype B invasion and favoured the spread of biotype Q in southern Italy, as well as in other Mediterranean Countries.

In this study, phylogenetic analysis of *COI* sequences revealed that the Italian Q-type haplotypes grouped separately (posterior probability, 1.00) from the Israeli Q-type haplotype and from Turkish, Egyptian and Syrian haplotypes suggesting a western Mediterranean origin and membership of the Q1 subgroup. Furthermore, digestion profiles of *COI* amplicons (866 bp) obtained by electrophoresis experiments using the restriction enzyme *Apo*I revealed a unique profile amongst all the Italian populations; this digestion profile was different from that of the Israeli reference biotype Q. The Italian Q-type digestion profiles were identical to those obtained in silico using Western Mediterranean haplotypes available in GenBank; analogously, digestion profile of the Israeli reference biotype Q was identical to profiles of the eastern Mediterranean haplotypes available in GenBank. Tsagkarakou et al. (2007) found that the endonuclease *Alu*I produced restriction patterns that are useful to distinguish the phylogeographic origin, namely, western Mediterranean (Q1) or eastern Mediterranean (Q2), of the biotype Q haplotypes. Here, restriction profiles obtained by in silico digestion with *Alu*I also confirmed the western Mediterranean origin of the Italian populations and the eastern Mediterranean origin of the Israeli reference population analysed (data not shown). Therefore, no invasion of eastern Mediterranean populations seems to have occurred in Southern Italy so far.

In this study, we have developed a PCR-RFLP method for discrimination of *B. tabaci* genotypes based on digestion of a *COI* fragment with the endonuclease *Apo*I. This enzyme produces banding profiles specific for each genetic variant recorded from the Mediterranean region, thereby being able to discriminate amongst the B, Q, M, S and T biotypes and the herein-discovered Ru haplotypes. Furthermore, *Apo*I is able to discriminate between Q1 (western Mediterranean) and Q2 (eastern Mediterranean) haplotypes of biotype Q. This diagnostic method could be useful, at least in the Mediterranean region, in population studies of *B. tabaci* aimed to monitor biotype distribution over time and space, and to find association between biotype distribution and occurrence of insecticide resistance or epidemics of *B. tabaci*-transmitted viral diseases.

Another result of the present study is the discovery in southern Italy of a new genetic variant of *B. tabaci*, which was named Ru (after *Rubus ulmifolius*). The Ru haplotypes were widely distributed as they were found in different localities, ranging from mild coastal to cool inner mountain areas. They were collected almost exclusively on the elmleaf blackberry *R. ulmifolius* (four populations) and once on the grapevine *V. vinifera*, although different species of cultivated and uncultivated plants were sampled nearby the colonized host plant species. This result suggests the possibility that the Ru haplotypes have a restricted host range.

Phylogenetic analysis revealed that Ru haplotypes obtained on elmleaf blackberry and grapevine form a distinct clade closely related to the clade including major genetic groups of Asia 1, Asia 2, Asia 2 India, China, Australia, Australia/Indonesia and Italy (Fig. 1). The closest relative of the Ru group (10.7% pairwise genetic distance) was the Italy group, this latter so far including only the haplotypes of the biotype T. Ru haplotypes and biotype T haplotypes share the geographic distribution (Italy) but differ for their ecology. While Ru haplotypes were found in agricultural districts and on at least two host plants (elmleaf blackberry and

grapevine), the biotype T has been collected only from restricted natural areas, where it is monophagous on *Euphorbia characias* L. (Demichelis et al. 2005; Bosco et al. 2006). On the basis of the >3.5% *COI* sequence divergence criterion for *B. tabaci* cryptic species separation (Dinsdale et al. 2010; De Barro et al. 2011), the clade including Ru haplotypes could represent a cryptic species of the *B. tabaci* species complex. However, before the completion of a consistent biological and morphological characterization showing the species status of the Ru group, this latter should be considered more properly as a genetic variant. Finally, as the Ru and the biotype T groups are basal to the clade including the Asian/Australian major genetic groups, our data suggest a possible origin of the Asian/Australian populations of *B. tabaci* from ancient introduction of whiteflies from the European continent.

Colonies of the Ru genetic variant occurred in the field at very low densities, especially on grapevine, where only one pupal case was found, leaving very unlikely the raising of pest status. However, additional studies are required to clarify its biology and ecology, in particular its actual host range, geographic distribution and relationships with plant viruses. This latter point is particularly interesting because, to our knowledge, the known hosts of the Ru haplotypes, namely *R. ulmifolius* and *V. vinifera*, have not been recorded as natural hosts of Geminiviruses, and consequently it is probable that the Ru haplotypes evolved in the absence of these viruses. Therefore, poor or no virus acquisition and transmission abilities are expected. In contrast, Geminivirus-vector genotypes, like the B and Q biotypes, have been subjected to complex interactions with viruses and plants leading in some cases to evolution of vector–virus mutualistic relationships favouring whitefly invasion and spreading of viral diseases (Jiu et al. 2007). Molecular and genomic comparative analyses between the Ru genetic variant and vector genotypes could be useful to understand the mechanisms involved in the acquisition and transmission of the Geminiviruses and develop new strategies for viral diseases control.

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