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In-vineyard population structure of '*Candidatus* Phytoplasma solani' using multilocus sequence typing analysis

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ABSTRACT

'*Candidatus* Phytoplasma solani' is a phytoplasma of the stolbur group (16SrXII subgroup A) that is associated with 'Bois noir' and causes heavy damage to the quality and quantity of grapevine yields in several European countries, and particularly in the Mediterranean area. Analysis of '*Ca.* P. solani' genetic diversity was carried out for strains infecting a cv. 'Chardonnay' vineyard, through multilocus sequence typing analysis for the *vmp*1, *stamp* and *secY* genes. Several types per gene were detected: seven out of 20 types for *vmp*1, six out of 17 for *stamp*, and four out of 16 for *secY*. High correlations were seen among the *vmp*1, *stamp* and *secY* typing with the *tuf* typing. However, no correlations were seen among the *tuf* and *vmp*1 types and the Bois noir severity in the surveyed grapevines. Grouping the '*Ca.* P. solani' sequences on the basis of their origins (i.e., study vineyard, Italian regions, Euro-Mediterranean countries), dN/dS ratio analysis revealed overall positive selection for *stamp* (3.99, $P = 0.019$) and *vmp*1 (2.28, $P = 0.001$). For *secY*, the dN/dS ratio was 1.02 ($P = 0.841$), showing neutral selection across this gene. Using analysis of the nucleotide sequencing by a Bayesian approach, we determined the population structure of '*Ca.* P. solani', which appears to be structured in 3, 5 and 6 subpopulations, according to the *secY*, *stamp* and *vmp*1 genes, respectively. The high genetic diversity of '*Ca.* P. solani' from a single vineyard reflects the population structure across wider geographical scales. This information is useful to trace inoculum source and movement of pathogen strains at the local level and over long distances.

1. Introduction

Bois noir (BN) is a grapevine disease that is associated to '*Candidatus* Phytoplasma solani' ('*Ca.* P. solani'; 16SrXII-A subgroup) (Quaglino et al., 2013), and it is most common and widespread in Euro-Mediterranean regions (Maixner, 2011; Zahavi et al., 2013). In recent years, rapid spreading of BN has been seen frequently, and this can be a limiting factor in several grapevine-growing regions (Maixner, 2011; Zahavi et al., 2013). BN spreading is influenced by the biology of the main vector, *Hyalesthes obsoletus*, together with other potential vectors (Imo et al., 2013; Landi et al., 2013; Cvrković et al., 2014), and their wide range of host plants (Kessler et al., 2011; Johannesen et al., 2012; Riolo et al., 2012; Cvrković et al., 2014). Disease management for BN is very difficult, as the phytoplasma and insect vectors are both not host specific, and thus their control is mainly based on agronomic approaches. Innovative perspectives to mitigate disease spread and severity have been based on the induction of host defences (Romanazzi et al., 2013). In cv. 'Chardonnay', which is particularly sensitive to BN infection, the drying up of grape bunches can result in production losses of about 50%, with lower sugar content in the grapes of symptomatic plants (Endeshaw et al., 2012).

'*Ca.* P. solani' isolates are characterised by different degrees of genetic variability according to the genes involved (Quaglino et al., 2009, 2013; Foissac et al., 2013). The most variable genes are those that code for surface membrane proteins, which have been sequenced for several phytoplasma, and have been classified into three types: immunodominant membrane protein (Imp); immunodominant membrane protein A (ImpA); and antigenic membrane protein (Amp) (Kakizawa et al., 2006a). Several genes that code for surface membrane proteins in '*Ca.* P. solani' have been sequenced and characterised (Cimerman et al., 2009; Fabre et al., 2011). In particular, in samples from the Euro-Mediterranean basin (e.g., vectors, different hosts), 23 restriction fragment-length polymorphism (RFLP) genotypes have been recorded in the gene coding for variable membrane protein-1 (*vmp*1), 14 in the gene coding for antigenic membrane protein (*stamp*), and 39 in the *secY* gene (Fabre et al., 2011; Foissac et al., 2013).

The study of phytoplasma population genetics can be based on genotyping by multilocus sequence typing analysis (Arnaud et al., 2007; Danet et al., 2011), and on the estimation of the dN/dS ratio,

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which is the ratio between the non-synonymous (dN) and the synonymous (dS) substitution rates in an alignment of amino-acid-coding sequences (Nielsen, 2005). Synonymous substitutions are usually regarded as having much smaller effects on fitness than non-synonymous substitutions. This ratio has been used to identify the types of selection in genes; i.e., dN/dS >1, =1 and <1, for positive, neutral and negative selection, respectively (Nielsen, 2005). This approach can reveal useful information on the molecular ecology, to trace the route of spread of phytoplasma strains, so as to better understand the epidemiology of phytoplasma diseases. Moreover, it can be helpful to hypothesise natural events that can provide pressure of selection, particularly for those microrganisms for which phenotypic differentiation is complicated by the difficulty of cultivation.

The aims of this study were: (i) to determine the genetic variability and strain composition of '*Ca.* P. solani' in the investigated vineyard, using multilocus sequence typing analysis of the *vmp*1, *stamp* and *secY* genes; (ii) to estimate the selection (i.e., positive, negative, neutral) on the same genes moving from the small scale (vineyard), through the national scale (Italian regions), to the international scale (Euro-Mediterranean countries); and (iii) to determine the population structure of '*Ca.* P. solani', considering the sequencing related to the grapevine and the main vectors in the vineyard.

2. Materials and methods

2.1. Sample sources

The present study included part of a DNA collection (329 samples) that was obtained from a total of 357 infected grapevines that were sampled in 2011. This collection was carried out in a commercial vineyard in central-eastern Italy (42° 59′ 00″ N, 13° 36′ 00″ E), with the infection previously assessed by molecular tools to belong to '*Ca.* P. solani' (Murolo et al., 2014). Based on the information of BN severity of each infected vine recorded according to an empirical scale, as proposed in a previous study (Murolo et al., 2014), the disease severity that occurred in the vineyard and that was induced by different strains of '*Ca.* Phytoplasma solani' was calculated according to the formula $S = \sum(c \times f)/n$, where c is the severity class, f is the frequency of the class, and n is the number of symptomatic plants.

2.2. PCR and molecular characterisation of 'Ca. P. solani' for the tuf gene using RFLP

The 329 DNA samples were amplified in nested PCR with tufAYf/r (Schneider et al., 1997), then molecular characterisation of the *tuf* gene was carried out by PCR-RFLP, according to the protocol described by Langer and Maixner (2004).

Moreover, the same samples were amplified with additional primer pairs designed on the *stamp* and *secY* regions of the '*Ca.* P. solani' genome, as available in the literature (Fialova et al., 2009; Fabre et al., 2011).

