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Molecular analysis of grapevine Pinot gris virus and its association with grapevine leaf mottling and deformation on 'Vermentino' grapevines in Sardinia

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Summary. In 2017–2018, grapevines of cultivar 'Vermentino' infected with grapevine Pinot gris virus (GPGV) in Sardinia, Italy, exhibited leaf symptoms of mosaic, chlorotic mottling, and curling, and stunted shoots. Disease incidence assessed in 2018 was greater (67%, 103 symptomatic plants out of 153 monitored) than in 2017 (26%, 40 of 153 plants). All symptomatic samples tested by RT-PCR were positive for GPGV in both years, while 70% (53 of 76) of the asymptomatic samples in 2017, and 42% (19 of 45) in 2018, were also positive for GPGV. Characterizing six GPGV isolates from 'Vermentino' by RT-PCR and sequencing of a genomic fragment covering the movement and coat protein genes showed high conservation at the nucleotide level (98.7% to 100.0%) among Sardinian isolates and isolates for which sequence information is available in GenBank. Phylogenetic analysis indicated that most Sardinian GPGV isolates grouped with other European isolates. This is the first characterization of GPGV in a Sardinian vineyard.

Keywords. GLMD, GPGV, symptoms, sequencing.

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INTRODUCTION

Grapevine Pinot gris virus (GPGV, Giampetruzzi *et al*., 2012) (*Trichovirus*, *Betaflexiviridae*) was identified by small RNA sequencing on the grapevine 'Pinot gris' in northern Italy. Since its discovery, GPGV has been reported in Korea (Cho *et al*., 2013), Slovakia and the Czech Republic (Glasa *et al*., 2014), Slovenia (Pleško *et al*., 2014), Greece (Martelli, 2014), France (Beuve *et al*., 2015), Turkey (Gazel *et al*., 2016), Georgia (Casati *et al*., 2015), Germany (Reynard *et al*., 2016), Canada (Xiao and Meng 2016), the United States

of America (Al Rwahnih *et al*., 2016), China (Fan *et al*., 2016b), Spain (Ruiz-García and Olmos 2017), Pakistan (Rasool *et al*., 2017), Brazil (Fajardo *et al*., 2017), Croatia (Vončina *et al*., 2017), Australia (Wu and Habili 2017), the United Kingdom (Silva *et al*., 2018), Chile (Medina *et al*., 2018), Ukraine (Eichmeier *et al*., 2018), Armenia (Eichmeier *et al*., 2019), Iran (Tokhmechi and Koolivand 2019), and Argentina (Debat *et al*., 2020). Plants infected by GPGV show a variety of symptoms that may include stunting, chlorotic mottling and leaf deformations (Giampetruzzi *et al*., 2012; Tarquini *et al*., 2018). Additionally, an association between GPGV and grapevine leaf mottling and deformation disease (GLMD) has been reported (Babini *et al*., 2018).

The GPGV genome is a single-stranded positive RNA of length 7,259 bp, excluding the 3' poly-A tail. The 5' untranslated region (UTR) is composed of 104 nucleotides in the SK13, SK01 and SK30 isolates, as determined by RT-PCR 5'-RACE. Furthermore, the 3' UTR is composed of 82 nucleotides in isolates ZA505-1A and SK30. GPGV is phylogenetically related to grapevine berry inner necrosis virus (GINV). The 5' UTR and 3' UTR of Slovak GPGV isolates share identities of 78 and 85%, respectively, with those of GINV (Giampetruzzi *et al*., 2012; Glasa *et al*., 2014). By comparing the genome of GPGV and other trichoviruses, identities of 69.0% with GINV (NC_015220), 49.0% with apple chlorotic leafspot virus (ACLSV, X99752), 48.7% with apricot pseudo-chlorotic leaf spot virus (APCLSV, AY713379), 47.7% with cherry mottle leaf virus (CMLV, NC_002500), and 48.6% with peach mosaic virus (PcMV, NC_011552), have been reported (Saldarelli *et al*., 2017). The GPGV genome is composed of three open reading frames regions (ORFs): ORF1 (214 kDa) encodes an RNA-dependent RNA polymerase (RdRP); ORF2 (42 kDa) encodes the movement protein (MP); and ORF3 (22 kDa) encodes the coat protein (CP) (Giampetruzzi *et al*., 2012). Different primer pairs were designed in the MP/CP (588 bp, Saldarelli *et al*., 2015), MP (302 bp, Glasa *et al*., 2014; 770 bp, Beuve *et al*., 2015) and CP (412 bp, Glasa *et al*., 2014; 430 bp, Bertazzon *et al*., 2016) genomic regions, and these are used for RT-PCR based diagnoses of GPGV.

