Plant Pathology (2014) **63**, 700–707 Doi: 10.1111/ppa.12122

Spatial and temporal stolbur population structure in a cv. Chardonnay vineyard according to *vmp1* gene characterization

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Bois noir is a grapevine disease caused by the stolbur phytoplasma. It is widespread in all European and Mediterranean viticultural areas, and it can induce severe damage to the quality and quantity of production. The recent disease recrudescence has encouraged studies on the use of molecular markers to assess the genetic diversity of stolbur strains. The aim of this study was to evaluate the presence of Bois noir symptoms and to monitor the spatial genetic structure of the stolbur population according to *vmp1* genotypes, through 2011 and 2012 in a cv. Chardonnay vineyard. In both years, there were increased vines with symptoms from July to September. The analysis of dispersal indices showed that the spatial distribution was uniform in the vineyard. However, the two-dimensional contour maps show that Bois noir severity was higher in plants located on the borders than in the central parts of the vineyard. Stolbur population was composed of two prevalent *vmp* genotypes (V14, V12) across both years, along with other minor haplotypes (V3, V4, V9, V11, V15, V18, in 2011; V3, V18 in 2012). The data indicate that the *vmp1* gene is an efficient marker to study the population structure of stolbur phytoplasma, to track the movement of the pathogen, and to identify the inoculum source, which will all serve in the planning of control strategies.

Keywords: grapevine, molecular characterization, molecular epidemiology, phytoplasma, variable membrane protein

Introduction

Phytoplasmas are widely spread prokaryotes that are responsible for severe diseases of many plants; overall they are known as the 'yellows'. In Europe, one of the most recurrent grapevine yellows is Bois noir (BN), the spread of which is usually endemic. Over recent decades, frequent Bois noir outbreaks have been recorded, which have resulted in severe perturbations to leaf gas exchange, chlorophyll a fluorescence, pigment content and maximum quantum efficiency of photosystem II, with the direct influence of decreased total berry production (Endeshaw *et al.*, 2012). The causal agent of BN is a phytoplasma that belongs to the stolbur group (16SrXII-A subgroup). It is transmitted by the polyphagous cixiid planthopper *Hyalesthes obsoletus* to a wide range of wild plants such as *Convolvulus arvensis*, *Calystegia sepium*, *Urtica dioica* and several other herbaceous hosts (Maixner *et al.*, 1995; Langer & Maixner, 2004). These all thus represent potential inoculum sources. On the other hand, grapevine is only occasionally infected by *H. obsoletus* and can be considered a dead-end host for the stolbur phytoplasma. The multiple interactions with wild and cultivated annual and perennial host plants and insect vectors in different ecosystems might be responsible for generating genetic diversity both in conventionally conserved genes (Langer & Maixner, 2004; Quaglino *et al.*, 2013), and in a highly variable gene (*vmp1*) encoding a putative membrane protein (*variable membrane protein 1;* Cimerman *et al.*, 2009). This membrane protein is in direct contact with the host cells, and can thus be particularly exposed to positive selection, which has generated its high variability (Kakizawa *et al.*, 2006).

A wide molecular characterization of stolbur based on the *vmp1* gene was carried out in the framework of the STOLBUR-EUROMED Consortium (SEE-ERA.NET; www.phytoplasma.eu), where 23 different genotypes were identified (Foissac *et al.*, 2013). The molecular tools generally applied to discriminate different *vmp1* genotypes are polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) followed by nucleotide sequencing for the representative types (Fialova *et al.*, 2009; Pacifico *et al.*, 2009; Murolo *et al.*, 2010). The hypervariability of the *vmp1* gene means that it has been considered as a suitable marker for molecular epidemiology, which is acquiring more and more importance for microbial identification and epidemiological studies (Li *et al.*, 2009). This approach is particularly relevant to uncultivable organisms, and it allows the wide implementation of tracking pathogen movements, identifying source populations, tracing epidemic history, and understanding dispersal routes of pathogens (Baric *et al.*, 2011). Using *vmp1* molecular typing, this study

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Published online 18 September 2013

has been able to monitor the potential spread and distribution of stolbur outbreaks even in a restricted area of study.