2.3. Multilocus sequence typing analysis

On the basis of the RFLP characterisation of the *tuf* gene (the present study) and the *vmp*1 gene (Murolo et al., 2014), representative samples amplified with specific primer pairs for the *vmp*1, *stamp* and *secY* genes were purified using Wizard SV gels and PCR Clean-Up kits (Promega Corporation, Madison, WI, USA), and quantified using a BioPhotometer (Eppendorf, Hamburg, Germany). These samples were sequenced using an ABI 3730XL DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) at Beckman Coulter Genomics (Essex, UK), edited using the Chromas version 2.33 software, and assembled using the GAP4 Staden Package (http://www.staden.sourceforge.net), to obtain a consensus sequence. The Bioedit software, v. 7.0.0 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) was used to cut off ~20–30 bp of the terminal end sequence. All of the consensus sequences for each gene were used as the query sequences in the BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to determine the nucleotide identities for the '*Ca.* P. solani' strains available in GeneBank NCBI (http://www.ncbi.nlm.nih.gov).

Fifteen *vmp*1, 17 *stamp*, and 13 *secY* nucleotide sequences from the samples of the study vineyard (Table 1) were aligned with the nucleotide sequences available in Genebank, with the selection of '*Ca.* P. solani' strains from the same host (grapevine) and from vectors and potential vectors only from the vineyard (*H. obsoletus*, *Reptalus panzeri, R. quinquecostatus*) (Table 1). The multiple alignment was carried out using the Clustal X v. 1.8 programme (Thompson et al., 1997).

2.4. Phylogenetic analysis

Distance-based trees were produced based on distance matrices generated by the Jukes–Cantor distance correction method (Jukes and Cantor, 1969), followed by tree construction using the Mega v. 5.1 software (Tamura et al., 2011). The percentage of replicate trees in which the associated strains clustered together in the bootstrap test (1000 replicates) was estimated, as shown next to the tree branches. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. All of the positions containing gaps and missing data were eliminated from the dataset. The nucleotide sequences were analysed with DnaSP version 5 (Librado and Rozas, 2009), to distinguish between the different types for each gene, to assess the '*Ca.* P. solani' strain composition in the context of the study vineyard, and that of the national and international phytoplasma infections of grapevine.

2.5. Genetic analysis and selective pressure of 'Ca. P. solani' from the study vineyard to the Euro-Mediterranean countries

DnaSP version 5 was used to estimate: number of polymorphic sites (S) (singletons and parsimony-informative sites), total number of mutations (η), number of molecular types (Hn), molecular type diversity (Hd), average number of nucleotide differences between sequences from the same population (k), and average pairwise nucleotide diversity (Pi) (Tajima, 1983).

The ratio between the proportion of non-synonymous and synonymous substitutions, known as the dN/dS ratio, was determined for the nucleotide sequences at the study vineyard level, with respect to the national (Italian regions) and international (other Euro-Mediterranean countries) contexts. Additionally, the dS and dN variances were studied, as Var(dS) and Var(dN), respectively. The dN/dS ratios and the null hypothesis of no selection (H0: dN = dS) *versus* the positive selection hypothesis (H1: dN > dS) were calculated using the Nei–Gojobori method in a codon-based Z-selection test (Nei and Gojobori, 1986). The analysis was carried out in MEGA5, and the variance of the differences was computed using the bootstrap method (1000 replicates) (Tamura et al., 2011). The synonymous and non-synonymous nucleotide substitution rates for the nucleotide sequences of the target genes (*vmp*1, *stamp*) with the housekeeping *secY* gene (Fabre et al., 2011) were calculated. Positive selection happens when dN/dS ratio >1.0 and p-value for the Z-test < 0.05, on the other hand a ratio <1.0 suggests purifying selection process (Nei and Kumar, 2000).

Table 1
'*Ca.* P. solani' nucleotide sequences used in the phylogenetic analysis for the *stamp*, *secY* and *vmp*1 genes from the study vineyard and for those available in Genebank for the Italian regions and the Euro-Mediterranean countries.

Isolate	Host	Country	Accession number		
			stamp	*secY*	*vmp1*
Study vineyard					
P10/11	*Vitis vinifera* cv Chardonnay	Italy, Marche	**KJ145342**	**KJ145368**	**KJ145353**
136/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145340**	**KJ145362**	**KJ145354**
P42/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145341**	**KJ145370**	**KJ145356**
166/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145343**	**KJ145366**	**KJ145355**
115/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145337**	**KJ145365**	**KJ145350**
P25/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145339**	**KJ145371**	ND
353/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145338**	ND	**KJ145352**
P51/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145331**	ND	ND
78/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145334**	**KJ145367**	**KJ145349**
215/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145329**	**KJ145372**	ND
P75/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145333**	ND	**KJ145357**
P136/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145336**	**KJ145373**	**KJ145358**
315/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145330**	**KJ145369**	**KJ145360**
60/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145345**	ND	**KJ145346**
266/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145344**	ND	ND
425/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145335**	**KJ145363**	**KJ145348**
P53/11	*V. vinifera* cv Chardonnay	Italy, Marche	ND	**KJ145364**	ND
149/11	*V. vinifera* cv Chardonnay	Italy, Marche	ND	**KJ145374**	**KJ145347**
411/11	*V. vinifera* cv Chardonnay	Italy, Marche	ND	ND	**KJ145359**
287/11	*V. vinifera* cv Chardonnay	Italy, Marche	**KJ145332**	ND	**KJ145351**
Available in Genebank – Italian regions					
Mca21	*V. vinifera*	Italy, Marche	**KJ145382***	–	HM008599
B51	*V. vinifera*	Italy, Basilicata	–	–	HM008600
Aaq1	*V. vinifera*	Italy, Abruzzi	**KJ145383***	–	HM008601
Aa16	*V. vinifera*	Italy, Abruzzi	**KJ145380***	**KJ145389***	HM008602
C3	*V. vinifera*	Italy, Campania	–	–	HM008603
B49	*V. vinifera*	Italy, Basilicata	–	–	HM008604
Ag4a	*V. vinifera*	Italy, Abruzzi	**KJ145377***	**KJ145393***	HM008605
Mp46	*V. vinifera*	Italy, Marche	**KJ145379***	**KJ145396***	–
Mp49	*V. vinifera*	Italy, Marche	**KJ145376***	**KJ145398***	HM008607
B7	*V. vinifera*	Italy, Basilicata	–	–	HM008608
Mca28	*V. vinifera*	Italy, Marche	–	**KJ145390***	HM008609
C1	*V. vinifera*	Italy, Campania	–	–	HM008610
B2035	*V. vinifera*	Italy, Basilicata	–	–	HM008611
Mvercer2	*V. vinifera*	Italy, Marche	**KJ145375***	**KJ145395***	HM008612
Mag1	*V. vinifera*	Italy, Marche	–	**KJ145400***	HM008613
Aa25	*V. vinifera*	Italy, Abruzzi	**KJ145387***	**KJ145399***	HM008614
Mri10	*V. vinifera*	Italy, Marche	–	–	HM008615
S13	*V. vinifera*	Italy, Sardinia	–	–	HM008616
B4	*V. vinifera*	Italy, Basilicata	–	–	HM008617
C6	*V. vinifera*	Italy, Campania	–	–	HM008618
Mcil	*V. vinifera*	Italy, Marche	**KJ145385***	**KJ145401***	–
Ate7	*V. vinifera*	Italy, Abruzzi	**KJ145381***	–	–
Ate17	*V. vinifera*	Italy, Abruzzi	**KJ145386***	**KJ145402***	–
Aaq29	*V. vinifera*	Italy, Abruzzi	**KJ145388***	–	–
Aaq37	*V. vinifera*	Italy, Abruzzi	–	**KJ145391***	–
Mdxsain	*V. vinifera*	Italy, Marche	**KJ145384***	–	–
B1	*V. vinifera*	Italy, Basilicata	**KJ145378***	**KJ145394***	–
Mur2	*V. vinifera*	Italy, Marche	–	**KJ145392***	–
Mn2	*V. vinifera*	Italy, Marche	–	**KJ145397***	–
CH-1	*V. vinifera*	Italy	–	AM992089	AM992105
400-05	*V. vinifera*	Italy	–	–	EF655660
Rome15	*H. obsoletus*	Italy	FN813268	–	–
Available in Genebank – Euro-Mediterranean countries					
BG4560	*V. vinifera*	Bulgary	FN813252	FN813271	–
GGY	*V. vinifera*	Germany	FN813256	AM992093	AM992102
GR13	*V. vinifera*	Greece	FN813264	FN813284	–
SB5	*V. vinifera*	Croatia	FN813266	FN813272	–
19-25	*V. vinifera*	Germany	FN813267	AM992094	AM992101
LOZA2	*V. vinifera*	Serbia	–	FN813282	
GR138	*V. vinifera*	Greece	–	FN813283	
PO	*Hyalesthes obsoletus*	France	FN813259	AM992082	AM992095
H299	*H. obsoletus*	France	FN813254	–	–
NGA9	*H. obsoletus*	Slovenia	FN813262	–	–
E	*H. obsoletus*	Germany	FN813263	–	–
OSESLO2	*H. obsoletus*	Slovenia	FN813269	–	–
H187	*H. obsoletus*	France	–	FN813289	–
Charente-1	*H. obsoletes*	France	–	AM992084	AM992098
H155	*H. obsoletus*	France	–	FN813287	–
H160	*H. obsoletes*	France	–	FN813288	–

