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Genetic variability of grapevine Pinot gris virus (GPGV) in an organically cultivated vineyard in Hungary

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Summary. *Grapevine Pinot gris virus* (GPGV) is a recently identified trichovirus infecting grapevines. Despite wide distribution, there is limited available information on epidemiology, transmission, and associated symptoms of grapevine leaf mottling and deformation. Occurrence and genetic diversity of GPGV variants were surveyed in an organically cultivated Hungarian vineyard that was planted between 1996 and 2014. Sequence analysis demonstrated the widespread presence and high variability of GPGV, and according to phylogenetic analyses, the Hungarian virus isolates were classified into three groups. Most of the identified variants clustered with the representative asymptomatic isolates, but all isolates from one grapevine cultivar grouped with representative isolates of clade B. Furthermore, one isolate clustered with representative isolates of clade C, and the identified clade C variant had previously undescribed polymorphisms.

Keywords. RT-PCR, phylogenetic analysis, sequence analysis.

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INTRODUCTION

Grapevine Pinot gris virus (GPGV; *Betaflexiviridae*) is a newly emerging trichovirus. Since its discovery in Northeastern Italian grapevines in 2012 (Giampetruzzi *et al.*, 2012), GPGV has been identified in many grape-growing countries in Europe (Glasa *et al.*, 2014; Morelli *et al.*, 2014; Pleško *et al.*, 2014; Beuve *et al.*, 2015; Casati *et al.*, 2015; Bertazzon *et al.*, 2016, 2021a; Eichmeier *et al.*, 2016, 2017, 2018; Gazel *et al.*, 2016; Reynard *et al.*, 2016; Ruiz-García and Olmos, 2017; Czotter *et al.*, 2018; Silva *et al.*, 2018; Abou

Kubaa *et al*., 2019; Massart *et al*., 2020; Shvets and Vinogradova, 2022), the Middle East and Asia (Fan *et al*., 2016; Rasool *et al*., 2017; Abou Kubaa *et al*., 2020; Tokhmechi and Koolivand, 2020; Abe and Nabeshima, 2021), North and South America (Jo *et al*., 2015; Al Rwahnih *et al*., 2016; Poojari *et al*., 2016; Xiao *et al*., 2016; Fajardo *et al*., 2017; Zamorano *et al*., 2019; Debat *et al*., 2020), Africa (Eichmeier *et al*., 2020; Bertazzon *et al*., 2021b), and Australia (Wu and Habili, 2017).

Several studies have analyzed the incidence and evolutionary history of GPGV (Saldarelli *et al*., 2015; Bertazzon *et al*., 2017; Tarquini *et al*., 2019a; Hily *et al*., 2020, 2021a). Bertazzon *et al*. (2016) showed via comparative analyses that grapevine samples from Northeastern Italy collected in 2002 lacked GPGV infections, despite high incidence (79.4%) in samples from 2014. A similar conclusion was reached by Gentili *et al.* (2017), where no GPGV infection was detected in >10-year-old grapevines in southern and central Italian vineyards. This was also supported by the observation that local cultivars were less infected with GPGV than national and internationally well-known cultivars, implying a recent introduction of the virus from outside of Italy. A Brazilian survey also determined an increase in GPGV incidence in recently imported grapevine cuttings, compared with older samples or germplasm collections (Fajardo *et al*., 2017). PCR assays carried out on European grapevine samples from 2005 showed that GPGV was originally concentrated in Eastern Europe before it was detected in Southern and Western Europe (Bertazzon *et al*., 2016). These observations were further supported by Hily *et al.* (2020), who conducted phylogenetic and diversity analyses of new and already available high throughput sequencing data of GPGV. That survey indicated that Asia (and with high probability, China) was a possible source of origin for GPGV.

The means of natural transmission of GPGV remains a matter of speculation. No natural transmission and low prevalence of the virus were documented in the United States of America and Australia (Al Rwahnih *et al*., 2016; Wu and Habili, 2017), while other studies have recorded active vine-to-vine spread of the virus in vineyards during a 3-year-long monitoring period (Martelli, 2014; Bertazzon *et al*., 2017; Hily *et al*., 2021b). Furthermore, successful GPGV transmission was observed by grafting (Saldarelli *et al*., 2015). Distribution patterns of GPGV suggest the involvement of the eriophyid mite *Colomerus vitis* as a transmission vector, which was also shown to be the vector of another trichovirus, *Grapevine berry inner necrosis virus* (GINV). While *C. vitis* is a monophagous mite of grapevine, GPGV has been identified in other woody and herbaceous hosts, implying the contribution of other vectors in GPGV transmission (Gualandri *et al*., 2017; Demian *et al*., 2022). Herbaceous hosts could serve as reservoirs for GPGV, facilitating the dissemination of the virus. More information is required on the molecular and epidemiological aspects of the virus-vector interactions to provide guidance for the development of appropriate virus management decisions.

