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Identification of grapevine virus G and grapevine virus H in Sardinia, Italy

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Summary. Grapevine virus G (GVG) and grapevine virus H (GVH) (genus *Vitivirus*) are recently discovered viruses. Analysis of 38 samples from Sardinian grapevine cultivars for the presence of GVG and GVH was carried out using RT-PCR. All samples were also tested for grapevine Pinot gris virus (GPGV) using RT-PCR, and for grapevine leafroll virus -1, -2 and -3, grapevine virus A (GVA) and B (GVB), arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV) and grapevine fleck virus (GFkV) using multiplex RT-PCR. GVG was confirmed in four vines, and GVH was detected in only one sample. In phylogenetic analyses of the coat protein (CP) region, the Sardinian GVG isolates clustered separately from isolates from Croatia and New Zealand. The Sardinian GVH isolate clustered with most sequences from other countries, but with greater affinity to isolates from California (USA) for the CP region, whereas it clustered with isolates from Croatia in the RNA-dependent RNA polymerase (RdRp) region. In addition to GVG and GVH, many samples were coinfected with GVA, viruses from the leafroll complex, and GPGV. This is the first record of GVG and GVH occurring in Italy.

Keywords. RT-PCR, sequencing, grapevine viruses, vitiviruses.

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More than 80 viruses have been identified in grapevine, which is the greatest number of intracellular pathogens reported in one economic crop host (Martelli, 2014; Martelli, 2018; Fuchs, 2020). The genus *Vitivirus* (family *Betaflexiviridae*) contains plant-infecting viruses (Adams *et al*., 2012). The known *Vitivirus* species infecting grapevines are: grapevine virus A (GVA), grapevine virus B (GVB), grapevine virus D (GVD), grapevine virus E (GVE), and grapevine virus F (GVF) (Minafra *et al*., 2017). Recently, new vitiviruses have been identified and named: grapevine virus G (GVG) (Blouin *et al*., 2018a), grapevine virus H (GVH) (Candresse *et al*., 2018), grapevine virus I (GVI) (Blouin *et al*., 2018b), grapevine virus J (GVJ) (Diaz-Lara *et al*., 2018), grapevine virus L (GVL) (Debat *et al*., 2019), grapevine

virus N (GVN), and grapevine virus O (GVO) (Read *et al*., 2022). Currently, GVG has been reported in New Zealand (Blouin *et al*., 2018a), the United States of America (Diaz-Lara *et al*., 2019), and Croatia (Vončina and Almeida, 2018), while GVH has been reported in Portugal (Candresse *et al*., 2018), the United States of America (Diaz-Lara *et al*., 2019), Greece (Panailidou *et al*., 2021), and Croatia (Jagunić *et al*., 2021).

In Italy, only GVA (Conti *et al*., 1980), the cause of Kober Stem Grooving (KSG) (Garau *et al*., 1994), GVB, the cause of Corky Bark (Boscia *et al*., 1993), and GVD associated with Grapevine Rugose Wood Disease (Abou-Ghanem *et al*., 1997) have been reported to date.

To verify occurrence of new grapevine viruses in Sardinia (Italy), particularly GVG and GVH, a survey was conducted of several grapevine varieties randomly collected from different vineyards in this region.

During winter of 2020 and spring of 2021, a total of 38 grapevine samples/vines were analyzed for the presence of grapevine Pinot gris virus (GPGV), GVG, GVH, grapevine leafroll associated virus 1, 2 and 3 (GLRaV-1, GLRaV-2, GLRaV-3), GVA, GVB, arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV) and grapevine fleck virus (GFkV). Of the vines analyzed, eight were 'Cannonau' from a vineyard near Alghero, ten were 'Vermentino' from a vineyard near Olmedo, and five each were of 'Cannonau', 'Vermentino' 'Vernaccia', and 'Carignano' from a vineyard near Narbolia. For each sampling, five to six 10 cm canes were taken from different parts of the vine canopy to assess for presence of the viruses using molecular assays.

To extract total RNA, 100 mg of phloem tissue from each sample was macerated in liquid nitrogen using a pestle and mortar, and then transferred to a 2.0 mL capacity tube containing on of 1.8 mL of grinding buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 2% (w/v) PVP40, 0.2% (w/v) BSA, 0.05% (v/v) Tween 20, pH 9.6), and vortexed briefly. After centrifugation for 10 min at 13,200 g using a high-speed Centrifuge 5415 R (Eppendorf) , 8 µL of clear supernatant was added to 0.1 mL of GES buffer (0.1 M glycine-NaOH pH 9.0, 50 mM NaCl, 1 mM EDTA pH 9.0, 0.5% (v/v) Triton X-100) (La Notte *et al*., 1997), containing 1% β-mercaptoethanol in a 0.2 mL PCR reaction tube. The sample tubes were then incubated in a Mastercycler (Eppendorf) at 95°C for 10 min.