(*continued on next page*)

Table 1 (*continued*)

Isolate	Host	Country	Accession number		
			stamp	*secY*	*vmp1*
Rpg47	*Reptalus panzeri*	Serbia	KC703020	KC703048	KC703034
Rqg50	*R. quinquecostatus*	Serbia	KC703019	KC703047	KC703033
Rqg31	*R. quinquecostatus*	Serbia	KC703017	KC703045	KC703031
Rqg42	*R. quinquecostatus*	Serbia	KC703016	KC703044	KC703030
Rpg39	*R. panzeri*	Serbia	KC703009	KC703007	KC703023
Rqg60	*R. quinquecostatus*	Serbia	KC703011	KC703039	KC703025

–, nucleotide sequence not available.
Accession numbers of nucleotide sequences determined in the present study are indicated in bold font.
ND, nucleotide sequence not determined.
* These isolates are part of the project "STOLBUR-EUROMED Consortium" (SEE-ERA.NET; www.phytoplasma.eu).

2.6. Analysis of the 'Ca. P. solani' population structure

A Bayesian-model-based approach was used to infer the hidden genetic population structure of the samples, and thus to assign the genotypes into genetically structured groups/populations. This approach was implemented in the software BAPS 5.4 (Corander et al., 2003) (http://web.abo.fi/fak/mnf/mate/jc/software/baps.html). A total of 46, 48 and 43 nucleotide sequences for *vmp*1, *stamp* and *secY*, respectively, were used in this analysis.

Genetic mixture analysis was carried out to determine the most probable number of populations (*K*) given the data. Under its default settings, BAPS includes *K* as a parameter to be estimated, and the best partition of the data into *K* clusters is identified as that with the highest marginal log-likelihood. The clustering with linked loci analysis was chosen to account for the linkage present between sites within aligned sequences. Ten iterations of *K* (from 1 to 20) were conducted, to determine the optimal number of genetically homogeneous groups.

3. Results

3.1. Molecular detection and tuf typing by RFLP analysis

From part of the DNA collection of the '*Ca.* P. solani'-infected grapevines (Murolo et al., 2014), 329 samples were used as the template for amplification with different primer pairs specific for '*Ca.* P. solani'. No amplification was obtained using any primer pair when the samples were analysed in the first round of the PCR. However, in nested PCR, there were different efficiencies for the detection of '*Ca.* P. solani' according to the genes analysed. The primer pair tufAYf/r yielded a 900-bp fragment in 84.2% of the symptomatic grapevines, followed by a 500-bp fragment with stampF1/R1 (60.4%), and an 870 bp fragment with PosecN2/R2 (56.3%).

The molecular characterisation was carried out on 194 out of 277 samples among those positively amplified with tufAYf/r. In the study vineyard, the molecular characterisation on the basis of the *tuf* gene revealed that the grapevines were mainly infected by '*Ca.* P. solani' *tuf*-type b (92.3%), with occasional *tuf*-type a infections (7.7%). In the study vineyard, the mean disease severity induced on the grapevines by '*Ca.* P. solani' *tuf*-type a (2.57) was not significantly different with respect to *tuf*-type b (2.45). Crossing the data from *vmp*1 typing with the data recorded during the survey in 2011, we calculated the BN severity; there were no significant differences recorded for the disease severity induced in the grapevines by the V14 (2.47), V12 (2.37) and V3 (2.57) types.

3.2. Multilocus sequence typing analysis

Representative isolates according RFLP analysis were sequenced in the considered genes (*vmp*1, *stamp*, and *secY*) (Table 1). The sequences from the phytoplasma strains infecting the study vineyard showed the highest nucleotide identity in the *secY* nucleotide sequence (98.6–100%), followed by the *stamp* (92.9–100%) and *vmp*1 nucleotide sequences (65.9–100%). For both *stamp*, and more consistently, *vmp*1, amplicons with different lengths were detected.

3.3. Phylogenetic analysis and 'Ca. P. solani' population structure according to the vmp1, stamp, and secY genes

For each of the *vmp1, stamp,* and *secY* genes, the phylogenetic relationships were reconstructed for the nucleotide sequence of '*Ca.* P. solani' that originated from the study vineyard, with respect to nucleotide sequences from other Italian regions and from Euro-Mediterranean countries that were available in Genebank (Table 1).