Symptoms of stunting, leaf chlorotic mottling and deformation were observed on cv 'Vermentino' grapevines in Sardinia by Gentili *et al.* (2017). The aims of the present study were to monitor progression of GPGV and GLMD in a vineyard of 'Vermentino', the second most cultivated variety in Sardinia covering an overall area of about 4,200 hectares (http://www.sardegnavinitaly.it/), and to characterize Sardinian GPGV isolates.

MATERIALS AND METHODS

Study vineyard

The study was conducted in a 15-year-old, 1 ha 'Vermentino' vineyard located in northern Sardinia (Italy), near Olmedo (SS, 40°36'57"N, 8°23'05"E). The vines were in 26 rows with plant spacings of 1.8 × 0.8 m.

Monitoring and sampling

A total of 153 vines were monitored in the vineyard in the spring of 2017 and spring 2018 for typical GLMD symptoms. These included leaf deformation and chlorotic mottling, and shoot stunting. Symptoms of other grapevine virus diseases were also assessed. Five to six leaves per plant were collected to examine the presence of GPGV and other viruses based on molecular analyses.

RNA extraction and polymerase chain reaction (PCR)

For each sample, total RNA was extracted from 100 mg of petioles using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) following the manufacturer's protocol. A partial movement protein (MP) and coat protein (CP) genomic regions were amplified in RT-PCR with primers DET-Fow and DET-Rev (Saldarelli *et al*., 2015). The MP and CP genomic regions were also amplified in RT-PCR with primers 5637F and 5939R for MP, and 6609F and 7020R for CP (Glasa *et al*., 2014). PCRs were each performed in 50 µL containing: 1× Green buffer, 10-25 µg of DNA template, 0.2 mM of dNTPs, 0.5 µM of each primer, and 1.25 U of *Taq* polymerase (G2-Go *Taq* polymerase, Promega). The PCR programme included one cycle at 94°C for 1 min, 35 cycles each at 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. Reactions were carried out in a T100 ™ Thermal Cycler (BioRad). In addition, all samples were tested by multiplex RT-PCR to simultaneously test for grapevine leafroll associated virus 1 (GLRaV-1), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine leafroll-associated virus 3 (GLRaV-3), grapevine virus A (GVA), grapevine virus B (GVB), arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), and grapevine fleck virus (GFkV), according to published protocols (Gambino and Gribaudo 2006; Faggioli *et al*., 2012).

GPGV sequence analysis

The CP and MP gene sequences of six Sardinian GPGV isolates were analysed with the Vector software

Table 1. Sequences used in this study to generate phylogenetic trees. All the sequences were used to construct the coat protein tree (see Figure 3 a), and the sequences in bold font were used for construction of the movement protein dendrogram (Figure 3 b).