All samples were tested for the presence of GVG and GVH using the OneStep RT-PCR kit (Qiagen), with 0.5 µM of each specific primer (Supplementary Table 1) and the supplied mastermix, following the manufacturer's recommendations. The RT-PCR conditions applied in the Mastercycler (Eppendorf) included the reverse transcription step at 52°C for 30 min, an initial activation step at 95°C for 15 min, 35 cycles at 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 7 min. In addition, all samples were tested for GPGV by RT-PCR using specific primers (Supplementary Table 1), and by multiplex RT-PCR, to simultaneously test the samples for the presence of GLRaV-1, GLRaV-2, GLRaV-3, GVA, GVB, ArMV, GFLV and GFkV. Multiplex PCR was carried out according to published protocols (Faggioli *et al*., 2012).

The PCR products obtained, including partial coat protein (CP) and partial RNA-dependent RNA polymerase (RdRp) genes, were Sanger sequenced in both directions at Macrogen Europe (Amsterdam, The Netherlands), and analyzed using MEGA X (Kumar *et al*., 2018) and BioEdit software, version 7.2.5 (Hall, 1999).

Phylogenetic trees for GVG and GVH were generated using the sequences from the Sardinian isolates and sequences available in GenBank, including the GVN isolate (GenBank. MZ68235) for rooting the GVG tree and the GVM isolate (GenBank. MK492703) for rooting the GVH trees (Supplementary Table 2). Phylogenetic analyses were carried out using the aligned sequences, and trees were generated using the maximum likelihood method with the MEGA X program. A bootstrap analysis with 1,000 replicates was performed to estimate statistical support for the different tree branches, using a minimum of 50% as a threshold.

Four of the 38 grapevines/samples tested were positive for GVG, and one sample was positive for GVH (Table 1). The four GVG-positive samples were detected only with primers specific to the CP region, whereas the same samples were negative with primer pairs targeting the RdRp region. The failure to detect the virus was probably due to the genetic variability of the Sardinian isolates in the respective regions of the genomes.

In several studies investigating the effects of GVA, GVB, and GVD on plants (Sciancalepore *et al*., 2006; Blouin *et al*., 2018b; Rosa *et al.* 2011), combined infections were found to have greater negative effects on symptoms than single virus infections. Sardinian grapevines positive for GVG were coinfected with GVA in two samples, GLRaV-1 in one sample, GLRaV-2 in one sample, and GPGV in three samples, whereas coinfections with GLRaV-3 were present in all the samples (Table 1). Presence of other viruses included in the study was not confirmed in the vine infected with GVH.

Phylogenetic analyses indicated that all Sardinian GVG CP sequences formed a separate clade. The four Sardinian GVG isolates had nucleotide similarities from 98.3% to 100%. In addition, CP sequences clustered in separate clades based on the location of the vineyards, in Narbolia and Olmedo (Figure 1).

Table 1. RT-PCR detection of different viruses in different grapevine in Sardinia, in cvs. 'Cannonau'(CN) located in Alghero (AHO) and Narbolia (NAR), 'Vermentino' located in Olmedo (OLM) and Narbolia (NAR), and 'Vernaccia' (VRN) and 'Carignano' (CRG) located in Narbolia (NAR). Vines positives for GVG and GVH are indicated in bold font.