For the *vmp*1 gene, the phylogenetic analysis was carried out on 15 nucleotide sequences, which were representative of the RFLP types of the study vineyard, and on 31 that were available in GenBank. In the resulting dendrogram, the sequences generally clustered according to the PCR-RFLP patterns: strains with the same RFLP pattern showed high nucleotide similarity (>99%) of sequences. The '*Ca.* P. solani' previously characterised as *tuf*-type a were clearly distinguishable from *tuf*-type b (Fig. 1A). The phylogenetic analysis confirmed these results obtained by applying RFLP analysis. The sequence analysis of the '*Ca.* P. solani' strains that were infecting the study vineyard were based on DNAsp tool, which identified seven out of 20 *vmp*1 types, two of which (Hap_7, 11) were exclusively detected in this vineyard. Hap_1, 6, 10, and 15 were shared with those of the other Italian regions, and Hap_4, was common to the other Italian regions and to the Euro-Mediterranean countries (Fig. 1B).

For the *stamp* gene, there were 17 nucleotide sequences from the BN-infected grapevines in the study vineyard, and 31 from the NCBI database or available from the "STOLBUR-EUROMED Consortium". In the phylogenetic tree, together with other isolates from the Italian regions and Euro-Mediterranean countries, isolate 60/11 (KJ145345) that was previously characterised as *tuf*-type a was grouped in a different cluster from the isolates characterised as *tuf*-type b. The isolates that originated from the study vineyard were located across both clusters (*tuf*-types a and b). Most of isolates from the study vineyard shared high nucleotide similarity (*ca.* 100%) with FN813256 (isolate GGY), FN813262 (isolate NGA9), and FN813252 (isolate BG4560), and fell into a branch of the tree that is supported by significant bootstrap values (77) (Fig. 2A). P42/11, 166/11, P10/11 and 136/11 formed a different group, and shared high nucleotide similarity with another two Italian isolates, Ate7 (KJ145381) and Mca21 (KJ145342). The DNAsp tool defined 17 *stamp* types: three were shared between the study vineyard and the Italian regions (Hap_1, 8, 14). Type 3 was common to the study vineyard and the Euro-Mediterranean countries

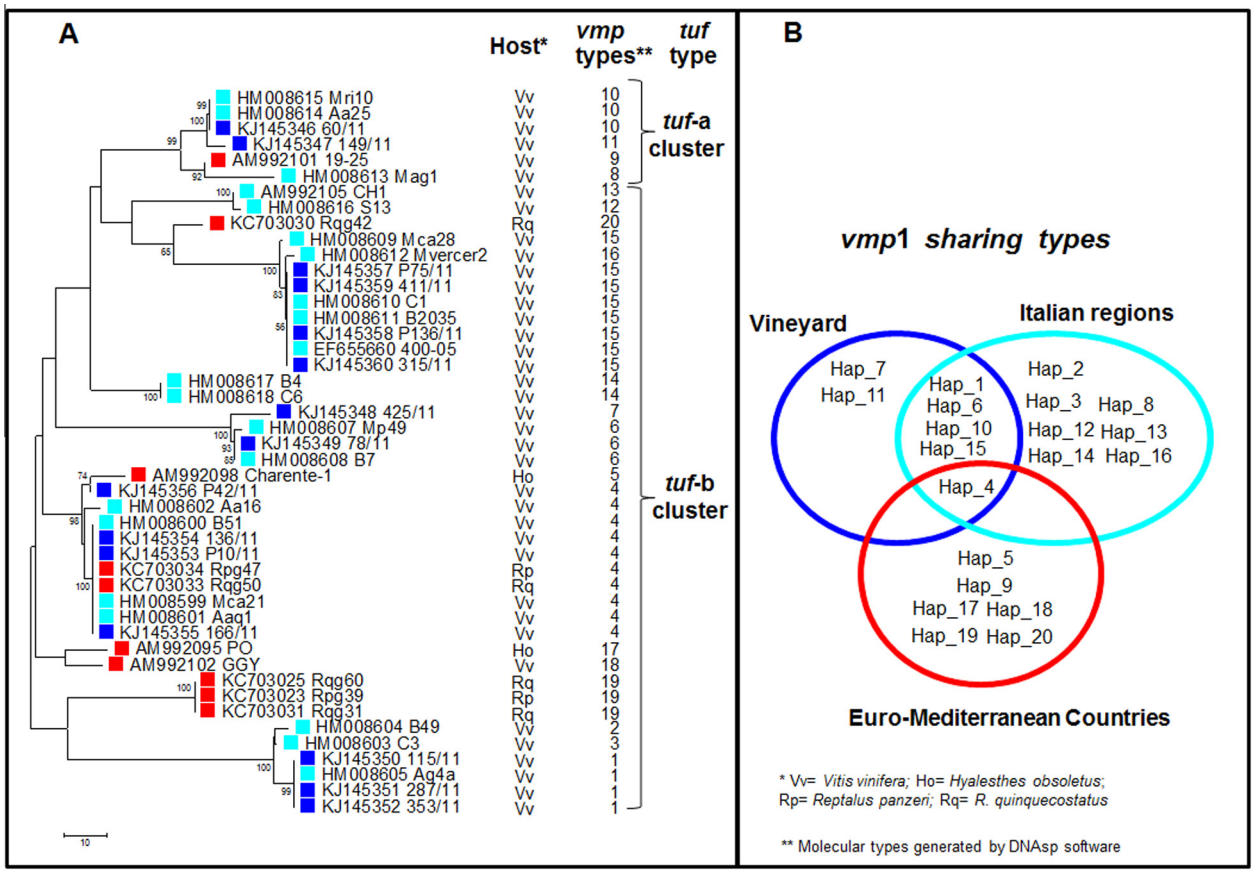

Fig. 1. (A) Phylogenetic tree for the *vmp*1 gene, using the minimum evolution method with 1000 bootstrap replicates. Constructed from the nucleotide sequences obtained for the study vineyard (blue), and from those available in the NCBI database for the Italian regions (sky blue) and the Euro-Mediterranean countries (red). Right: relationships between the types based on SNPs, using the DNAsp software, and *tuf*-typing characterisation (Langer and Maixner, 2004). (B) Types detected for the stolbur populations in the study vineyard (blue), the Italian regions (sky blue), and the Euro-Mediterranean countries (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2B), and it was the most recurrent in the study vineyard. Hap_6 was shared in all three contexts.

For the *secY* gene, 13 nucleotide sequences from the study vineyard and 30 sequences from GenBank or from the "STOLBUR-EUROMED Consortium" were analysed (Fig. 3A). The DNAsp software defined 16 *secY*-types, three of which (Hap_4, 7, 10) were only detected in the study vineyard, and one of which (Hap_3) was shared among all of the studied strains and was the most representative (Fig. 3B).

3.4. Genetic parameters and polymorphisms of the 'Ca. P. solani' populations

The genetic parameters of '*Ca.* P. solani' were analysed for the three genes (i.e., *vmp*1, *stamp*, *secY*) and according to the different levels of complexity. These analyses were carried out on the basis of the geographical origins of the samples, from a restricted area (study vineyard), up to the wider areas (Italian regions, other Euro-Mediterranean countries).

Overall, there were gradual increases in the values of the genetic parameters of *S*, η, Hd, *k* and Pi in the *stamp* and *secY* sequences in moving from the study vineyard up to the Euro-Mediterranean populations. Conversely, for the *vmp*1 gene, the highest values of these parameters were in the Italian population (Table 2).