The main aims of this study were therefore to: (i) record the in-vineyard disease incidence and severity; and (ii) monitor the genetic structure of the stolbur population according to the *vmp1* genotypes over 2 years in a cv. Chardonnay vineyard.

Materials and methods

Features of the vineyard

The trials were carried out through 2011 and 2012 in a cv. Chardonnay vineyard of about 0·6 ha located in Montalto delle Marche (Ascoli Piceno, Italy), central-eastern Italy (42°59′00″N, 13°36′00″E; 513 m a.s.l.). It had an average annual temperature of 13·7°C and precipitation of 668 mm. The vineyard was planted in 2005 as cv. Chardonnay plants grafted onto Kober 5BB (*Vitis berlandieri* × *V. riparia*) rootstock; these were trained as a single curtain, with a low cordon. The planting density was 2·5 m between the rows and 1·5 m along the rows. The vineyard was not irrigated, and fertilizers were distributed in winter. Control of the main fungal diseases (downy mildew and powdery mildew) and any additional green pruning were carried out in spring and summer, as normal practices for the area.

Climate data

During the vegetation period until harvest, the climate data of the area (minimum and maximum weekly temperatures, rainfall) were provided by the local weather station of ASSAM (Marche Region, Ancona, Italy).

Disease assessment

Visual inspections were carried out at the beginning of July, August and September in 2011 and 2012 to assess the incidence of BN symptoms on the canopy. Moreover, in the September assessments, the disease severity was recorded using an empirical scale of 1–5, where 0 = symptomless/healthy plant; 1 = plant showing 1–2 leaves with symptoms; 2 = plant showing more than 2 leaves with symptoms on one shoot; 3 = plant with leaves with symptoms on more than one shoot; and 4 = plant with more than 50% of canopy with symptoms.

DNA extraction and phytoplasma identification

During the visual inspections in September, 357 leaf samples with symptoms were collected in 2011, and 164 in 2012, in order to get representative samples of the entire vineyard. These were labelled and stored in plastic bags at 4°C for further analysis. Detection of phytoplasma was carried out by extracting the total DNA from 0·5 g leaf petioles. The DNA was extracted using the DNeasy Plant Mini kit (QIAGEN), and 10-fold dilutions were used as templates for PCR. Amplifications were performed in final reaction volumes of 25 μL, which contained *c.* 30–50 ng DNA template, 50 ng each primer, 125 μM each dNTP (Promega Corporation), 1 U *Taq* DNA polymerase (Promega), and 1 × PCR buffer with 1·5 mM $MgCl_2$. The PCR was performed in a programmable Bio-Rad Cycler (Bio-Rad), using the universal P1/P7 phytoplasma primer pair, followed by nested PCR with specific ribosomal primer pairs for the 16SrI, III and V phytoplasma groups, according to the conditions described by Lee *et al.* (1994). The samples from the healthy plants were used as negative controls. Amplicons obtained with R16(I)F1/R1 followed by *Mse*I-RFLP, allowed samples belonging to 16SrI to be distinguished from those belonging to the 16SrXII group. To further verify that the isolates belong to the 16SrXII-A subgroup, the samples were amplified with the rStol/fStol specific primer pair (Maixner *et al.*, 1995). Seven microlitres of each PCR product were analysed by electrophoresis through 1·5% agarose (Sigma-Aldrich) gels, with 1 × TAE (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid, pH 8·0) as running buffer. The gels were stained with GelRed (Biotium Inc.), visualized under UV light at 312 nm using a transilluminator, and imaged with the Gel Doc XR imaging system (Bio-Rad). The expected lengths of the amplified DNA fragments were estimated by comparisons with a 100 bp DNA ladder (New England BioLabs).

Molecular characterization of stolbur isolates for the *vmp1* gene

The isolates amplified with the stolbur group specific primer pair rStol/fStol were subjected to further molecular characterization, to investigate the non-ribosomal gene that encodes the VMP1 protein. This *vmp1* gene was amplified with the StolH10F1/R1 primer pair (Cimerman *et al.*, 2009), followed by a second round of PCR with the TYPH10F/R primer pair (Fialova *et al.*, 2009). The products of the nested PCRs were verified by electrophoresis through 1% agarose gels, and then an aliquot was digested with 2·5 U *Rsa*I restriction enzyme (New England BioLabs) at 37°C, according to the manufacturer's instructions. The digested products were analysed by electrophoresis on 2·5% agarose gels, which were resolved and recorded as described above, with a 100 bp DNA ladder (New England Biolabs) used as the marker. Finally, the picture captured by the Gel Doc XR imaging system (Bio-Rad) was analysed by QUANTITY ONE software (Bio-Rad).

