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Development of RT-PCR assays for the detection and the resultant phylogenetic analysis of four grapevine vitiviruses based on the coat protein sequences

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ABSTRACT

Four sets of primers were designed based on the alignment of the complete coat protein (CP) gene sequences of several isolates of four different vitiviruses, *i.e.* grapevine virus B (GVB), GVD, GVE and GVF, and tested for their efficiency in RT-PCR assays to detect vitiviruses infections in grapevine. The resultant RT-PCR amplicons were sequenced and analyzed for their genetic variability and phylogenetic studies. The results of the RT-PCR assays showed that these primers were highly efficient in detecting different vitivirus isolates in grapevine material originating from ten different Mediterranean countries. In particular, 76 out of 218 tested samples (*ca.* 35%) were infected with at least one vitivirus. GVE was the most detected (14.7%), followed by GVF (11.5%), GVB (6.9%), and GVD (2.8%). Nucleotide (nt) sequence analysis of the CP genes from this study and Genbank showed that the sequence identity matrixes among isolates of GVB and GVE were the most variable, with nt identity ranging from 77% to 100%, whereas isolates of GVD and GVF showed more conserved nt identities ranging between 82% to 100% and 86.4% to 99.8%, respectively. The phylogenetic trees constructed based on the CP sequences distinguished two main groups of isolates for each vitivirus species, except for the GVD isolates, which did not show any particular subdivision. In general, the distributions of the isolates in the phylogenetic tree were associated with their geographical origin, thus suggesting limited movement of grapevine materials between the different countries. This study reported for the first time: (i) the development of primers based on the complete CP gene sequences for RT-PCR assays for the universal detection of vitivirus species, (ii) the high genetic variability among Mediterranean isolates of vitiviruses and (iii) the presence of GVD in Jordanian vines, of GVE in grapevines from Hungary, Italy, Jordan, Malta and Palestine, and of GVF in grapevines from Afghanistan, Bulgaria, China, France, Hungary, Italy, Jordan, Lebanon and Malta.

The genus *Vitivirus* represents one of the most important groups of viruses infecting grapevine. This consideration is based on the severity of symptoms that these viruses induce and on their widespread distribution in the world (Minafra et al., 2017). Till 2008, only three vitiviruses were reported on grapevine, *i.e.* grapevine virus A (GVA), grapevine virus B (GVB) and grapevine virus D (GVD). Subsequently, two additional members, namely GVE and GVF, were added to the list (Nakaune et al., 2008; Al Rwahnih et al., 2012). An important turning point in the identification of vitiviruses dates back to 2018, when, thanks to the use of innovative molecular techniques, the list of vitiviruses was extended to include several other new species classified as putative vitiviruses in the newly established *Betaflexiviridae* family (Adams et al., 2011), *i.e.* 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 K (GVK, Jo et al., 2017), grapevine virus J (GVJ, Diaz-Lara et al., 2018), grapevine virus L (GVL, Debat et al., 2019) and grapevine virus M (GVM, Alabi et al., 2019).

Vitiviruses are flexuous, non-enveloped, filamentous particles with a length of 725–785 nm and 12 nm in diameter, comprising a single coat protein (CP) polypeptide species of 18–21.5 kDa and a linear single-strand, positive-sense genome of about 7.3–7.6 kb (Martelli et al., 2007). The complete genome consists of five distinct open reading frames (ORFs): ORF1 (RNA-dependent RNA Polymerase), ORF2 (unknown protein, with a hypothetical role in the mealybug transmission), ORF3 (movement protein), ORF4 (coat protein) and ORF5 (nucleic acid-binding protein) (Galiakparov et al., 2003; Du Preez et al., 2011). In general, vitiviruses are transmitted in nature by pseudococcid mealybugs and/or scale insects (GVA, GVB, GVE) (Minafra et al., 2017;

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Martelli, 2014) and are considered to be the putative causal agents of certain specific syndromes of rugose wood complex, *i.e.*, GVA of Kober stem grooving (Digiaro et al., 1994; Garau et al., 1994; Chevalier et al., 1993) and GVB of corky bark (Bonavia et al., 1996; Boscia et al., 1993). Little is known about the exact etiological role of the other vitiviruses, except that GVD was detected in vines with corky rugose wood symptoms (Abou-Ghanem et al., 1997) and GVF in vines with graft incompatibility (Al Rwahnih et al., 2012).