The largest mean number of differences among the sequences from the same population (*k*) was recorded for *vmp*1 (31.7 nt), followed by *stamp* (8.9 nt), and *secY* (3.1 nt) (Table 2). Nucleotide insertions and deletions were frequent in the *vmp*1 gene, the amplicons of which are consistently different in length (ca. from 1450 bp up to 1700 bp). The highest overall nucleotide diversity (Pi) was recorded for *vmp*1 (0.0381 ± 0.0036), which was characterised by a high number of segregating sites (119 S), followed by *stamp* (0.0189 ± 0.0012; 28 S) and *secY* (0.0043 ± 0.0006; 26 S) (Table 2). This behaviour was confirmed in the study vineyard, where the sequences of *vmp*1 and *stamp* were more variable than *secY*.

The highest number of types (Hn) in the vineyard was recorded for *vmp*1 (7), followed by *stamp* (5), and with the lowest for *secY* (4) (Table 2). The molecular type diversity (Hd) can range from zero, meaning no diversity, to 1.000, which indicates the highest level of type diversity (Nei and Tajima, 1981). For all three examined genes, the samples from the study vineyard showed consistent Hd, which was higher for *vmp*1 (0.857 ± 0.057), slightly lower for *stamp* (0.728 ± 0.083), and lowest for *secY* (0.526 ± 0.153). Calculating the Hd in the overall population, the values were similar for these three genes, and they ranged from 0.819 ± 0.051 (*secY*) up to 0.914 ± 0.025 (*vmp*1), which indicated very high levels of diversity for each locus.

3.5. Selective pressure

The genetic variability and the selective pressure in the *vmp*1, *stamp* and *secY* genes were estimated for the '*Ca.* P. solani' strains according to the abundance of non-synonymous mutations. For the *secY* gene, the overall dN/dS ratio was 1.02 ($P = 0.841$), which suggested low neutral selection across this gene.

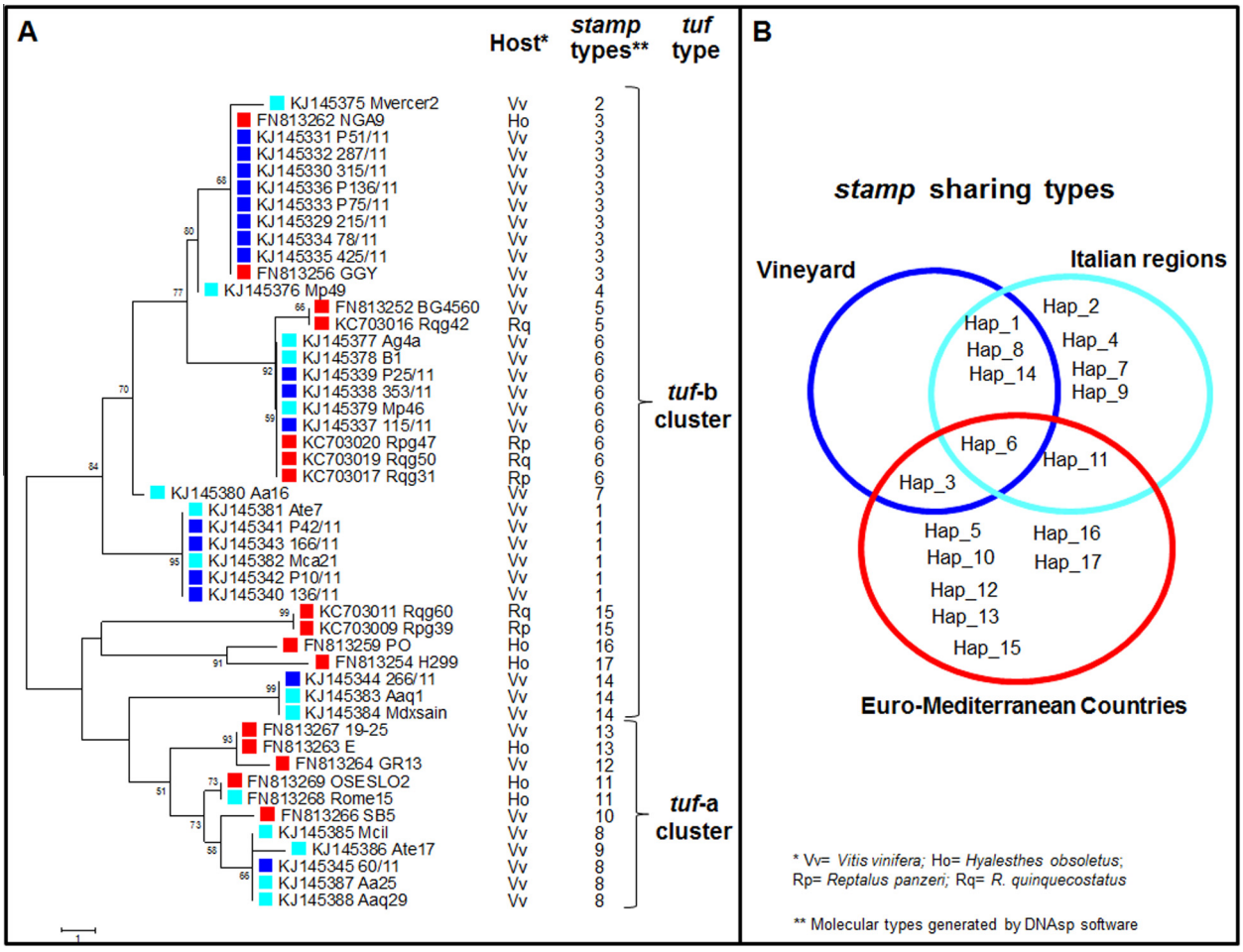

Fig. 2. (A, B) Phylogenetic tree (A) and types (B) for the *stamp* gene, as indicated in the legend to Fig. 1.

The overall ratio between the non-synonymous to the synonymous mutations (dN/dS) was >1.0 for *vmp*1 (2.28; $P = 0.001$) and *stamp* (3.99; $P = 0.019$). These high values of dN/dS (i.e., >1) indicated detection of a high number of non-silent (dN) mutations.

For the *stamp* gene, we can assume that the wider the origin of the '*Ca.* P. solani' nucleotide sequences, the less intensive the positive selective pressure (dN/dS) will be, which ranged from 3.04 ($P = 0.019$) (Euro-Mediterranean countries), through 4.64 ($P = 0.013$) (Italian regions), up to 7.05 ($P = 0.004$) (study vineyard). An opposite trend was seen for *vmp*1 with respect to *stamp*, where the dN/dS ratio gradually decreased from 2.99 ($P = 0.000$) (Euro-Mediterranean countries) to 2.28 ($P = 0.000$) (Italian regions) and 2.23 ($P = 0.000$) (study vineyard).