GPGV presence in grapevines is often connected with the appearance of grapevine leaf mottling and deformation (GLMD) symptoms (Tarquini *et al*., 2019b, 2021a). GLMD generally includes chlorotic mottling, leaf deformation, shortened internodes, as well as reduced yields (up to 50%) and berry quality (Bianchi *et al*., 2015; Saldarelli *et al*., 2015; Bertazzon *et al*., 2017). GPGV-infected grapevines show variable GLMD symptoms, ranging from mild to severe, or they can remain symptomless (Tarquini *et al*., 2023). This contrast in symptom expression remains to be explained. In some cases, the connection between GPGV presence and the severity of the symptoms was equivocal (Bianchi *et al*., 2015; Tarquini *et al*., 2018), while several studies have proposed a connection between molecular characteristics of GPGV variants and resulting host symptoms (Glasa *et al*., 2014; Saldarelli *et al*., 2015; Bertazzon *et al*., 2017; Tarquini *et al*., 2019a, 2021a). The ability of GPGV to trigger and suppress antiviral post-transcriptional gene silencing (PTGS) has also been demonstrated (Tarquini *et al*., 2021b), and boron deficiency, agronomic and abiotic factors may also contribute to GLMD symptom formation (Bertazzon *et al*., 2020; Kiss *et al*., 2021).

The GPGV genome consists of three overlapping open reading frames (ORFs), encoding the RNA-dependent RNA polymerase (RdRp) (ORF1), the movement protein (MP) (ORF2), and the coat protein (CP) (ORF3) (Giampetruzzi *et al*., 2012). Multiple classifications have been introduced based on phylogenetic analyses of partial sequence data of the movement protein and coat protein (MP/CP) regions of GPGV. Saldarelli *et al.* (2015) showed that MP/CP sequences partition into two groups, which was further supported by the asymptomatic and symptomatic phenotypes of these isolates. Bertazzon *et al.* (2017) classified three clusters, named clades A, B and C. Isolates derived from asymptomatic plants were classified as clade A; isolates with low (<1%) symptom incidence were clade B, and isolates with >1% symptom incidence were in clade C (Bertazzon *et al*., 2017). This three-cluster classification was further supported by full-genome phylogenetic analyses of GPGV, where the isolates were grouped into α-, β- and γ-clades (Tarquini *et al*., 2019a).

Although the symptom presence was not directly linked with the genetic variability of GPGV, distinctive molecular differences were identified in the MP/CP

and RdRp sequences (Tarquini *et al.*, 2019a). Tarquini *et al.* (2019a) demonstrated differentiating amino acid (aa) alterations between the clades, and suggested putative phosphorylation events which could play significant roles in symptom development. These single nucleotide polymorphisms (SNPs) were also shown to affect virus-derived siRNA production (Tarquini *et al.*, 2021a). Shvets and Vinogradova (2022) also investigated the putative role of detected SNPs in Russian grapevines.

Knowledge of the genetic variability of different GPGV strains has the potential to provide information on the evolutionary history of the virus. Research on GPGV is mainly focused on Western European countries with grape cultivation histories, and there is limited information available on vineyard GPGV spread in Eastern Europe. The present study aimed to verify the occurrence and diversity of GPGV isolates in one vineyard located in the southern wine region of Hungary.

MATERIALS AND METHODS

Plant material

The vineyard selected for this study is located in Szajk, in the Pécs wine region of southern Hungary. The vineyard was planted between 1996 and 2014 (Figure S1) and is maintained under organic cultivation. In the early summer of 2021, 20 different cultivars were sampled from the vineyard. The cultivars included the internationally well-known 'Cabernet sauvignon', 'Sauvignon blanc', and 'Traminer', traditional Hungarian cultivars such as 'Juhfark', 'Hárslevelű', and 'Olaszrizling', the recently bred Hungarian cultivars 'Pamerzs', 'Silver', and 'Jázmin', and experimental hybrid lines. Five plants were randomly selected and sampled from each grapevine cultivar, with a total of 100 samples collected and without distinction between the presence or absence of symptoms.