Currently, commercial ELISA kits are available for GVA and GVB, but not for the other vitiviruses for which molecular techniques seem to be the only means of detection. Furthermore, for the newly discovered viruses (GVE and GVF), there is a lack of detection techniques, and data regarding their distribution and possible genetic variability in the Mediterranean region. Consequently, the present study focused on the development of RT-PCR assays using newly designed degenerate primers for the detection of GVB, GVD, GVE and GVF in grapevines originating mainly from different Mediterranean countries, and the subsequent sequence analysis of the CP amplicons to discern the genetic relationships existing among vitiviruses from the Mediterranean and other origins. GVA, for which numerous data and information already exist in literature (Minafra et al., 2017), was excluded from this study.

In 2016, mature canes (218 samples) of different grapevine varieties originating mainly from the Mediterranean countries (Table 1) were collected from two collection plots at Locorotondo and Valenzano (Apulia region, South Italy), which host grapevine self-rooted varieties collected over the past fifty years and that were free from quarantine pests after a previous isolation in quarantine facilities. Phloem scrapings were used for proteins and total nucleic acids (TNAs) extractions that were afterward assayed in DAS-ELISA (Boscia et al., 1993) using antisera from Agritest (Valenzano-Bari, Italy) and RT-PCR (Minafra and Hadidi, 1994), respectively.

The two comparative detection methods (ELISA and RT-PCR) were possible only for GVB, since no commercial antisera were available for the other vitiviruses considered in this study (GVD, GVE and GVF).

TNAs were extracted from 0.1 µg of cortical scrapings using silica particles according to Foissac et al. (2001). The phloematic tissue was ground in liquid nitrogen and homogenized in 1 ml of extraction buffer (4.0 M guanidine isothiocyanate, 1 M potassium acetate, 0.2 M sodium acetate, 25 mM EDTA, 2.5% PVP-40). The extracted sap was mixed with 6 M sodium iodide, 0.15 M sodium sulphite, 150 µl ethanol and 25 µl silica particles suspension (1 g/ml, pH 2.0). After stripping by heat treatment in sterile water (70 °C for 3 min) and centrifugation for 3 min at 16,000 *g*, the TNAs were recovered and stored at −20 °C until use.

TNAs extracted from GVB- and GVD-infected vines maintained at the premises of the Mediterranean Agronomic Institute of Bari (MAIB, Italy), together with TNAs from GVE- and GVF-infected vines provided by Dr. H. Maree from the University of Stellenbosch, South Africa, were used as positive controls in the respective RT-PCR assays.

Two genes of the vitivirus genome, *i.e.*, the RNA-dependent RNA Polymerase (RdRP) and the coat protein (CP), were used for designing the universal primers that can detect as many isolates as possible of each vitivirus species. Accordingly, genomic sequences of several isolates (accession numbers are reported between brackets in Fig. 1) for each vitivirus under study were recovered from the NCBI public database and aligned using CLUSTALX 1.8 (Thompson et al., 1997). Stretches of nucleotide (nt) sequences covering regions conserved among the isolates were chosen to design several sets of primers for both genes. Nucleotide degeneracy found at each of the primers sites was incorporated into the primers (Table 2).

TNAs were randomly reverse-transcribed into cDNA in the presence of reverse-transcriptase enzyme, according to the manufacturers' instructions (ThermoFisher, Italy). cDNA was added to 2.5 µl Taq polymerase buffer 10x (Promega) containing a final concentration of 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM each specific sense and antisense primers, 0.2 µl Taq polymerase enzyme (5 U ml^{-1}) in a final volume of 25 µl. RT-PCR cycling consisted of an initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation, annealing and elongation, respectively, at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 40 s. Final extension was at 72 °C for 7 min. Amplification products of 594 bp, 486 bp, 600 bp and 597 bp in sizes for GVB, GVD, GVE and GVF, respectively, were visualized after electrophoresis in Agarose gels in TAE buffer (Table 2).

RT-PCR assays conducted with both RdRP- and CP-based primers (Table 1) on isolates from different origins showed different levels of specificity. In general, the RdRP-based primers proved to be less successful to detect GVB, GVD, GVE and GVF isolates. In fact, RdRP-based primers detected only 42 positives out of 218 samples tested, with an infection rate of 19% *versus* 30% obtained when CP-based primers were

Table 1
The country of origin and number of grapevine plant samples assessed by RT-PCR assays for the presence of four vitiviruses.