Spatial distribution of BN infected plants

The occurrence of disease gradients within the vineyard for these BN-affected grapevines was studied. For this purpose, the percentages of diseased plants in the 13 rows and the 24 plots across each row were calculated. The percentages of diseased plants in each plot were plotted and the regression curve calculated in the vineyard for 2011 and 2012. Using PASSAGE software, v. 2 (Rosenberg & Anderson, 2011), the aggregation of adjacent vines and the more complex spatial relationships over longer distances were examined. Seven different dispersion indices were used: the index of dispersion and the variance-to-mean ratio (Campbell & Madden, 1990), the index of cluster size (David & Moore, 1954), Green's index (Green, 1966), the index of cluster frequency (Douglas, 1975), the index of mean crowding (Lloyd, 1967), the index of patchiness (Lloyd, 1967) and Morisita's index (Morisita, 1959). To compute these indices of dispersion, the vineyard was subdivided into transects of different sizes with increasing numbers of vines (2 × 2, 3 × 3, 2 × 5, 3 × 5, 2 × 10, 6 × 5).

The data regarding the positioning of the vines with symptoms and symptomless/healthy vines and the *vmp1* genotyping were plotted on a two-dimensional map, using the SYSTAT program, v. 11 (SyStat software Inc.) to spatially monitor the epidemiology of grapevine stolbur in 2011 and 2012.

The data of symptom severity (z) that were recorded in 2011 and 2012 were defined with respect to plant position (x, y). The SYSTAT software first computes its own square grid of interpolated or directly estimated values. From this grid, contours were followed using the method of Lodwick & Whittle (1970), combined with linear interpolation. The plot automatically determines the number of contours to draw, so that the surface is delineated and the contour labels can be characterized by different colours.

Results

Climate data

The analysis of the climate data showed that for June and July, average temperatures were lower in 2011 than 2012 by −1°C and −3°C, respectively, and rainfall was greater in 2011 than 2012 by 10 and 47 mm, respectively (Fig. 1).

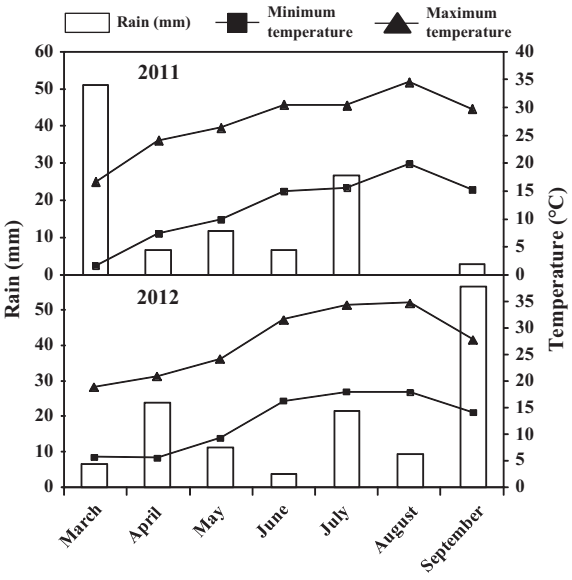

Figure 1 Main climatic data for rain and minimum and maximum temperatures recorded from March to September in 2011 and 2012 in the vineyard with cv. Chardonnay, located in Montalto delle Marche (AP, Italy).

Symptom assessment

The symptom status of vineyard cv. Chardonnay was assessed in July, August and September 2011 and 2012. In 2011, the number of BN-infected vines, estimated on the basis of leaf symptoms, increased from 157 in July up to 775 in September, whilst in 2012, 99 plants with symptoms were observed in July, and 400 in September.