RNA extraction

Total RNA was extracted from the 100 samples, using a simplified version of the extraction method described by Xu *et al.* (2004). For each sample, 3 g of leaf tissue was ground in liquid nitrogen with a mortar and pestle, and 1 mL of lysis buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 1.5 M NaCl, 3% CTAB, 2% PVP, and 4% of β-mercaptoethanol added just before use) was added to the ground tissues. The mixture was then incubated at 65°C for 30 min, and the sample was vortexed every 10 min. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) and 100 µL of 5 M potassium acetate were added to the sample, and gentle shaking was applied until homogenization. After a centrifuge step (6810 g for 5 min), the supernatant was transferred to a new microcentrifuge tube, and a new extraction step was performed by adding 800 µL of chloroform:isoamyl alcohol, followed by homogenization by inverting and centrifuging at 6810 g for 5 min. The supernatant was then transferred to a new microcentrifuge tube, and 750 µL isopropanol and 80 µL of 3 M sodium acetate were added and the sample was homogenized by inverting. The sample was then incubated at room temperature for 20–30 min and centrifuged (18 000 g for 8 min). The resulting pellet was washed twice with 70% ethanol, air-dried, and resuspended in 25 µL of sterile nuclease-free water. The DNase treatment was carried out as described by Oñate-Sánchez and Vicente-Carbajosa (2008). Three µL of 10× DNase buffer and 2 µL of DNase I (Thermo Fisher Scientific) were added and the mixture was incubated at 37°C for 30 min. Seventy µL of DEPC-treated water, 50 µL of 7.5 M sodium acetate, and 400 µL of ethanol were then added to the solution, which was thoroughly mixed, and then centrifuged at 4°C for 20 min. After a washing step with 70% ethanol and air-drying, the RNA was resuspended in 20 µL of sterile RNase-free water.

RT-PCR

Reverse transcription (RT) reaction was carried out using a RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RT-PCR was carried out using primer pairs for amplification of the MP/CP region of GPGV: DetF (5'-TGGTCTGCAGCCAGGGGACA-3') and DetR (5'-TCACGACCGGCAGGGAAGGA-3') (Morelli *et al.*, 2014). RT-PCR was carried out with a One-Step RT-PCR Kit (Qiagen), with the following conditions: an initial denaturation step of 94°C for 2 min; followed by 40 cycles each of 94°C for 30s, 58°C for 40 sec and 72°C for 45 sec, and a final extension of 72°C for 5 min. A control amplification of the *Vitis* 18S rRNA gene was carried out with primers 18S-H325 (5'-AAACGGCTAC-CACATCCAAG-3') and 18S-C997 (5'-GCGGAGTC-CTAAAAGCAACA-3') (Gambino and Gribaudo, 2006). The nucleotide sequence of the amplified products was determined either directly as a PCR product or cloned into pGEM®-T Easy vector (Promega).

Cloning procedure

Sequence analysis of isolate HU-27 showed multiple peaks in the chromatogram, indicating the pres-

ence of distinct isolates in the individual plant samples. For this reason, cloning of the amplified PCR products was carried out to clarify the exact nucleotide sequences. The PCR products were cloned into pGEM®-T Easy vector (Promega) and then transformed into *Esherichia coli* C53. After miniprep plasmid isolation, the nucleotide sequences of the amplified products were determined.

Phylogenetic studies and analyses of sequence diversity

For the phylogenetic and molecular analyses, one GPGV-positive sample was selected from each grapevine cultivar for nucleotide sequencing of the amplified MP/CP regions.

To investigate the diversity between the newly identified Hungarian sequences, an unrooted ML tree was also constructed, including the previously described sequences. The GenBank accession numbers of the these sequences are: KF134123, KF134124, KF134125, KF686810, KM491305, KT894101, KU194413, KU949328, KX522755, KY706085, LN606703, LN606705, LN606739, MH087439, MH087440, MH087441, MH087442, MH087443, MH087444, MH087445, MH087446, MH087447, and MH802023. The sequence diversity analysis was completed by using the Maximum likelihood method based on the JTT matrix-based model with MEGA6 (Tamura *et al*., 2013). The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 40% of bootstrap replicates are collapsed. Detection of amino acid polymorphisms was carried out by multiple alignments of the protein sequence of MP/CP region, which were performed using JalView program (Waterhouse *et al*., 2009).

RESULTS

Virus detection via RT-PCR

Five samples per cultivar were assayed by RT-PCR to detect GPGV. In almost all cultivars (except 'Merlot'), all 5 out of 5 samples showed virus infection (100%), while in the case of 'Merlot', only 2 out of 5 samples were tested positive for GPGV (40%) (Table 1). All in all, the RT-PCR assays detected 97% GPGV infection among the sampled grapevines.