Accession number	Isolate	Country	Reference
KF686810	**SK 30-01**	**Slovakia**	**Glasa *et al.* (2014)**
KF134123	**SK-30**	**Slovakia**	**Glasa *et al.* (2014)**
KM491305	**Mer FR**	**France**	**Beuve *et al.* (2015)**
KR528581	**Tannat UY**	**Korea**	**Direct submission[a]**
KT894101	**TN US**	**California**	**Al Rwahnih *et al.* (2016)**
KX522755	**25-3 DE**	**Germany**	**Reynard *et al.* (2016)**
KU312039	**FEM01**	**Italy**	**Gualandri *et al.* (2017)**
KT345219	BJ-MGX	China	Fan *et al.* (2016b)
KF134125	**SK13**	**Slovakia**	**Glasa *et al.* (2014)**
FR877530	**IT**	**Italy**	**Giampetruzzi *et al.* (2012)**
KT345218	BJ-MLZ	China	Fan *et al.* (2016b)
KT345217	LN-HDQ	China	Fan *et al.* (2016b)
AB731567	KR	Korea	Direct submission[a]
KT345221	LN-MGX	China	Fan *et al.* (2016b)
KT345222	LN-PLZ	China	Fan *et al.* (2016b)
KU234316	**LN-Beta-RS**	**China**	**Fan *et al.* (2016a)**

[a] Sequences available in Genbank.

(Invitrogen). A CP phylogenetic tree was created using the sequences of 16 GPGV isolates and one GINV isolate (Table 1). An MP phylogenetic tree was constructed using the sequences of nine GPGV isolates and one GINV isolate (Wu and Habili 2017).

Two phylogenetic trees (Figure 3) were constructed from pairwise distance matrix by the Neighbor Joining method, applying MEGA version X software. Bootstraps analysis with 1000 replicates was carried out to estimate the statistical support of different tree branches. In addition, GPGV MP and CP sequence identities were compared to other sequences deposited in GenBank, using BLAST (NCBI).

Statistical analyses

All statistical analyses were carried out using R statistical software version 3.10 (R Development Core Team, 2016). A Chi-square test was used to assess differences in distribution of symptoms between the two years of monitoring. Pearson's standardized residuals were calculated to test observed *vs* expected values. Logistic regression model was used to test the probability that plants with specific symptoms was related to the presence of virus infections. Generalized linear models (GLMs) with binomial error distribution were considered to assess differences in probability of viral infection among plants showing different levels of symptoms (i.e. symptomatic, asymptomatic, or other symptoms). GLMs were carried out separately in 2017 and 2018 considering implicit and explicit bias reduction method suggested by Kosmidis and colleagues (2019) and using the brglmFit function in the "brglm2" package in R (Kosmidis 2020). Significance of predictor (i.e. symptoms) was tested using Wald test followed by multiple pairwise comparison (at $P = 0.05$) for means separation.

RESULTS

The main symptoms detected on 'Vermentino' leaves were typical of GLMD. These included mosaics and chlorotic mottling, reduced vein distension inducing leaf curling and folding of the margins. Stunted shoots with short internodes and apical shoot necrosis were also observed (Figure 1). Symptoms appeared in May-July in both years, and sometimes negatively affected plant growth and final grape yields.

To infer associations between symptoms and virus entities, 153 'Vermentino' vines were monitored and grouped into three symptom classes, of asymptomatic, symptomatic for GLMD or other symptoms (i.e. symptoms of other virus diseases). Symptom expression was different between the two years of vineyard monitoring ($\chi^2 = 60.08$, df = 2, $P < 0.01$). The numbers of asymptomatic vines and vines with other symptoms were significantly less in 2018 than in 2017. In contrast, the number of vines with specific GLMD symptoms was greater in 2018 than in 2017 (Table 2).

All vines exhibiting GLMD symptoms tested positive for GPGV in 2017 (40 of 40) and in 2018 (103 of 103), but 70% (53 of 76) of the asymptomatic plants also tested positive for GPGV in 2017, and 42% (19 of 45) of asymptomatic plants also tested positive for GPGV in 2018. The numbers of GPGV positive samples from symptomatic plants was greater ($P < 0.05$) in 2018 than in 2017 (Figure 2). The number of asymptomatic plants and plants with symptoms of other virus diseases decreased in 2018. Among all samples tested by multiplex RT-PCR, 20 were infected by GVA and 52 by grapevine GFkV. No samples were positive for GLRaV-1, GLRaV-2, GLRaV-3, GVB, GFLV or ArMV.