Samples	Cultivar	Site	GVG	GVH	GPGV	GLRaV 1	GLRaV 2	GLRaV 3	GVA	GFkV
AHO_1_CN	Cannonau	Alghero	-	-	+	-	-	+	-	-
AHO_2_CN	Cannonau	Alghero	-	-	+	-	-	+	+	+
AHO_3_CN	Cannonau	Alghero	-	-	+	-	+	+	-	+
AHO_4_CN	Cannonau	Alghero	-	-	+	-	+	+	+	+
AHO_5_CN	Cannonau	Alghero	-	-	+	-	+	+	-	+
AHO_6_CN	Cannonau	Alghero	-	-	+	-	-	-	-	+
AHO_7_CN	Cannonau	Alghero	-	-	+	-	-	+	+	-
AHO_8_CN	Cannonau	Alghero	-	-	-	-	-	-	+	+
OLM_9_VRM	Vermentino	Olmedo	-	-	+	-	-	+	+	-
OLM_10_VRM	Vermentino	Olmedo	-	-	+	-	-	-	-	-
OLM_11_VRM	Vermentino	Olmedo	-	-	+	-	-	+	+	-
OLM_12_VRM	Vermentino	Olmedo	-	-	+	-	-	+	-	-
OLM_13_VRM	Vermentino	Olmedo	-	-	+	-	-	-	-	-
OLM_14_VRM	Vermentino	Olmedo	+	-	+	-	-	+	+	-
OLM_15_VRM	Vermentino	Olmedo	-	-	+	-	-	+	+	-
OLM_16_VRM	Vermentino	Olmedo	-	-	+	-	-	+	-	-
OLM_17_VRM	Vermentino	Olmedo	+	-	+	-	-	+	-	+
OLM_18_VRM	Vermentino	Olmedo	-	-	+	-	-	+	-	-
NAR_19_CN	Cannonau	Narbolia	-	-	-	-	-	+	-	-
NAR_20_CN	Cannonau	Narbolia	-	-	+	-	-	+	-	-
NAR_21_CN	Cannonau	Narbolia	-	-	+	-	-	+	-	-
NAR_22_CN	Cannonau	Narbolia	-	-	+	-	-	+	-	-
NAR_23_CN	Cannonau	Narbolia	-	-	-	-	-	+	-	-
NAR_24_VRN	Vernaccia	Narbolia	-	-	-	-	-	-	-	-
NAR_25_VRN	Vernaccia	Narbolia	-	-	+	-	-	-	-	-
NAR_26_VRN	Vernaccia	Narbolia	-	-	+	-	-	+	-	-
NAR_27_VRN	Vernaccia	Narbolia	-	-	-	-	-	-	-	-
NAR_28_VRN	Vernaccia	Narbolia	-	-	-	-	-	-	-	-
NAR_29_VRM	Vermentino	Narbolia	-	-	+	-	-	-	-	-
NAR_30_VRM	Vermentino	Narbolia	-	-	+	-	-	-	-	-
NAR_31_VRM	Vermentino	Narbolia	-	-	+	-	-	-	-	-
NAR_32_CRG	Carignano	Narbolia	-	-	+	+	-	-	-	-
NAR_33_CRG	Carignano	Narbolia	-	-	-	-	-	-	-	-
NAR_34_CRG	Carignano	Narbolia	-	-	+	+	-	+	+	-
NAR_35_CRG	Carignano	Narbolia	-	-	+	-	-	+	+	-
NAR_36_CRG	Carignano	Narbolia	+	-	+	+	+	+	-	-
NAR_37_CRG	Carignano	Narbolia	+	-	-	-	-	+	+	-
NAR_38_CRG	Carignano	Narbolia	-	+	-	-	-	-	-	-

Sardinian GVG isolates showed the greatest nt identity (93.57%) with the Croatian VD-102 isolate (Vončina and Almeida 2018).

The GVH phylogenetic trees (Figures 2 and 3) generated using the partial CP and RdRp sequences showed the greatest CP nucleotide similarity of 99.72% with isolate GC5462 (GenBank acc. no. MK838926) from the United States of America, but a grapevine accession originating from Romania (Diaz-Lara *et al*., 2019). In the RdRp region, the Sardinian GVH isolate showed greatest nucleotide identity (98.31%) with the Babica plosnata isolate from Croatia (Jagunić *et al*., 2021).

This study is the first to record the presence of GVG and GVH in Italy. GVG was found in two different areas of Sardinia, in one area in the red grape 'Carignano', and one in the white grape 'Vermentino'. GVH