Spatial distribution of BN infected plants

Using the SYSTAT software, the positions of the healthy vines and vines with symptoms recorded in 2011 and 2012 were plotted, to obtain a two-dimensional map for each year of investigation (Fig. 2). In both years, there was a higher frequency of vines with symptoms along the borders of the vineyard than in its central part. The regression curve that overlapped the percentage of diseased vines compared with the distance from the border of the rows was in the form of a binomial curve in both 2011 ($y = 0{\cdot}0144x^2 - 0{\cdot}1182x + 0{\cdot}685$; $R^2 = 0{\cdot}9627$) and 2012 ($y = 0{\cdot}0086x^2 - 0{\cdot}0766x + 0{\cdot}3942$; $R^2 = 0{\cdot}7015$).

From the analysis of the various dispersion indices calculated considering the different transect sizes, the distribution of the vines with symptoms in both 2011 and 2012 showed a uniform pattern. In particular, the index of dispersion, index of patchiness and Morisita's index were <1, and the index of cluster size was negative (Table 1).

In the disease assessment carried out in September 2011, more than 70% of the plants with symptoms showed mild symptoms, represented by one or a few yellow leaves (severity classes 1, 2) on one shoot per vine. About 20% of the plants with symptoms showed symptoms on some shoots (severity class 3) and only 6% showed systemic expression (Fig. 3a). In 2012, more than 50% of the vines with symptoms showed yellows on several shoots (severity class 3) and systemic symptoms (severity class 4), with the others that showed milder symptoms (Fig. 3a). The data for symptom severity (z) recorded in 2011 and 2012 were analysed according to the plant positions (x, y) and are illustrated as two-dimensional mosaic contour maps in Figure 3b.

Phytoplasma molecular detection

Molecular detection was carried out on 521 samples with symptoms (357 in 2011, 164 in 2012). No amplicons were yielded with direct PCR using the universal phytoplasma primer pair P1/P7, nor in the nested PCR with specific primers for the 16SrV and 16SrIII groups (data not shown). The rStol/fStol primer pair amplified a 500 bp fragment in about 90% of the samples with symptoms collected in 2011 and 2012, confirming the results obtained by *Mse*I-RFLP carried out on R16F1/R1 amplicons, which showed a typical profile of stolbur phytoplasma reference strain.

Molecular characterization and spatial distribution of the stolbur *vmp1* genotypes

The StolH10F1/R1 primer pair followed by TYPH10F/R primer pair, which is specific for a putative membrane protein-coding region, allowed the amplification of a specific fragment in 53·8% of the samples with symptoms, according to the visual assessment carried out in 2011. The specific amplicon was dimorphic: 160 out of 192 (83·3%) of positive samples showed a fragment of about 1450 bp, while the remaining samples showed a fragment of about 1700 bp (data not shown).

From the *Rsa*I-RFLP assay, eight different *vmp1* types (V3, V4, V9, V11, V12, V14, V15 and V18) were