Country of origin	Tested samples (no.)	Infected samples (no.)*	Infected vines by vitivirus species (no.)			
			GVB	GVD	GVE	GVF
Afghanistan	2	1	0	0	0	1
Albania	5	0	0	0	0	0
Bulgaria	3	1	0	0	0	1
China	7	6	1	0	6	1
Cyprus	2	0	0	0	0	0
France	6	1	0	0	0	1
Greece	4	4	0	0	2	4
Jordan	17	3	0	1	2	2
Hungary	3	3	0	0	3	1
Italy	23	12	4	1	5	3
Lebanon	23	3	1	0	0	2
Malta	3	3	2	0	2	2
Nigeria	1	0	0	0	0	0
Palestine	24	5	1	0	4	0
Romania	2	0	0	0	0	0
Tunisia	85	21	4	4	8	7
Turkey	8	2	2	0	0	0
Total	**218**	**65 (29,8%)**	**15 (6,9%)**	**6 (2,8%)**	**32 (14,7%)**	**25 (11,5%)**

* Infected varieties : AF (Kishimish Maramorni), BU (Bolgar), CH (unknown cvs.), FR (Chardonnay), GR (Corinto b., Razaki, Sultanina), JO (Black Pearl, Baladi, Dabouqi), HU (Mirs, Jubileum 75, Dimont Pomaric), IT (Primus, Italia, Pascale, Verdeca, Montonico, Malvasia n., Pampanuto, Sangiovese, Montepulciano, Trebbiano b, Greco b., Cardinal), LE (Aabaidi, Hifawi, Tfaifhi), MA (Buskett, Sultanina b., Gellowza), PA (Dabouqi, Halawani, Marrawi, Romi, Darawishi), TU (Superior seedless, Saint nero, Rich Baba Sam, Italia, Cardinal, Kizil, Cilores, Kabaczik, Muskat di Kelibia, Aledo, Sangiovese, Cinsaut, and unknown cvs.), TK (Besni, Dimrit).