One isolate from each cultivar was selected for nucleotide sequence determination of the amplified PCR product. For isolate HU-27, two distinct sequences were identified, which are indicated as HU-27.1 and HU-27.2 (Figure 1, Table 2). The acquired nucleotide sequence data were deposited in the NCBI GenBank database (accession numbers OP56859 to OP56887).

Phylogenetic analyses

The sequence diversity analysis was carried out based on the nt sequences of the MP/CP region (564 nt), which includes the carboxyl-terminal region of the MP and the amino-terminal region of the CP (Figure 1). The analysis included the nt sequences of the Hungarian isolates identified in this study and 23 sequences available in the NCBI GenBank database (All acc. numbers are shown in Figure 1). The phylogenetic analysis demonstrated the diversity of the collected GPGV isolates and indicated their distribution between the three distinct clusters (clades A, B, and C) identified previously (Bertazzon *et al*., 2017).

The majority of the Hungarian sequences (from 18 of the 20 cultivars) clustered with "asymptomatic" GPGV isolates, belonging to clade A (according to the classifi-

Table 1. Detection of GPGV by RT-PCR from 100 grapevine samples collected from different grapevine cultivars in 2021.

Sample ID	Cultivar	GPGV	Sample ID	Cultivar	GPGV
HU-1 - HU-5	Pinot regina	5/5	HU-51 - HU-55	Cabernet sauvignon	5/5
HU-6 - HU-10	Castellum	5/5	HU-56 - HU-60	Traminer	5/5
HU-11 - HU-15	Jåzmin	5/5	HU-61 - HU-65	Hårslevelü	5/5
HU-16 - HU-20	Silver	5/5	HU-66 - HU-70	Olaszrizling SK	5/5
HU-21 - HU-25	Borsmenta	5/5	HU-71 - HU-75	Juhfark	5/5
HU-26 - HU-30	Olaszrizling BB20	5/5	HU-76 - HU-80	Sauvignon blanc	5/5
HU-31 - HUI-35	Pamerzs	5/5	HU-81 - HU-85	Hybrid #1	5/5
HU-36 - HU-40	Merlot	2/5	HU-86 - HU-90	Hybrid #2	5/5
HU-41 - HU-45	Blauer Portugieser	5/5	HU-91 - HU-95	Hybrid #3	5/5
HU-46 - HU-50	Cabernet franc	5/5	HU-96 - HU-100	Hybrid #4	5/5

The number of GPGV positive samples out of the total samples is reported for each cultivar.