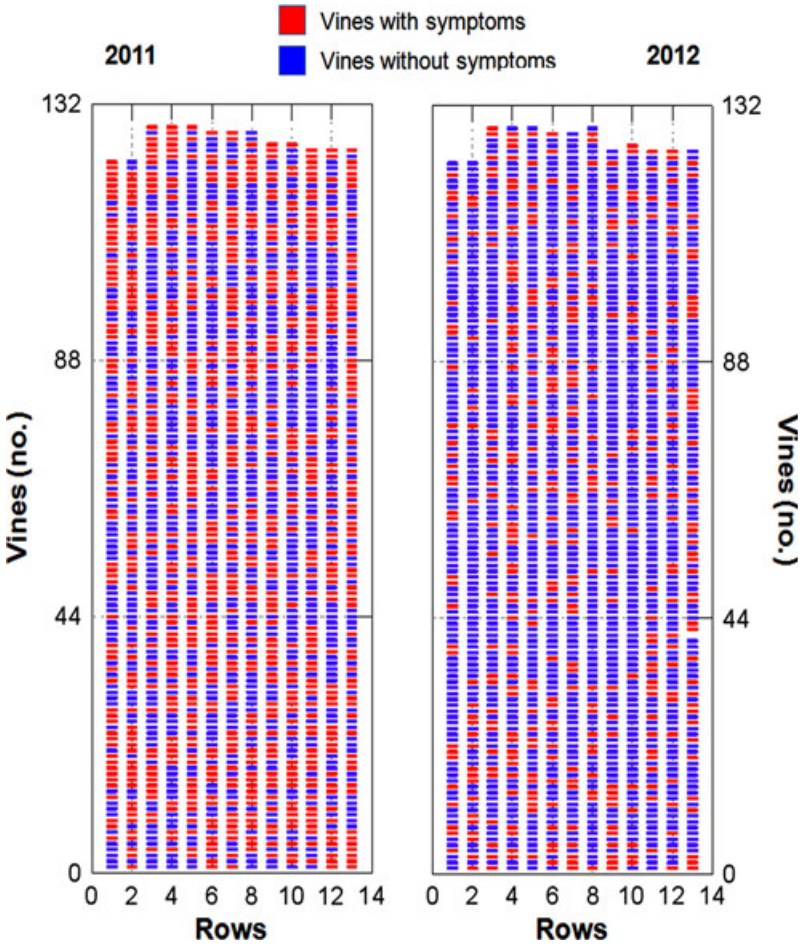

Figure 2 Maps of the vineyard cv. Chardonnay grapevines located at Montalto delle Marche (AP, Italy), surveyed in September 2011 and 2012, showing vines with (red) and without (blue) symptoms.

Table 1 Indices of dispersion for Bois noir-infected cv. Chardonnay grapevines in the vineyard at Montalto delle Marche (AP, Italy) in 2011 and 2012

			Index of dispersion[a]						
Quadrat size (vines)	Diseased plants/quadrat (mean)	No. of plants/quadrat	ID	ICS	GI	ICF	IMC	IP	MI
Year 2011									
2 × 2	1·95	4	0·631	−0·368	−0·0001	−5·293	1·581	0·811	0·811
3 × 3	4·38	9	0·657	−0·342	−0·0020	−12·816	4·044	0·921	0·922
2 × 5	4·92	10	0·635	−0·364	−0·0024	−13·500	4·561	0·925	0·926
3 × 5	7·13	15	0·673	−0·326	−0·0033	−21·828	6·803	0·954	0·954
2 × 10	9·32	20	0·776	−0·223	−0·0029	−41·627	9·096	0·975	0·976
6 × 5	14·62	30	0·697	−0·302	−0·0062	−48·354	14·317	0·979	0·979
Year 2012									
2 × 2	1·06	4	0·8327	−0·167	−0·0001	−6·3254	0·890	0·841	0·841
3 × 3	2·35	9	0·7596	−0·240	−0·0014	−9·7827	2·110	0·897	0·898
2 × 5	2·65	10	0·9776	−0·022	−0·0001	−118·008	2·617	0·991	0·991
3 × 5	3·92	15	1·0239	0·023	0·0002	163·930	3·943	1·006	1·006
2 × 10	5·01	20	0·9559	−0·044	−0·0005	−113·816	4·968	0·991	0·991
6 × 5	7·62	30	0·9207	−0·079	−0·002	−96·120	7·540	0·989	0·989

[a]ID, index of dispersion; ICS, index of cluster size; GI, Green's index; ICF, index of cluster frequency; IMC, index of mean crowding; IP, index of patchiness; MI, Morisita's index.

detected in the vineyard, with different frequencies (Fig. 4). In 2011, the dominant *vmp1* genotypes were V14, in 68·7% (132/192) of the positive samples, followed by V12, in 16·7% (32/192), which was obtained from the digestion of the largest fragment (about 1700 bp). The remaining *vmp1* genotypes were