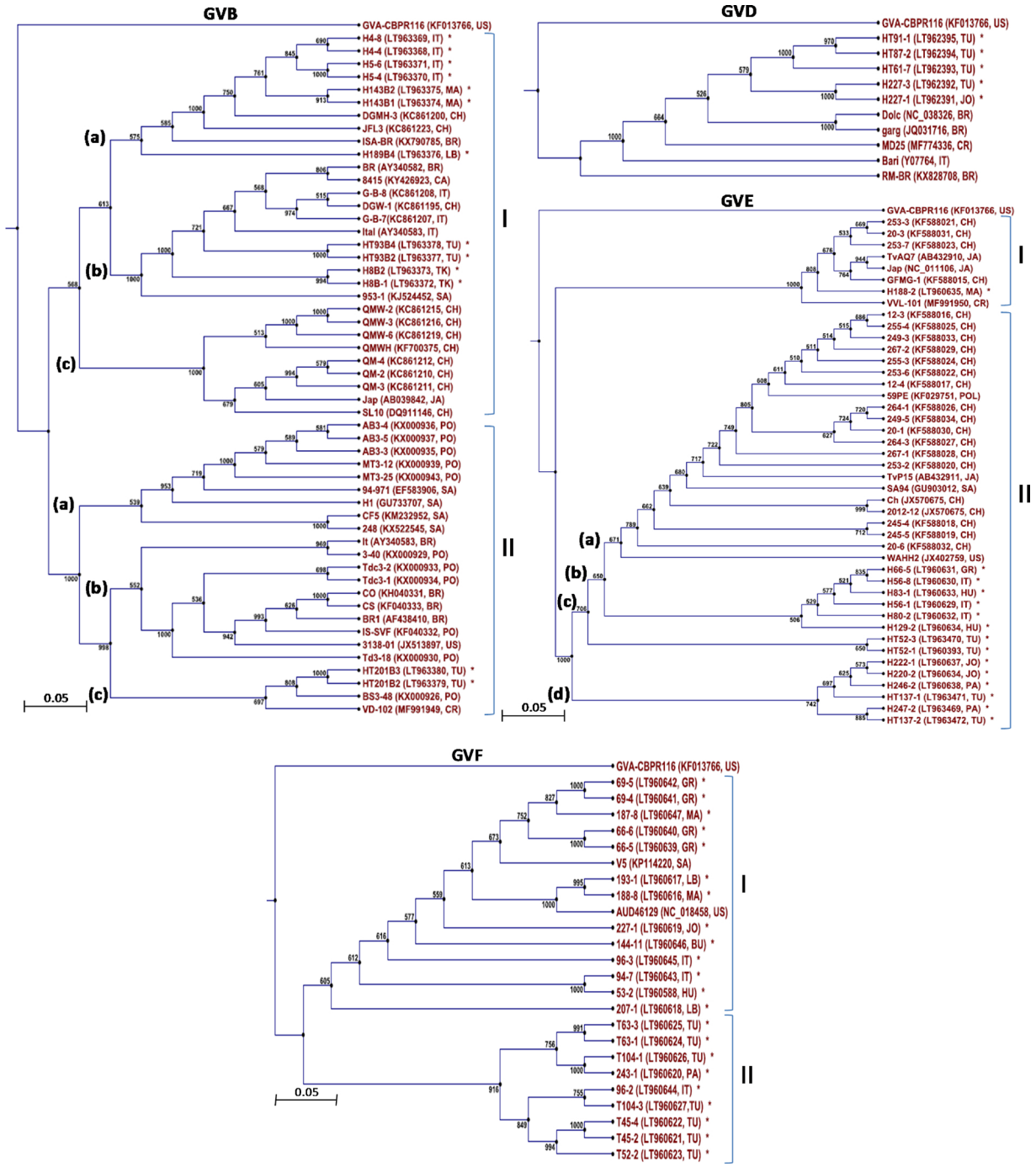

Fig. 1. Phylogenetic trees constructed based on nucleotide sequences of the CP gene of GVB, GVD, GVE and GVF. Sequences obtained in this study are marked with asterisk. Accession numbers of sequences used and their countries of origin are reported between brackets, respectively. Abbreviations used to indicate countries of origin of analyzed vitiviruses isolates are: AF (Afghanistan), AL (Albania), BR (Brazil), BU (Bulgaria), CA (Canada), CH (China), CR (Croatia), CY (Cyprus), FR (France), GR (Greece), HU (Hungary), IT (Italy), JA (Japan), JO (Jordan), LB (Lebanon), MA (Malta), PA (Palestine), PO (Poland), SA (South Africa), TU (Tunisia), TK (Turkey), US (USA). I\II and a–c represent the repartition of vitiviruses isolates into groups and subgroups, respectively. GVa–cBPR116 isolate was used to root the phylogenetic tree. Numbers on branches indicate percentage of support out of 1000 bootstrap replications.

used. This difference is most likely due to the high sequence variability known to encompass in the RdRP gene (Shi et al., 2004), a limitation apparently inconsistent in the most conserved region of the CP. Consequently, RdRP primers were discarded and the CP gene was adopted as the only target region for amplification. RT-PCR assays using these primers on a large number of samples successfully amplified the expected products from 65 out of 218 samples (*ca.* 30%) of different origins, *i.e.* 23 samples besides to the 42 samples identified by the RdRP primers. In particular, 54 samples were infected by a single vitivirus species (83%), 11 by mixtures of two or three different vitiviruses (17%). GVE was the most detected vitivirus (14.7% of samples), followed by GVF (11.5%), GVB (6.9%), and GVD (2.8%). On the other hand, DAS-ELISA was able to detect GVB only in 9 of 15 samples found positive by RT-PCR.

Through the use of these CP-based primers and the examination of hundreds of vines mainly coming from Mediterranean countries, it was possible to draw preliminary epidemiological and genetic scenarios on the potential diffusion of the four grapevine-infecting vitiviruses and on

Table 2
List of primers used in the RT-PCR assays for the detection of vitiviruses. The degenerate nucleotides employed are indicated.

Virus	Primers names	Primers sequences (5'to 3')	Genes	PCR product (bp)
GVB	RdRP-B-s	GAAAGGGGTWCAYACGAGT	RdRP	605
	RdRP-B-a	GTCCAWGTYTGGCCRTGCACA		
	CP-s	ATGGAAAATATATCCCGGATGGC	CP	594
	CP-a	CTATATYTCRACAGAYTGCTC		
GVD	RdRP-D-s	GATTCGGACCYAAATTGAGG	RdRP	300
	RdRP-D-a	AAGCGCATTATCGCCAAATT		
	CP-s	ATGTAYCTKAGGACSCTSTTCGG	CP	486
	CP-a	TTATATCTCAACTGCCTGCTCTCC		
GVE	RdRP-E-s	CTAARGATAGTACTTTTRTT	RdRP	420
	RdRP-E-a	AGAGGTTGATGKGACCCWAYRCT		
	CP-s	ATGGAGTCAAAAGCGATCMGRTC	CP	600
	CP-a	CTAGACYTCCACCGAGYTTTC		
GVF	RdRP-F-s	AAGAATRCAAGGTACAAYAGG	RdRP	600
	RdRP-F-a	AGRTACAACAATACRGGCTTMAC		
	CP-s	TGGCTCAGATATCAAGAAGGATG	CP	597
	CP-a	CAGATCTCAGCTGCTTGTTCACCG		

B (C + G + T); D (A + G + T); H (A + C + T); K (G + T); M (A + C) ; R (A + G); S (G + C); V (A + C + G); W (A + T); Y (C + T); s = sense ; a = antisense; deg = primer with nt degeneracy; CP = coat protein; RdRP = RNA-dependent RNA Polymerase.

their genome variability. The results showed that GVB is present in vines with a limited prevalence, whereas GVE and GVF are significantly diffused and present in many countries. GVD seems to be quite rare given that it was found only in a few Italian, Tunisian and Jordanian vines. In addition, this study allowed to report, for the first time, the presence of GVD on vines from Jordan, of GVE on vines from Greece, Hungary, Italy, Jordan, Malta and Palestine, and of GVF on vines from Afghanistan, Bulgaria, China, France, Greece, Hungary, Italy, Jordan, Lebanon and Malta.