3.6. Analysis of the population structure

Genetic clusters of '*Ca.* P. solani' were investigated using the BAPS software, considering all of the nucleotide sequences related to the grapevines or the main vectors. The estimated numbers of populations with the most statistical support were $K = 3$, 5 and 6 for the *secY*, *stamp*, and *vmp*1 genes, respectively (Fig. 4). Considering the *vmp*1, *stamp* and *secY* genes of '*Ca.* P. solani', the population structure recorded in the vineyard was similar to that of the Italian regions, which lacked, however, some of the clusters with respect to the Euro-Mediterranean population (Fig. 4). This result showed that the population structure of the '*Ca.* P. solani' infecting the grapevines and the main vectors was related to the geographical origin.

4. Discussion

Based on the information acquired during the survey carried out in September 2011, the symptomatic grapevines represented about 50% of all of the grapevines in the vineyard (Murolo et al., 2014), for which the average BN severity was 2.32. In the studied vineyard, '*Ca.* P. solani' *tuf*-type b was predominant over *tuf*-type a; these were both distributed throughout the field, and hence no specific distribution patterns were recorded for these isolates. Several studies have described different distributions of *tuf* types according to geographical areas. In north-western Europe and the regions of northern Italy, the reported BN epidemics show increasing spread of '*Ca.* P. solani' *tuf*-type a (Johannesen et al., 2012; Quaglino et al., 2013). Conversely, a wider distribution of '*Ca.* P. solani' *tuf*-type b has been reported for the regions of southern and central Italy (Pacifico et al., 2007; Pasquini et al., 2007; Murolo et al., 2010). This finding can be linked to the different compositions of the weeds around and within the vineyards, as locations with more rain and higher organic matter content in the soil favour nettle growth, which is the main host plant for *tuf*-type a (Maixner, 2011). Occasionally, as seen by Fialova et al. (2009), *tuf*-type b infection has been detected in nettle. More recently, a particular '*Ca.* P. solani' *tuf*-type b was recorded in nettle in Austria that showed a specific *Hpa*II-restriction profile that had been previously attributed to bindweed-associated strains; this showed a different sequence with respect to the reference *tuf*-type b strains, and was assigned as *tuf*-type b2 (Aryan et al., 2014). In the Marche region, where the present study was based, and where nettle is rarely found around vineyards, the BN-infected grapevines

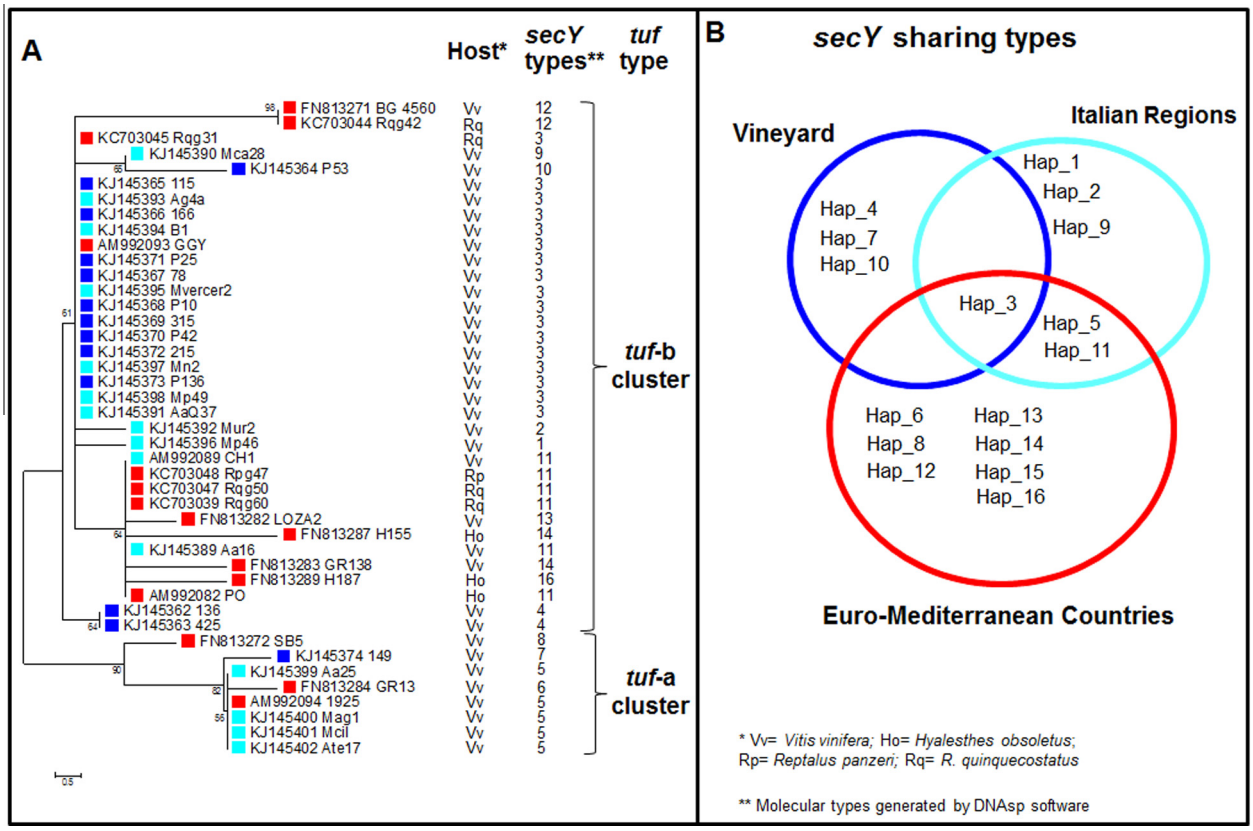

Fig. 3. (A, B) Phylogenetic tree (A) and types (B) for the *secY* gene, as indicated in the legend to Fig. 1.

Table 2
Genetic parameters calculated using the DNAsp software for the '*Ca.* P. solani' population at the study-vineyard, Italian, and Euro-Mediterranean levels for the *vmp*1, *stamp* and *secY* genes.