Results from GLM analyses showed that the probabilities of a vine testing positive for GPGV significantly differed among plants with or without GLMD symptoms, both in 2017 ($\chi^2 = 9.2$, $P < 0.01$) and 2018 ($\chi^2 = 44.1$, $P < 0.01$). The probability of GPGV infection in asymptomatic plants was 69.53 ± 5.2% in 2017, but was

Figure 1. Typical symptoms of chlorotic leaf mottling and deformation on a GPGV-infected 'Vermentino' grapevine. Stunted shoots with short internodes (a), mosaics, chlorotic pitting and curling of leaves (b).

Table 2. Occurrence of grapevines without (asymptomatic) or with GLMD symptoms (symptomatic), or with symptoms of other virus diseases (other symptoms) in 2017 and 2018. Results of Pearson's standardized residuals, indicating statistical differences between observed and expected values, are indicated in parentheses.

Plant appearance	2017	2018	Total
Asymptomatic	76 (+)	45 (−)	121
Symptomatic	40 (−)	103 (+)	143
Other symptoms	37 (+)	5 (−)	42
Total	153	153	306

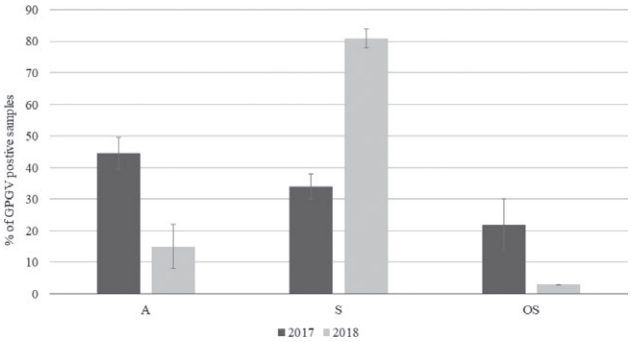

Figure 2. Mean proportions (%) of GPGV infected plants in 2017 and 2018 in different symptom classes of asymptomatic (A), symptomatic for GLMD (S) or with symptoms of other virus diseases (OS). Percentages were calculated for total numbers of positive samples in the two years of survey.

less in 2018 at 42.4 ± 7.4%. In contrast, the probability of a vine with symptoms of other viruses testing positive for GPGV was slightly less in 2017 (69.8 ± 7.5%) than in 2018 (75.9 ± 19.1%), but this was influenced by the very few plants with other symptoms detected in 2018 (Table 2). The probability of symptomatic plants testing positive for GPGV was high both in 2017 (98.9 ± 1.6%) and 2018 (97.7 ± 1.5%).

The GPGV MP and CP gene sequence analyses showed slight differences between the Sardinian sequences and sequences available in GenBank. The sequence identity ranged from 98.7% (VRM 4 MP) to 100.0% (VRM 9 MP). Both phylogenetic trees indicated that most Sardinian GPGV CP and MP gene sequences grouped with European isolates in a separate clade from sequences of Korean or Chinese isolates (Figure 3). The only exception was VRM5 MP, which grouped with isolates from Korea and the United States of America.

Sardinian GPGV isolates were 98.0% identical at the nucleotide level to most GPGV sequences available in GenBank. The identities of Sardinian GPGV CP and MP sequences were, respectively, approx. 70.0% and 82.0% with those of GINV.

DISCUSSION

GLMD symptoms were observed for the first time in 2001 in Slovenia (Pleško *et al.*, 2014) and then in