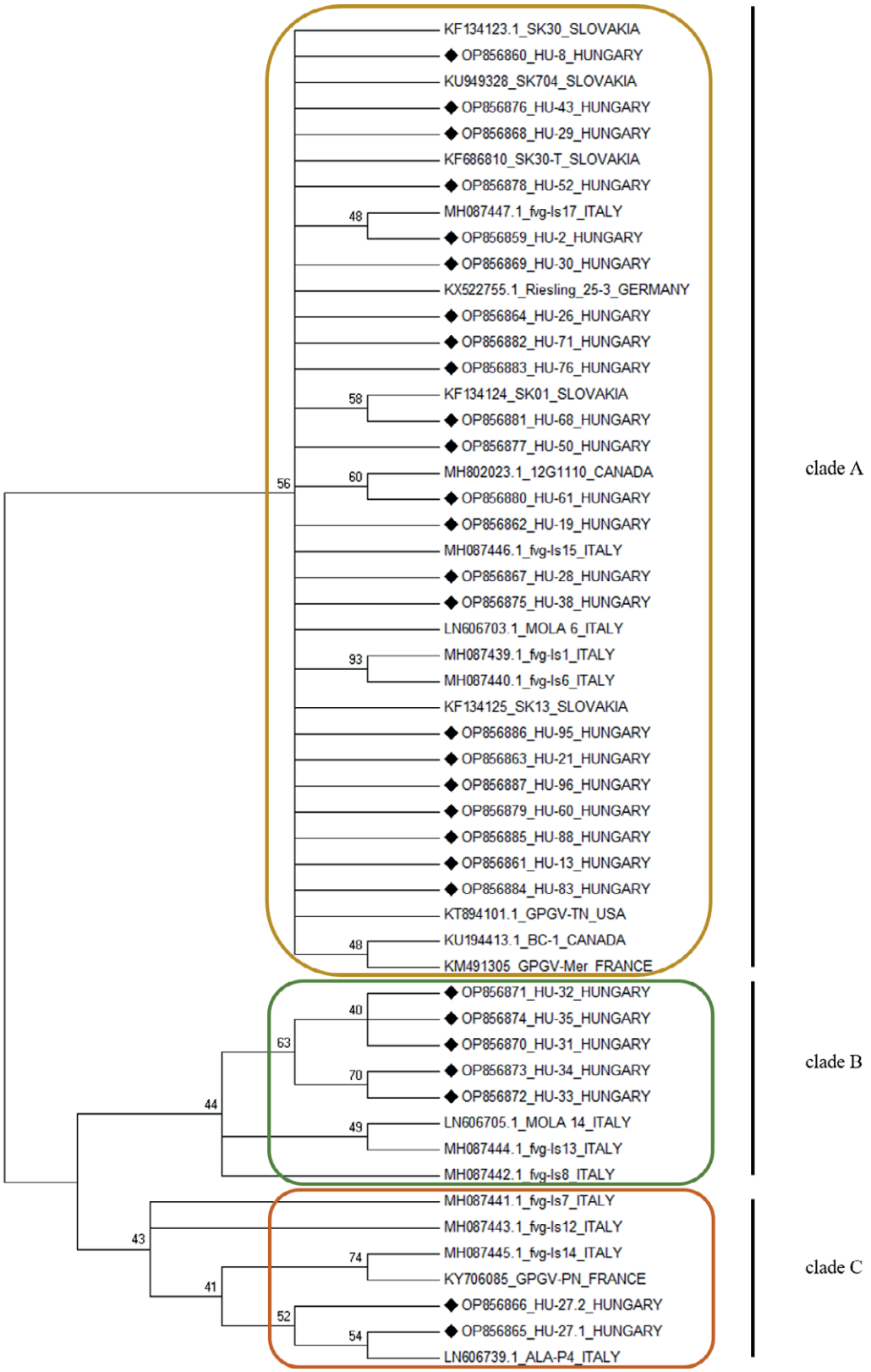

Figure 1. Unrooted ML tree of GPGV MP/CP sequences. The samples determined in the present study are marked with ♦. The sequences are available in the NCBI GenBank under the indicated accession numbers.

cation of Bertazzon *et al.* (2017). The nt sequence of the initially selected 'Pamerzs' sample (HU-31) clustered with Italian isolates classified as members of clade B. The HU-27.1 and HU-27.2 sequences (from 'Olaszrizling BB20') grouped in clade C with "symptomatic" isolates from Italy and France, showing close similarity between HU-27.1, HU-27.2 and the Italian ALA-P4 isolate, which is used as a representative isolate of clade C (Bertazzon *et al.*, 2017). Since only HU-31, HU-27.1 and HU-27.2 clustered into different clades than the majority of the identified sequences (which all grouped into clade A), the nt sequences of the rest of the PCR products (derived from the four different GPGV-positive, not yet sequenced plant samples of 'Pamerzs' and 'Olaszrizling BB20' cultivars) were also determined (included in Table 2).

After including the four additional sequences from the four different 'Pamerzs' vines in the sequence diversity analysis, all five isolates (HU-31, 32, 33, 34, 35) clustered in clade B, indicating close similarity between them. For the 'Olaszrizling BB20' samples, only the originally selected and analyzed isolates (HU-27.1 and HU-27.2) grouped in clade C, while all the other isolates (HU-26, 28, 29, 30) clustered in clade A.

Detection of amino acid polymorphisms in the MP/CP region of GPGV

The GPGV isolates differed in their MP/CP nucleotide sequences with sequence similarities between 90 and 99%. Multiple differences were identified in the examined 152 aa region of the 3'-end of the MP (Figure 2A). Based on Tarquini *et al.* (2019a), six aa alterations were proposed to distinguish between the three clades in

Table 2. The GPGV isolates analyzed in the present study, and their characteristics, showing the names of source grapevine cultivars, the year of planting and grafting, and the isolate GenBank accession numbers. The classification is based on Bertazzon *et al.* (2017), and the result of the sequence diversity analysis.