Population	No. seq	Genetic parameter								
		S	*η*	Hn	Hd	*k*	Pi	dS	dN	dN/dS*
vmp1 gene										
This study	15	133	138	7	0.857 ± 0.057	47.7	0.0453 ± 0.0054	0.02438	0.05450	2.23 (*P* = 0.000)
Italian	21	200	207	12	0.962 ± 0.026	66.2	0.0512 ± 0.0038	0.02716	0.06205	2.28 (*P* = 0.000)
Euro-Med.	10	79	83	7	0.911 ± 0.066	33.6	0.0403 ± 0.0043	0.01661	0.04974	2.99 (*P* = 0.000)
Combined	46	119	128	20	0.914 ± 0.025	31.7	0.0381 ± 0.0036	0.02003	0.04573	2.28 (*P* = 0.001)
stamp gene										
This study	17	21	21	5	0.728 ± 0.083	5.9	0.0126 ± 0.0028	0.00224	0.01581	7.05 (*P* = 0.004)
Italian	15	23	24	9	0.924 ± 0.044	9.2	0.0194 ± 0.0015	0.00525	0.02438	4.64 (*P* = 0.013)
Euro-Med.	16	24	28	10	0.945 ± 0.045	10.2	0.0216 ± 0.0019	0.00862	0.02618	3.04 (*P* = 0.019)
Combined	48	28	33	17	0.904 ± 0.023	8.9	0.0189 ± 0.0012	0.00588	0.02348	3.99 (*P* = 0.019)
secY gene										
This study	13	9	9	4	0.526 ± 0.153	1.6	0.0022 ± 0.0011	0.00000	0.00287	nd
Italian	15	9	9	6	0.790 ± 0.079	2.7	0.0039 ± 0.0007	0.00260	0.00421	1.62 (*P* = 0.855)
Euro-Med.	15	20	20	10	0.924 ± 0.053	4.4	0.0061 ± 0.0011	0.00797	0.00567	0.71 (*P* = 0.762)
Combined	43	26	26	16	0.819 ± 0.051	3.1	0.0043 ± 0.0006	0.00428	0.00436	1.02 (*P* = 0.841)

S, number of polymorphic (segregating) sites; *η* (Eta), total number of mutations; Hn, number of molecular types; Hd, molecular type diversity; *k*, mean number of nucleotide differences between sequences; Pi, nucleotide diversity according to the Jukes and Cantor method (29); dS, synonymous nucleotide substitution rate; dN, non-synonymous nucleotide substitution rate. dS and dN values were calculated according to Nei and Gojobori (33).

* *P*, probability of rejecting the null hypothesis of strict neutrality (dN = dS), with values <0.05 are considered significant at the 5% level.

showed mainly (~80%) '*Ca.* P. solani' *tuf*-type b (Murolo et al., 2010). The random presence of grapevines in the study vineyard that were infected by *tuf*-type a, which was not confined to the borders, indicated that the phytoplasma might have already been present in the propagation material. In the study vineyard, both *tuf*-types were found, and also in BN-infected grapevines that grew a few metres from each other, although with different frequencies. The same situation was recorded in a few other studies, although with minor significance (Berger et al., 2009; Mori et al., 2012), maybe because of the low numbers of samples analysed from the same vineyard in most of the other investigations.

The relatively low percentage of samples amplified for the *stamp* and *secY* genes (~60%) confirmed the high genetic variability that has been reported in previous studies on *vmp*1 (Pacifico et al., 2009; Murolo et al., 2010, 2013). In the same vineyard, Murolo et al. (2014) detected several already known *vmp*1 types. The wide

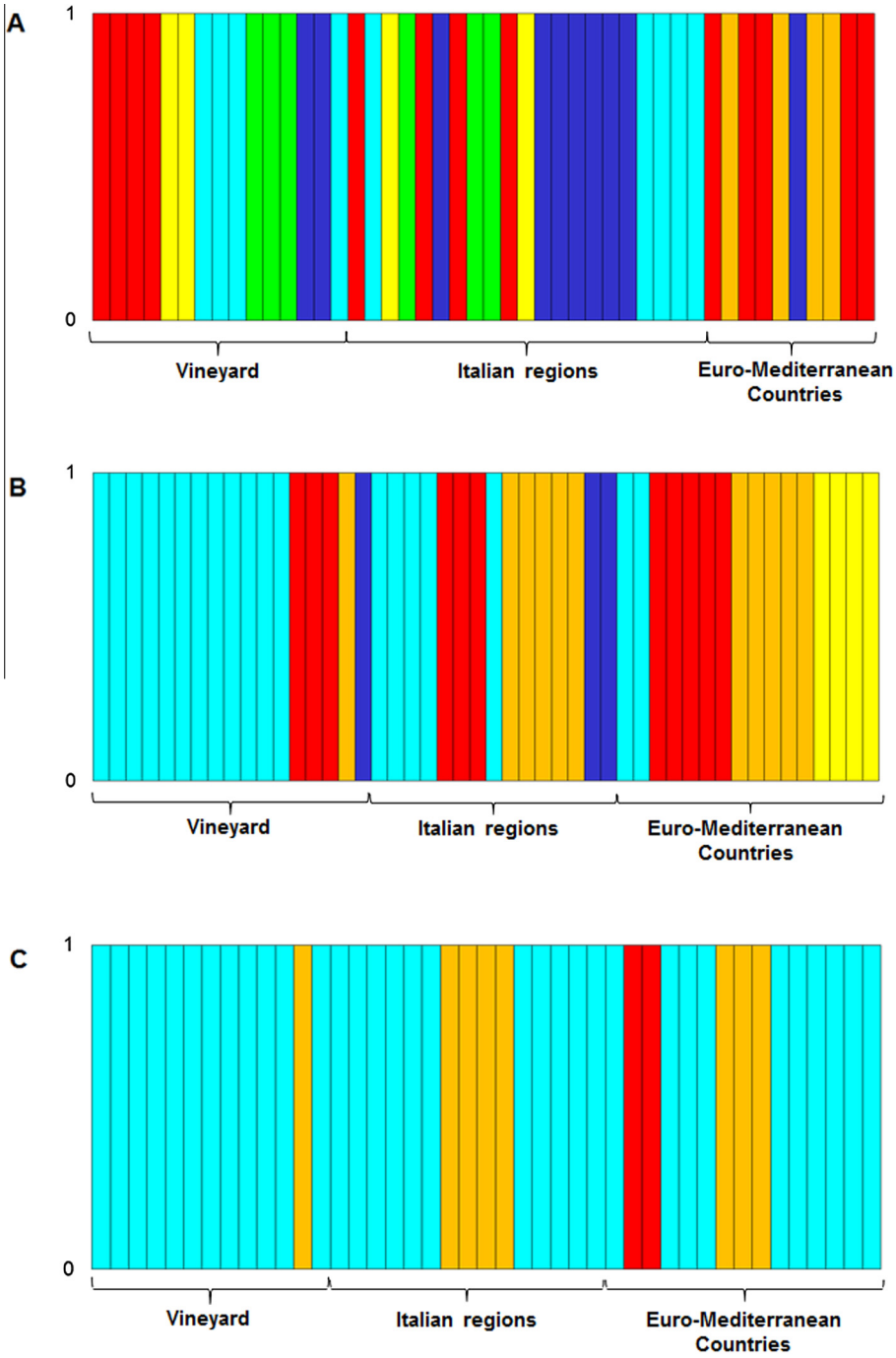

Fig. 4. Population structure of '*Ca.* P. solani' according to the *vmp*1 (A), *stamp* (B), and *secY* (C) genes from the vineyard up to the Euro-Mediterranean countries, using the BAPS software. Each isolate is the colour corresponding to the cluster where it was placed. Shared colours denote individuals resembling each other as much as possible genetically. The colour ordering in the different gene analyses was arbitrary, and hence the colours between the analyses cannot be compared. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genetic diversity of '*Ca.* P. solani' has been reported and generally related to complex interactions between the vector and the wide range of wild host plants (Kessler et al., 2011; Riolo et al., 2012).