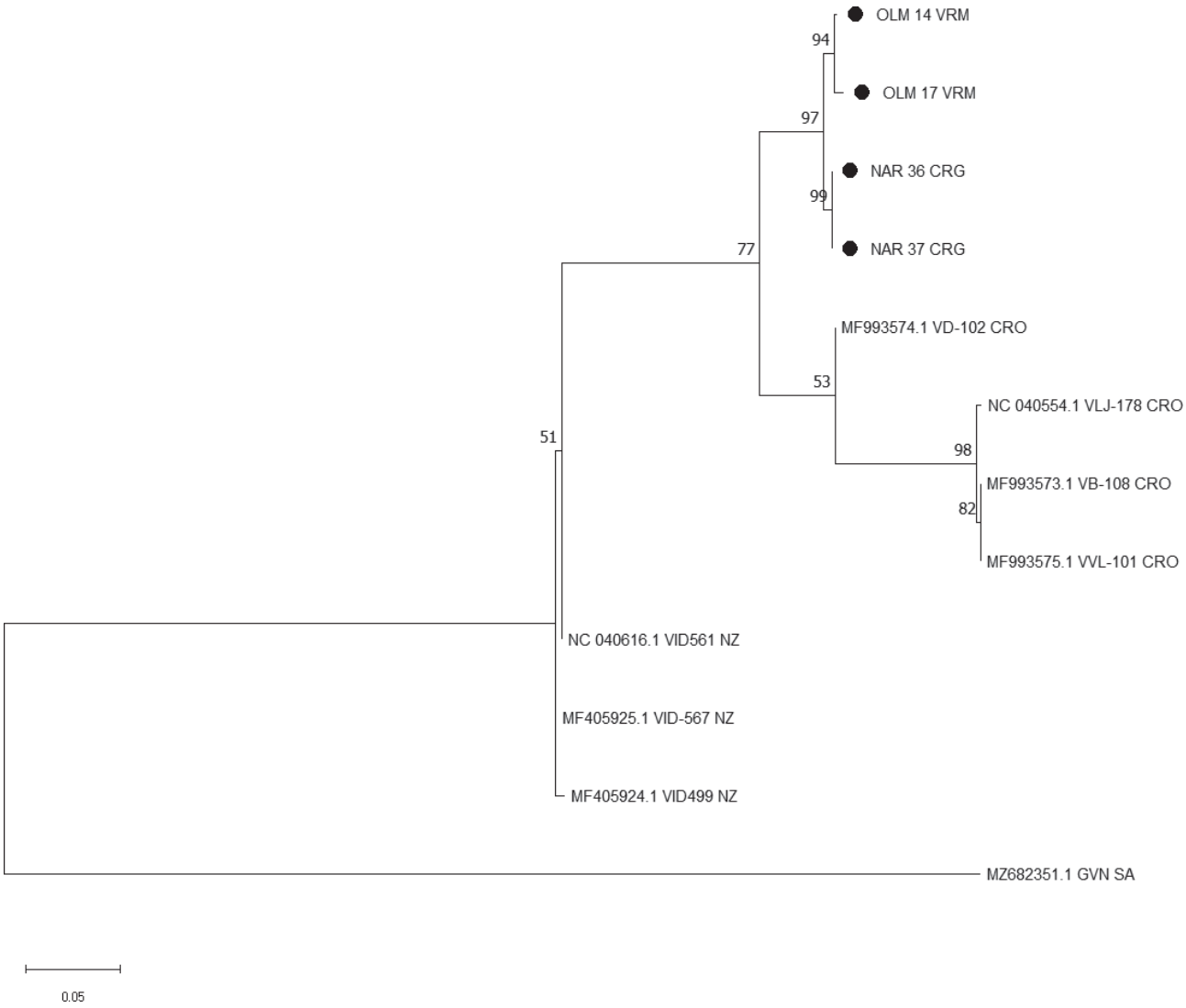

Figure 1. Phylogenetic tree generated from partial nucleotide sequences of the CP region of GVG isolates. Bootstrap values were obtained using 1000 replicates. Only branches with support greater than 50% are indicated. Sardinian isolates detected in this study are indicated by black dots. The phylogenetic tree was reconstructed using the maximum likelihood algorithm implemented in MEGA X software. Four isolates from Croatia (CRO) and three from New Zealand (NZ) were selected from the GenBank database. The GVN sequence (MZ682351) was used as the rooting outgroup.

was detected only in one sample of 'Carignano'. As mentioned by Diaz-Lara *et al*. (2019), none of the novel grapevine vitiviruses have been associated with diseases. It may be important, however, to monitor infection by these viruses in grapevines, to check for any impacts on vine performance and production.