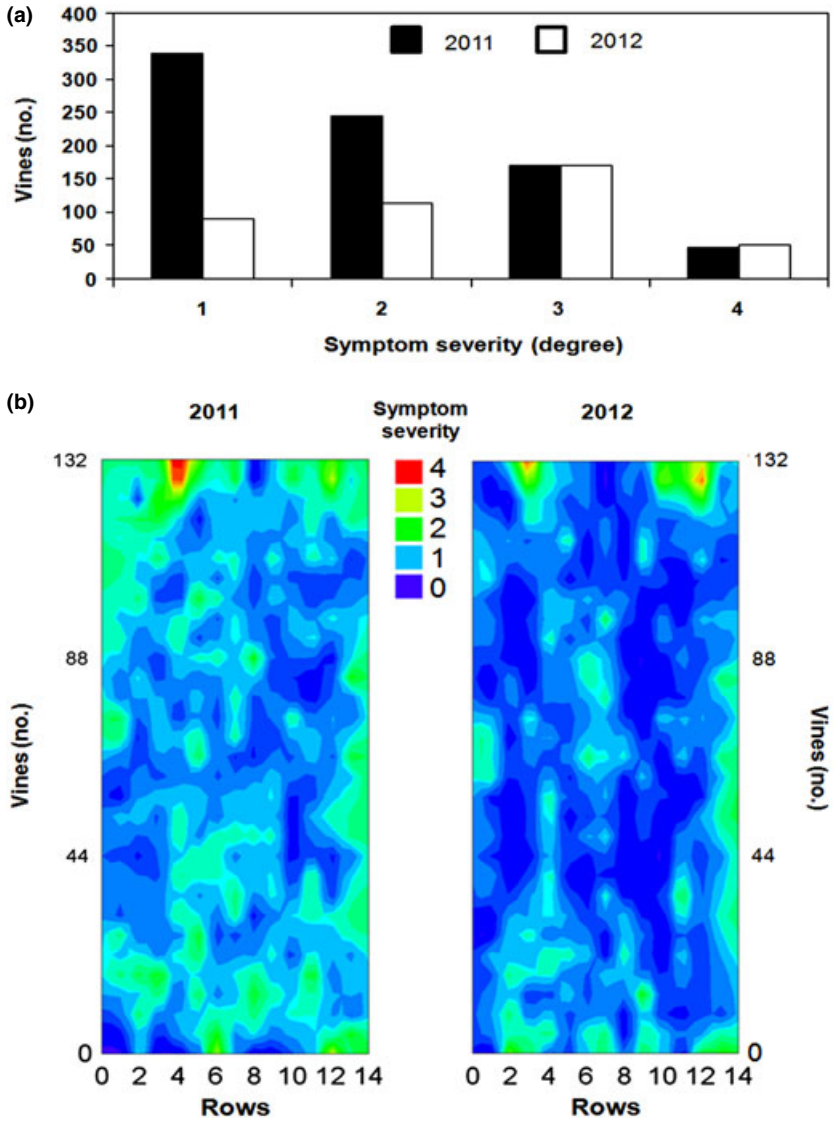

Figure 3 (a) Number of vines showing different degrees of symptom severity in September 2011 (■) and 2012 (□). (b) Maps of the vineyard with cv. Chardonnay located at Montalto delle Marche (AP, Italy) surveyed in September 2011 (left) and 2012 (right), according to reported disease severity of symptoms.

sporadically present with low frequencies, as: V3 (8·3%), V18, V15, V11 (1·5% each), V4 (1·0%) and V9 (0·5%). In 2012, 72·6% (119/164) of all samples with symptoms that were analysed were only infected by four *vmp1* genotypes: again, V14 was the most frequent, in 78·8% of the positive samples, followed by V12 (9·3%), V3 (6·8%) and V18 (5·1%; Fig. 4).

Discussion

Several surveys have been carried out in different wine-growing regions to assess the incidence of grapevine BN (Belli *et al.*, 2010; Maixner, 2011). However, there is no information on investigations on limited areas (i.e. at the single vineyard level) regarding the spatiotemporal distribution of BN-infected vines and its relationship with the stolbur population structure in terms of the highly variable *vmp1* gene, which has proven useful as a genetic marker. The present study starts from observations of leaf symptoms induced by BN in a 7-year-old-vineyard of cv. Chardonnay, which were carried out in 2011 and 2012. The surveys were in September, to obtain a more realistic picture of the symptom status. In September, symptoms appear unambiguously, and the titre of phytoplasma is generally high, which allows the easy and reliable detection of plants with symptoms, as has been confirmed by previous studies on different grapevine yellows phytoplasma species that have been carried out with traditional and more sensitive molecular tools (Prezelj *et al.*, 2013; Zahavi *et al.*, 2013).