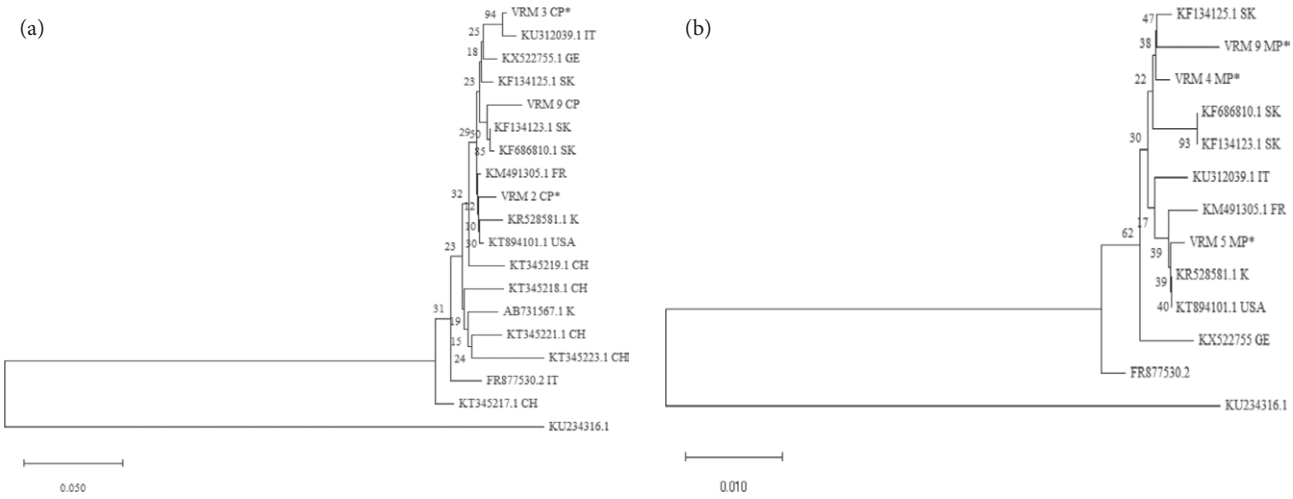

Figure 3. Phylogenetic trees generated from partial nucleotide genome sequences of the coat protein region (a) and movement protein region (b) of GPGV isolates. Bootstrap values were obtained using 1000 replicates. CP and MP sequences of GPGV isolates determined in this study are indicated with an asterisk. The phylogenetic trees were reconstructed using the neighbor joining algorithm implemented in MEGA X. Sixteen isolates from China (CH), France (FR), Italy (IT), Korea (K), Slovakia (SK), Germany (GE) or California (USA) were selected from GenBank database and used to construct the CP dendrogram (a). Nine isolates from Korea (K), France (FR), Italy (IT) or California (USA) were used for the MP dendrogram (b). The GINV sequence (KU234316) was used as the outgroup. The number on each tree node represents the bootstrap value.

2003 in Trentino (Giampetruzzi *et al*., 2012). In agreement with the observations by Giampetruzzi *et al.* (2012), GPGV on 'Vermentino' was also previously associated with GLMD in Sardinia (Gentile *et al*., 2017). In the 'Vermentino' vineyard selected for the present study, GLMD symptoms were observed for the first time in 2014. The present study confirmed the correlation between GLMD symptoms and the presence of GPGV in 'Vermentino' plants. Nonetheless, GPGV was also found in symptomless grapevines, as has been reported by Glasa *et al.* (2014). Several studies have suggested the existence of two GPGV strains, one causing disease symptoms and the other not associated with symptoms (Bianchi *et al*., 2015; Saldarelli *et al*., 2015; Bertazzon *et al*., 2017; Spilmont *et al*., 2018; Bertazzon *et al.,* 2020). In the present survey, several plants that were asymptomatic in 2017 became symptomatic in the following year. This may have been because of an increase of virus concentrations in the plant tissues from one year to the next, or due to the incubation period between mite-transmitted inoculation of GPGV and onset of host symptoms.

It would therefore be interesting to test samples by quantitative real time PCR to check whether clear differences in virus titre can be detected between symptomatic and asymptomatic vines that are infected by GPGV. In addition, virus isolates from asymptomatic samples should be sequenced to check for any difference in sequence composition.

Phylogenetic analyses indicated that Sardinian GPGV isolates mostly grouped in the clade of European strains, suggesting that the use of infected propagation material could be involved in the spread of the virus.

The emergence of new virus diseases is often associated with factors related to ecological changes or intensive agronomic practices (Elena *et al*., 2014), which are commonly and frequently occurring in some premium wine production regions where GPGV has been found. To reduce the expansion of GPGV, collaborative efforts are needed to clarify disease biology and epidemiology, and to include this virus in grapevine certification programmes.

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