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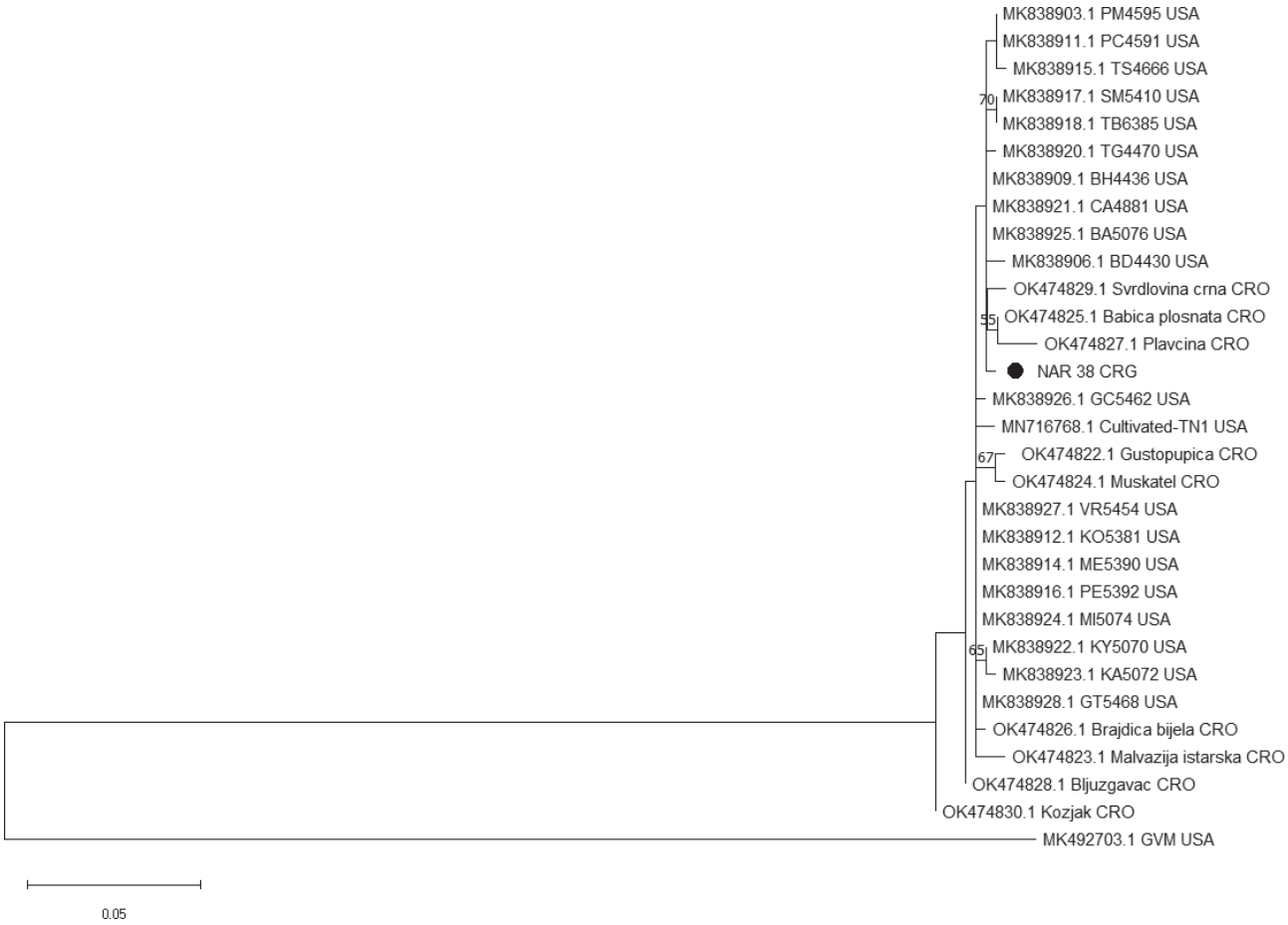

Figure 2. Phylogenetic tree generated from partial nucleotide sequences of the GVH CP region. Bootstrap values were obtained using 1000 replicates. Only branches having a support greater than 50% are indicated. The Sardinian isolate detected in this study is indicated by a black dot. The phylogenetic tree was reconstructed using the maximum likelihood algorithm implemented in the MEGA X software. Nineteen isolates from different countries located in the collection in the United States of America (USA) and eight isolates from Croatia (CRO) were selected from GenBank and used to construct the tree. The GVM sequence (MK492703) was used as the rooting outgroup.

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Figure 3. Phylogenetic tree generated from partial nucleotide sequences of the GVH RdRp region. Bootstrap values were obtained using 1000 replicates. Only branches having a support above 50% are indicated. The Sardinian GVH isolate detected in this study is indicated by a black dot. The phylogenetic tree was reconstructed using the maximum likelihood algorithm implemented in the MEGA X software. Seventeen isolates from different countries (United States of America - USA, Portugal - PT and Croatia - CRO) were selected from GenBank and used to construct the tree. A GVM sequence (MK492703) was used as the rooting outgroup.

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