The disease severity was different in the two seasons examined, with the symptoms being milder in 2012 compared to 2011. June and July 2011 were slightly colder than the same months in 2012, and in 2012 the amount of rainfall in the same months was lower. It can be assumed that the weather conditions in 2012 induced

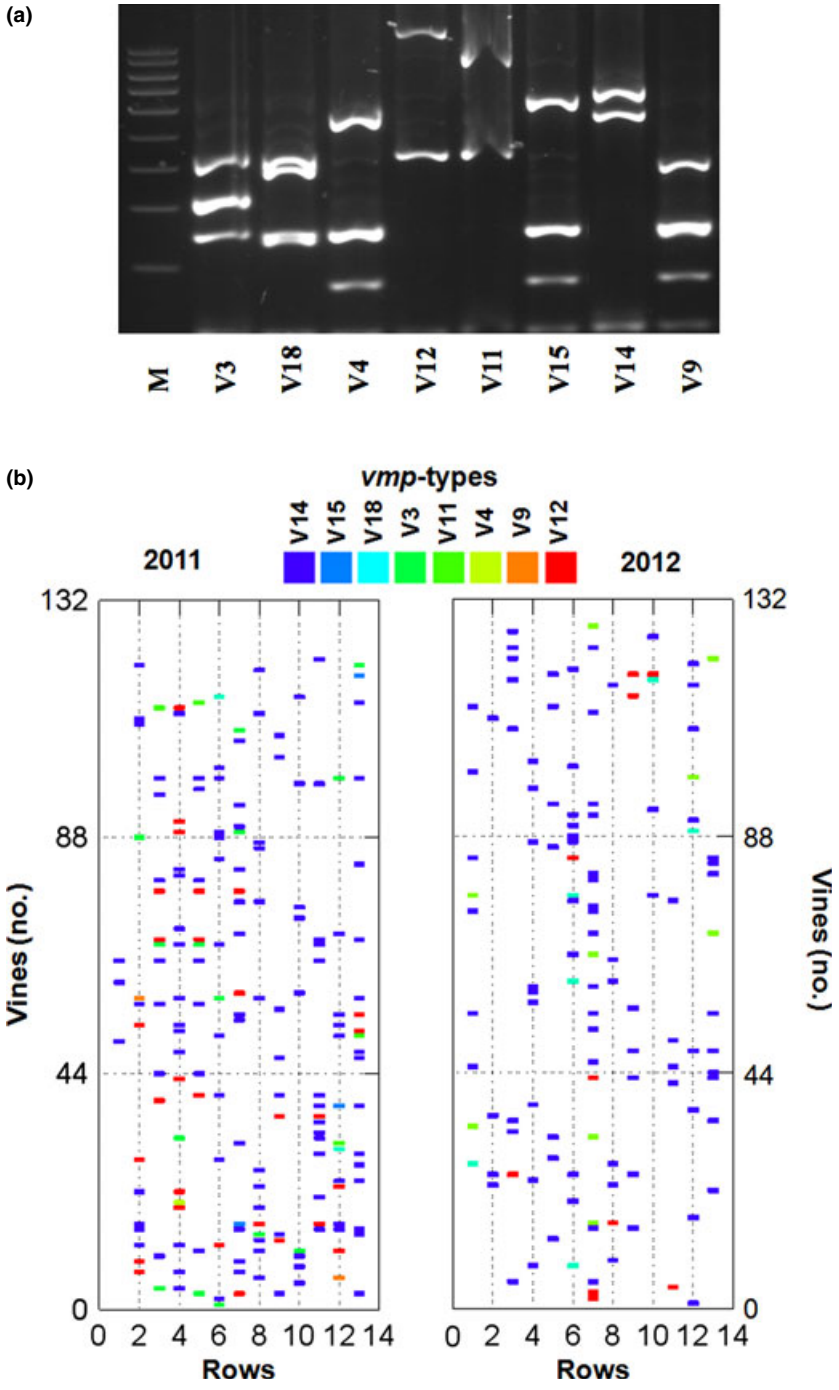

Figure 4 (a) Molecular genotypes of the stolbur *vmp1* gene characterized by digesting the TYPH10F/R amplicon with *Rsa*I in PCR-RFLP. (b) Maps of the vineyard with cv. Chardonnay located at Montalto delle Marche (AP, Italy) according to the spatial distributions of the *vmp1* genotypes identified and characterized in the vines with symptoms collected in September 2011 (left) and 2012 (right).

higher stress in the plants, and so the disease symptoms were milder. A similar situation was described by Hren *et al.* (2009), which related colder temperatures with a greater severity of BN symptoms in cv. Chardonnay vines, and the up-regulation of the gene encoding alcohol dehydrogenase 1.