Sample ID	Cultivar	GenBank acc. number	Year planted/regrafted	Classification
HU-2	Pinot regina	OP56859	2014	clade A
HU-8	Castellum	OP56860	2003	clade A
HU-13	Bianka/Jåzmin	OP56861	2003/2018	clade A
HU-19	Silver	OP56862	2003	clade A
HU-21	V. vinifera hybrid#5/Borsmenta	OP56863	2003/2015	clade A
HU-26	Olaszrizling BB20	OP56864	2003	clade A
HU-27.1	Olaszrizling BB20	OP56865	2003	clade C
HU-27.2	Olaszrizling BB20	OP56866	2003	clade C
HU-28	Olaszrizling BB20	OP56867	2003	clade A
HU-29	Olaszrizling BB20	OP56868	2003	clade A
HU-30	Olaszrizling BB20	OP56869	2003	clade A
HU-31	Blauer Portugieser/Pamerzs	OP56870	2010/2017	clade B
HU-32	Blauer Portugieser/Pamerzs	OP56871	2010/2017	clade B
HU-33	Blauer Portugieser/Pamerzs	OP56872	2010/2017	clade B
HU-34	Blauer Portugieser/Pamerzs	OP56873	2010/2017	clade B
HU-35	Blauer Portugieser/Pamerzs	OP56874	2010/2017	clade B
HU-38	Merlot	OP56875	1997	clade A
HU-43	Blauer Portugieser	OP56876	2010	clade A
HU-50	Cabernet franc	OP56877	2008	clade A
HU-52	Cabernet sauvignon	OP56878	2000	clade A
HU-60	Traminer	OP56879	2008	clade A
HU-61	Hårslevelü	OP56880	2003	clade A
HU-68	Olaszrizling SK	OP56881	2008	clade A
HU-71	Juhfark	OP56882	2000	clade A
HU-76	Sauvignon blanc	OP56883	1996	clade A
HU-83	Pinot gris/Hybrid #1	OP56884	2005/2020	clade A
HU-88	Pinot gris/Hybrid #2	OP56885	2005/2020	clade A
HU-95	Pinot gris/Hybrid #3	OP56886	2005/2020	clade A
HU-96	Pinot gris/Hybrid #4	OP56887	2005/2020	clade A