The phylogenetic analyses that were carried out for the *vmp*1, *stamp*, and *secY* genes were useful to study the relationships that the '*Ca.* P. solani' that originated in the study vineyard had with respect to the nucleotide sequences from the other Italian regions and from the Euro-Mediterranean countries. The phylogenetic analysis of the *vmp*1 gene confirmed the results obtained in our previous study using RFLP analysis (Murolo et al., 2014), thus showing the accuracy of this technique. However, RFLP is a preliminary tool for the estimation of genetic diversity, and it requires the support of a more informative tool, such as sequence analysis (Pacifico et al., 2009; Papura et al., 2009; Murolo et al., 2010; Foissac et al., 2013). In the phylogenetic trees of *vmp*1, *stamp*, and *secY*, and independent from the genes analysed, we recorded a robust correlation between *tuf* characterisation and the isolation of a branch that clustered the isolates characterised as *tuf*-type a, as already demonstrated in previous studies (Fabre et al., 2011; Cvrković et al., 2014; Kostadinovska et al., 2014).

Based on DNAsp tool, we identified different molecular types according to the genes analysed. In particular, we focused our attention on Hapl_4 of the *vmp*1 gene and Hapl_6 of the *stamp* gene. Both of these haplotypes of the two membrane proteins were shared in all three contexts (i.e., study vineyard, Italian regions, Euro-Mediterranean countries). This was a relevant finding, considering that both of the haplotypes were also shared with all of the phytoplasma types (Rpg47 *R. panzeri* Serbia KC703020, Rqg50 *R. quinquecostatus* Serbia KC703019, Rqg31 *R. quinquecostatus* Serbia KC703017) that were found in established (*R. panzeri*) or putative (*R. quinquecostatus*) vectors of '*Ca.* P. solani' (Pinzauti et al., 2008; Cvrković et al., 2014). This suggests a new scenario for possible *R. panzeri*-related BN diffusion, which has been demonstrated in Serbia, and recently hypothesised also in the Republic of Macedonia (Kostadinovska et al., 2014).

The analysis of the genetic parameters related to genetic diversity (*S*, η, Hd, *k* and Pi) revealed higher values in *vmp*1 and *stamp*, with respect to *secY*. Indeed, the genes that encode membrane proteins, which are expected to have important functions in host–phytoplasma interactions (Hogenhout et al., 2008), showed significant differences among closely related strains of other phytoplasma belonging to the 16Sr I and 16Sr III groups (Arashida et al., 2008; Galetto et al., 2011; Neriya et al., 2011). In particular, nucleotide insertions and deletions are frequent in the *stamp* gene (Fabre et al., 2011) but more consistent in the *vmp*1 gene, where the amplicons can have different lengths (Pacifico et al., 2009; Murolo et al., 2010).

The higher genetic variability in the *vmp*1 and *stamp* genes with respect to the *secY* gene arose from the estimation of the rate of non-silent mutation (dN). According to this parameter, which is an indication of selective pressure, Fabre et al. (2011) defined the *secY* gene as a housekeeping gene, while the *vmp*1 and *stamp* genes were under positive selection.

As phytoplasma lack cell walls and are intracellular parasites, these proteins (*vmp*1, *stamp*) are in direct contact with the host-cell cytoplasm (Kakizawa et al., 2006a). Here they are involved in specific interactions, such as the adhesion to the host cell that has been demonstrated for other *Mollicutes* (Chaudhry et al., 2007). Moreover, a role in species-specific recognition between vector and phytoplasma was proposed for Amp of '*Ca.* P. asteris', an onion yellows strain (Suzuki et al., 2006). This was based on the *in vitro* interaction of this onion yellows Amp with the actin of the vector, and on the co-localisation of the phytoplasma with actin filaments in the insect gut.

These direct and intense interactions can generate genetic variability, which has an important role in adaptation, as their host environments are extremely variable, and include the intracellular environments of the phloem tissues of plants, and the gut, salivary glands, and other organs and tissues of their insect hosts (Bai et al., 2006). The selective pressure that acts on *vmp1* and *stamp* might be explained by these complex interactions. Cloning of the *amp* genes from several strains of aster yellows group phytoplasma showed that the Amp proteins were under positive selection, and that the positively selected amino acids were encoded in the central hydrophilic domain of Amp (Kakizawa et al., 2006b).

Positive selection has also been demonstrated for several phytoplasma proteins in terms of their interactions with insect tissue (Kakizawa et al., 2009). In particular, it is known that Amp of '*Ca.* P. asteris' specifically interacts with vector proteins (Suzuki et al., 2006; Galetto et al., 2011), while Imp of '*Ca.* P. mali' was demonstrated to specifically interface with the plant actin (Boonrod et al., 2012). For '*Ca.* P. solani', natural transmission has been demonstrated for at least three species of planthoppers in the *Cixiidae* family (Fos et al., 1992; Gatineau et al., 2001; Jović et al., 2009; Imo et al., 2013; Cvrković et al., 2014). However, the main vector remains *H. obsoletus*, for which different ecotypes have been described as living on the nettle *Urtica dioica* or on the bindweed *Convolvulus arvensis* (Langer and Maixner, 2004), and as being able to transmit the '*Ca.* P. solani' *tuf*-types a or b, respectively (Johannesen et al., 2012). The complex, continuous and dynamic interactions with their hosts and vectors is the basis for the recurrent selection of adaptive mutations. This is confirmed by the detection of elevated polymorphism for both the *vmp*1 and *stamp* genes, even in a restricted area such as a single vineyard.

The positive selective pressure for the *stamp* gene was particularly high in the isolates that originated in the study vineyard. The very high dN/dS ratio among very closely related bacterial genomes has been considered as a statistical artefact (Jordan et al., 2002), or has been interpreted as evidence of positive or relaxed selection (Read et al., 2002; Baker et al., 2004). Rocha et al. (2006) demonstrated that the dN/dS ratio is time dependent. Hence, we can hypothesise an initial stage of sequence diversification in the vineyard. With time, only those few mutations that result in pernicious selective consequences are removed. Consequently, almost all of this variability will be lost, and only a tiny minority of it will become fixed.

In conclusion, we have confirmed a correlation of variability for the *vmp*1, *stamp* and *secY* genes with the *tuf* gene. In the study vineyard, we did not record any correlations for BN severity. We have demonstrated that the genetic diversity and population structure of '*Ca.* P. solani' sampled from a homogenous single crop (i.e., the grapevine cv. 'Chardonnay') in a restricted geographical area (i.e., the study vineyard) reflects the stolbur population structure across the wider geographical scale (i.e., Italian regions, other Euro-Mediterranean countries). Moreover, the different frequencies within a restricted location of the molecular types in *tuf* and *vmp*1, as well as the high genetic variability in the *stamp* gene of '*Ca.* P. solani', provide useful information to trace an inoculum source and the movement of pathogen strains over local and long distances.

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