Analysing diseased vines with respect to their distance from the vineyard borders revealed that the pressure of BN was higher along the edges of the vineyard than in the central part. A similar situation was observed in vineyards for the epidemiology of flavescence dorée (Pavan *et al.*, 2012). The occurrence of decreasing gradients of BN-infected grapevines from the vineyard borders shows that external sources of infectious *H. obsoletus*, or other potential vectors, have an important role in the epidemiology of BN. Most of the vineyards affected by this phytoplasma disease are characterized by external sources of infectious vectors (usually the growth of nettle plants along ditches surrounding the vineyards), the occurrence of decreasing gradients of infected grapevines,

and the ineffectiveness of insecticide treatments when applied to these vineyards (Maixner, 2011; Mori *et al.*, 2012).

The indices of dispersion show that the distribution of the vines with symptoms follows a uniform or regular pattern, without clustering of infected plants or clustering of healthy plants. This applies across the data recorded for both of these years, and even in the season when the BN incidence was particularly high. Indeed, it is well known that the spread of BN in the vineyard does not occur from plant to plant, but is instead mediated by the weeds that represent potential inoculum sources. This type of spatial pattern recorded for BN is similar to the distribution in the vineyard of plants showing esca disease (Romanazzi *et al.*, 2009).

Considering the distribution of vines in the vineyard according to the severity of the BN leaf symptoms, the construction of the two-dimensional contour maps provides a clearer graphical visualization of the vines that were more severely affected by BN along the borders of the vineyards in 2011, when the incidence of vines with symptoms was higher. This picture of disease severity appears to confirm a natural source of inoculum and the activity of potential vectors in spreading BN in this vineyard.

A high genetic variability of the stolbur phytoplasma was recorded in the vineyard through the monitoring of the *vmp1* gene. Eight different *vmp1* genotypes were detected in 2011, and four in 2012, all of which have already been identified in stolbur-infected grapevine samples grown in Italian regions (Murolo *et al.*, 2010). The occurrence of the minor *vmp*1 types in such a restricted area in the presence of two dominant *vmp1* genotypes (V14 and V12), indicates that the inoculum source was not introduced solely with infected propagating material. If this was the case, typically less genetic variation would be expected because of genetic bottlenecks and its homogeneity. The wide genetic diversity appears to support the active role of vector-borne generalist pathogens, which are not necessarily host-specific and have different ecological dynamics, rather than specialist vectors that infect a single host (Coletta-Filho *et al.*, 2011). The dominance of one specific *vmp1* genotype with respect to the others might depend on the long list of annual, pluriannual, woody and herbaceous, and cultivated, ornamental and wild hosts (Kessler *et al.*, 2011) which, with their volatile compounds, will influence the behavioural responses of the vectors (Riolo *et al.*, 2012).

When generalist pathogens are dispersed by polyphagous vectors that visit many hosts, the number and diversity of host–pathogen interactions can be overwhelming. This will generate a large pathogen population size (of high genetic diversity) that is generally expected to adapt faster under natural selection, and consequently, represents a higher risk of circumventing the effects of the host resistance genes.

The present study on the monitoring of the spatiotemporal distribution of these plants with BN symptoms contributes to the further understanding of how complex the population structure of stolbur phytoplasma is, whilst also raising questions about the different abilities of the different vectors to acquire these *vmp1* genotypes, particularly in light of recent BN epidemiological studies (Johannesen *et al.*, 2012). These data can also contribute to better management of phytoplasma disease, together with weed control and increased plant resistance (D'Amelio *et al.*, 2011; Maixner, 2011; Romanazzi *et al.*, 2013), thus also further promoting sustainable agricultural practices.

Acknowledgements

This study was carried out within the projects 'Varenne', funded by the Fondazione Cariverona (Bando 2007) and MIUR PRIN 2005074429_002 'Factors associated with recovery in Bois noir affected vines in central and southern Adriatic sea coast and in Sardinia and search for innovative means to increase this phenomenon'. The authors gratefully acknowledge Doctor Erica Feliziani, Federico Patrizio and Nello Petrelli for technical assistance during the surveys.

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