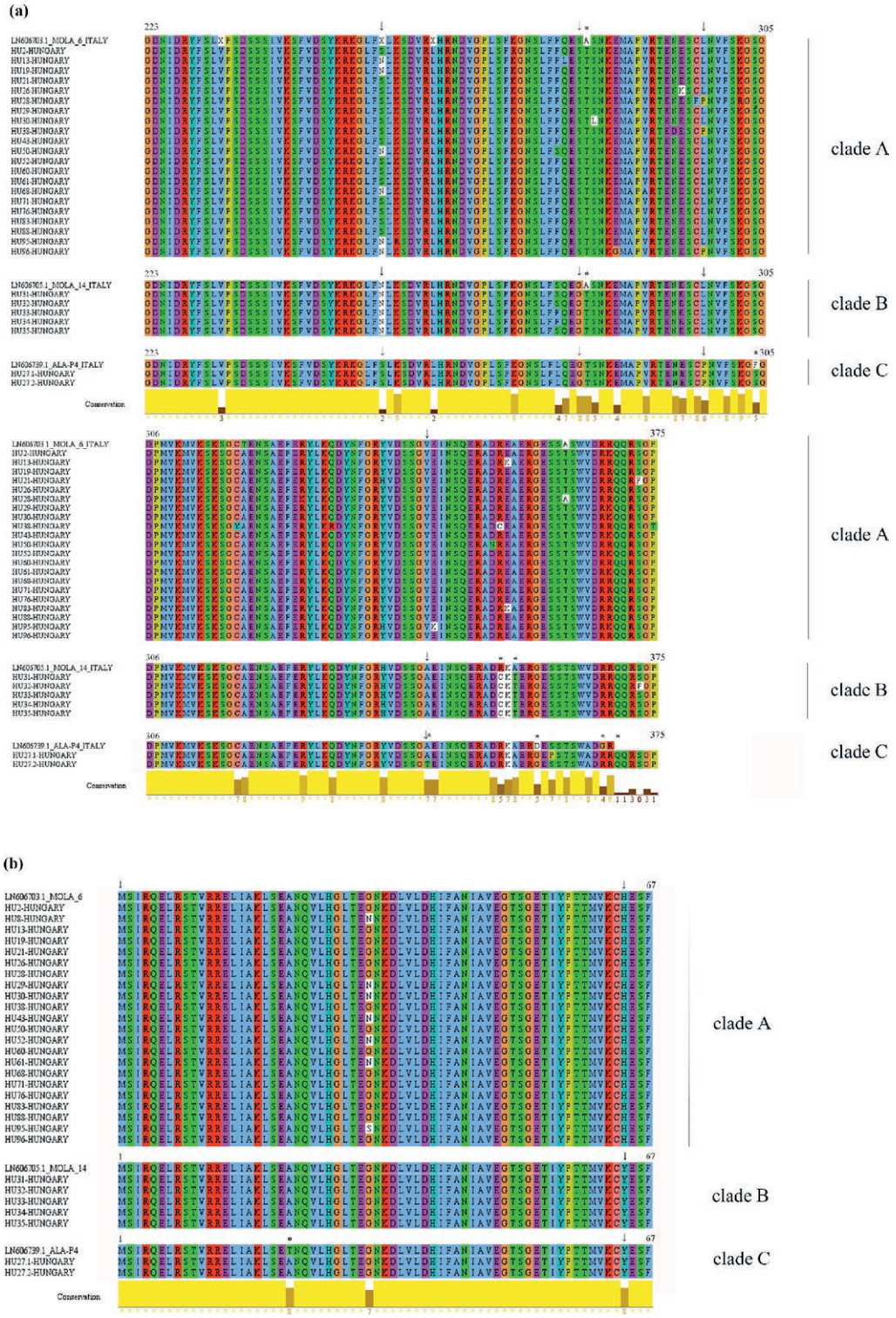

Figure 2. Amino acid alignments of the MP and the CP region of GPGV. (a) The multiple sequence alignment of the carboxyl-terminal region of GPGV MP, and (b) the amino-terminal region of the GPGV CP. The scale numbers indicate the aa positions according to the complete protein. The reference sequences for the classification were: MOLA 6 for clade A, MOLA 14 for clade B, and ALA-P4 for clade C (Saldarelli *et al.*, 2015). The corresponding GenBank accession are, respectively, LN606703, LN606705, and LN606739. The aa alterations of the isolates are indicated by asterisks, and the differentiating aa positions proposed by Tarquini *et al.* (2019a) are marked by arrowheads. The colours of the aa are according to the Clustal X Color Scheme. Conservation values quantify the similarity between the aa.

this region, five of which were detected in the Hungarian isolates as well: three in the MP and one in the CP.

At position 255 of GPGV MP, asparagine (N) was identified in isolates of clade B, but serine (S) in clade C. Both N and S were present in isolates belonging to clade A. Similar aa alteration was also observed in aa position 299; in clade A both leucine (L) and proline (P) were present, while clade B only contained L residue, and P was detected only in clade C. Moreover, discriminating aa changes between clade A and the 'symptomatic' clades (B and C) were present in two aa positions. Alterations were identified at position 282 serine-glycine (S-G), and at aa position 344 valine-alanine (V-A).

The aa alignment of the MP/CP region revealed multiple differences between the newly identified isolates and the three reference isolates, MOLA 6, MOLA 14, and ALA-P4, representing, respectively, clades A, B, and C (Bertazzon *et al.*, 2017). At three aa positions, all the Hungarian isolates of clade B showed differences from the MOLA 14 reference isolate. At positions 283, 354, and 356, threonine (T), cysteine (C), and another T residue were observed, instead of, respectively, aa alanine (A), arginine (R), and A. The two isolates belonging to clade C (HU-27.1 and HU-27.2) displayed significant alterations from the representative ALA-P4 isolate. HU-27.1 and HU-27.2 both lacked the SNPs at aa position 370, which resulted in a premature stop codon and a 6 amino acid short MP, which has been suggested as an important distinction between the clades and a putative determinant in severe symptom formation (Bertazzon *et al.*, 2017; Tarquini *et al.*, 2021a). Furthermore, HU-27.2 had a T residue instead of A at position 344, and HU-27.1 had a P residue at position 361, instead of S. Both these Hungarian isolates differed at positions 304, 359, and 368, where S, G, and R residues were present instead of, respectively, F, aspartic acid (D) and G. Both of these results indicate a wider variety in aa sequences within clade C than previously described.

Regarding the aa variance of the amplified CP region, a highly conservative 5'-end region was observed with only three aa alterations in the 67 aa long CP fragment (Figure 2B). A differentiating aa alteration was present at position 64, since in clade A only histidine (H) was present, while in the two other clades (B and C) tyrosine (Y) was detected. At position 22, both of the isolates HU-27.1 and HU-27.2 showed A residues instead of T. The diversity at position 32 was also only detected in isolates grouped within clade A (residues G, N, and S).

DISCUSSION

The internationally well-known and Hungarian grapevine cultivars included in this study were a mixture of young and older vines that were planted between 1996 and 2014. Later, in 2015-2020, some of the grapevines were regrafted onto the original cultivars (e.g., 'Pamerzs', 'Borsmenta', 'Jázmin') (Table 2). This study found that the surveyed vineyard is highly infected with GPGV (97% of the sampled grapevines). Because the identified GPGV variants displayed significant differences and showed similarity to all three GPGV clades, there is a high probability of multiple independent introduction events of GPGV into this vineyard. Previously, GPGV was reported in several Hungarian vineyards as shown by high-throughput sequencing methods, and the presence of the virus was also verified in rootstock cultivars (Czotter *et al.*, 2018), but no comprehensive evaluation of the GPGV isolates was carried out.