The amplified CP products of 55 vitivirus isolates (13 GVB, 5 GVD, 15 GVE and 22 GVF) were ligated into the StrataClone™ PCR Cloning vector pSC-A (Stratagene, USA) and custom sequenced (Eurofins Genomics, Germany). Three DNA clones from each isolate were sequenced. The resulting CP nucleotide and protein sequences were analyzed with the assistance of the DNA Strider 1.1 program (Marck, 1988). Multiple alignments of the nucleotide and amino acid sequences and identity matrixes were obtained using the default options of CLUSTALX 1.8 (Pearson and Lipman, 1988; Thompson et al., 1997). Searches for homologies with proteins from the Protein Information Resources database (PIR, release 47.0) were done with the FASTA (Altschul et al., 1990), BlastX and BlastP (Tamura et al., 2013) programs. Tentative phylogenetic trees were constructed using the Neighbor-Joining (NJ) method in the CLC Genomics workbench v.6.5 analysis package.

Nucleotide sequence identity matrix of the CP gene of vitivirus isolates sequenced in this study (55 isolates) and of those retrieved from Genbank (accession numbers are reported in Fig. 1) showed that GVB and GVE have the widest variability between isolates, with nucleotide identity ranging from 77% to 100%, a value above the threshold of 72%, considered as one of the demarcating criteria for species in the genus *Vitivirus* (Adams et al., 2011). GVD and GVF were the most conserved, with a nt identity ranging from 82% to 100% and 86.4% to 99.8%, respectively. At the amino acids level, these differences were less consistent with minimum identity values ranging from 85.4% for GVE to 85.7% for GVB, 88.8% for GVD and 93.4% for GVF.

The irregular distribution of the infection in the two collection plots analyzed in this study together with the genomic differences observed between the sequenced viral isolates exclude that a natural secondary spread of the same viruses by vectors may have occurred in those fields.

In the phylogenetic tree of GVB, two main clusters (group I and II) were distinguished (Fig. 1). With very few exceptions (2 Tunisian isolates in the subgroup c of group II), all Mediterranean GVB isolates sequenced in this study clustered in the two subgroups a and b of group I, together with very few isolates from Brazil, China, South Africa and Canada. Almost all the isolates from China and Japan clustered in the subgroup c of group I, whereas those from South Africa and Brazil clustered in the subgroups a and b of group II. All Portuguese isolates of GVB were distributed in the subgroups a, b and c of group II (Fig. 1).

The phylogenetic tree of GVD constructed based on few sequences available in the Genbank showed a less distinct separation of isolates. However, the Mediterranean isolates originating from Tunisia and Jordan were closer to the Italian and Croatian isolates and slightly distant from the Brazilian one (Fig. 1).

The phylogenetic tree of GVE separated the isolates into two main groups (I and II), with the group II subdivided in at least 4 subgroups (a, b, c, and d). The Mediterranean isolates were all allocated in the subgroups b, c and d of the group II, with the exception of the Maltese isolate H188-2 that was allocated in the group I. The Croatian isolate and all the other non-Mediterranean isolates (China, Japan, South Africa, USA and Poland) were instead allocated in the group I and in the subgroup a of the group II (Fig. 1).

The phylogenetic tree of GVF similarly repartitioned the isolates into two groups (I and II), with the group I including isolates mostly from the Mediterranean countries (Greece, Malta, Lebanon, Jordan and Italy) and the group II mainly gathering the Tunisian isolates (Fig. 1).

This study has highlighted the high level of genetic variability existing within each vitivirus species, especially for GVB and GVE, contrarily to previous notes that emphasized the high sequence homogeneity of GVE (Nakaune et al., 2008; Vargas-Asencio et al., 2016). In addition, the phylogenetic analyses showed that vitiviruses could be repartitioned to groups based on their geographical origin, suggesting that substantially there has been a reduced movement of grapevine material, especially among Mediterranean countries.

Declaration of Competing Interest

None.

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