In the present study, 20 cultivars were evaluated for GPGV, and all but one was 100% infected by the virus. The exception was the 'Merlot' cultivar, for which two out of the five assayed plants were GPGV-positive (Table 1), which could be due to low virus titre. According to previous studies, 'Merlot' is one of the more GPGV-susceptible cultivars capable of displaying strong GLMD symptoms, compared with 'Pinot gris', 'Traminer', 'Sauvignon blanc', and others (Beuve *et al.*, 2015; Bianchi *et al.*, 2015; Gentili *et al.*, 2017).

Limited data are available on the dynamics of GPGV spread within vineyards. Two recent studies addressed the spatiotemporal spreading of GPGV in the major European wine-producing regions of Italy and France (Bertazzon *et al.*, 2020; Hily *et al.*, 2021b). During a 3-year monitoring period of two Italian vineyards, high disease occurrence and consistently increasing presence of GPGV (up to 76%) were observed. Patchy dissemination patterns of newly infected and symptom-bearing plantlets (clades B and C) were observed around the originally infected grapevines, while the asymptomatic plants (mainly clade A) occurred in a more scattered pattern throughout the vineyard. Over the monitoring period, only a small proportion of the newly infected plantlets showed GLMD symptoms, despite the drastic increase in the number of GPGV-positive samples (Bertazzon *et al.*, 2020). Hily *et al.* (2021b), who investigated GPGV spread in a vineyard in southern France, identified several major transmission events that increased the genetic diversity of GPGV by the end of an 8-year monitoring period. These studies raised questions about the mechanisms of GPGV transmission, and differences between transmission dynamics of symptomatic and

asymptomatic GPGV variants. In the present study, the correlation between symptoms and nucleotide sequence classifications was not addressed, because the observed symptoms (e.g., the strong GLMD of sample HU-27) may have been due to possible synergistic effects of multiple viruses.

Genetic determination of GLMD symptoms connected with some strains of GPGV is still unclear, despite the convincing results of other investigations (Tarquini *et al*., 2019a, 2021a). It has generally been observed that GPGV strains belonging to clade A do not induce severe symptoms, unlike isolates of clade C, which have been frequently shown to contribute to the presence of strong GLMD symptoms. Symptom formation was also suggested to be affected by virus titer. Studies have confirmed the hypothesis that increased GPGV concentration resulted in the elevated presence of severe GLMD symptoms (Bianchi *et al*., 2015; Bertazzon *et al*., 2017). To achieve high virus titer and overcome host defense mechanisms, the activation of PTGS was also demonstrated: Tarquini *et al*. (2021b) successfully identified GPGV CP as the viral suppressor of RNA silencing of GPGV. Furthermore, a putative role of the 3'-end of GPGV MP was also identified as a virulence determinant. The effect of the premature stop codon of the MP was first proposed by Saldarelli *et al*. (2015), when all the symptomatic variants of GPGV in clade C possessed the six aa shorter MP. Also, by replacing the 365 nt long 3'-end region of the MP gene in a symptomatic variant (previously linked with severe symptoms and inducing high virus titer) with the homologous region of a variant inducing milder symptoms, the chimeric construct elicited characteristics resembling the mild clone (Tarquini *et al*., 2021a).

In the present study, two GPGV isolates were identified which classified as clade C but lacked the SNP at the 3'-end of MP. New polymorphisms in the MP/CP region were previously described by Morán *et al.* (2018), but no clade C-member GPGV isolates were identified with six aa longer MPs before. This result is in accordance with the hypothesis that post-translational modifications (PTMs) could be the link between the genetic variability and symptom formation of GPGV (Tarquini *et al*., 2019a; Shvets and Vinogradova, 2022). There is, as yet, no direct proof of phosphorylation of GPGV MP, but it can be hypothesized that differences between elicited symptoms may originate from the presence or absence of phosphorylatable residues instead of the premature stop codon.

In the future, analysis of the sanitary status and the changes in the distribution of the different virus isolates in this vineyard could be worthwhile, since isolates from all three clades of GPGV were shown to be present. Further investigation of synergism/antagonism between different GPGV strains could also be important because of the possibilities of cross-protection from mixed virus infections.

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