Phytopathologia Mediterranea (2016) 55, 1, 73–88
DOI: 10.14601/Phytopathol_Mediterr-16378

RESEARCH PAPERS

Genetic variants of *Grapevine leafroll-associated virus 2* infecting Portuguese grapevine cultivars

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Summary. Genetic variability of 19 isolates of *Grapevine leafroll-associated virus 2* (GLRaV-2) from Portuguese grapevine cultivars was characterized by sequencing the entire capsid protein (CP) gene of the virus. Global phylogenetic analysis of the CP gene, which included nucleotide sequences obtained in this study and complete homologous sequences from GenBank, showed segregation of GLRaV-2 variants from Portuguese isolates into three major phylogroups (PN, 93/955 and H4). The novelty of these phylogenetic results is the evidence of well-supported subdivision within H4 as well as within PN, with subgroup PN3 composed exclusively of variants from a Portuguese isolate. These findings and the genetic analysis of global phylogroups indicate demographic expansion, mainly within PN and 93/955. Because the existence of a mixture of variants from different phylogroups was detected in some of the isolates, a typification assay based on reverse transcription reaction followed by polymerase chain reaction and restriction fragment length polymorphism analysis, was developed to complement molecular detection assay of the virus. This protocol discriminates variants from the phylogroups identified in this study, and is appropriate for routine testing for GLRaV-2.

Key words: Closterovirus, genetic variants, Grapevine leafroll disease, *Vitis vinifera.*

Introduction

Grapevine leafroll disease (GLRD) is probably the most economically important grapevine disease, responsible for accumulated yield losses of up to 30–68% during the productive life of vineyards (Walter and Martelli, 1997). It is a widespread disease reported in all grape-growing countries (Martelli and Boudon-Padieu, 2006; Fuchs *et al*., 2009; Golino *et al*., 2009; Atallah *et al*., 2012). GLRD delays fruit ripening and reduces soluble solids both in red and white grape varieties, which adversely affects the alcohol content of the resulting musts and results in penalties for the producers (Martelli and Boudon-Padieu, 2006; Martinson *et al*., 2008).

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The phloem-limited filamentous viruses associated with GLRD are named grapevine leafroll-associated viruses (GLRaVs). The majority have been classified within the genus *Ampelovirus* of the *Closteroviridae* family, while GLRaV-7 has been provisionally assigned to genus *Velarivirus* and GLRaV-2 is placed in genus *Closterovirus*. Contrary to *Beet Yellow Virus* (BYV), the type species of the genus, and the well-studied species, *Citrus Tristeza Virus* (CTV), GLRaV-2 has no known natural vectors. The genome of GLRaV-2 encompasses nine open reading frames (ORFs) (Zhu *et al*., 1998). Putative products of ORF1a/1b are methyltransferase (MT), helicase (HEL) and RNA-dependent RNA polymerase. ORF2 encodes a 57 AA protein analogous to a small hydrophobic protein found in other *Closteroviridae*, presumably forming transmembrane helices. ORF3 encodes a 65 kDa protein, which is homologous to the HSP70 cellular heat shock protein. The HSP70

homologue (HSP70h) is the defining character in *Closteroviridae* taxonomy, as it is specific for this family and is highly conserved. ORF4 encodes a 63 kDa protein homologous to the p64 of BYV and p61 of CTV. It has been shown for CTV that the heat shock protein homolog (HSP70h) and p61 are needed for efficient virion assembly, apparently working in concert (Satyanarayana *et al*., 2000, 2004). ORF5 encodes the minor capsid protein (CPm), upstream of the ORF6, which encodes the capsid protein (CP). ORFs 7 and 8 respectively encode proteins P19 and P24, and are similar in size and location to those described in the genomes of other closteroviruses such as BYV (P20, P21). However, no conserved regions have been found between these two genes and the corresponding genes in other closteroviruses.

The taxonomic and genomic characteristics of GLRaV-2, and the fact that some of its isolates are mechanically transmissible to herbaceous hosts (Goszczynski *et al*., 1996), a feature not described for the other viruses implicated in GLRD, distinguish GLRaV-2 from the other GLRaVs. To date, six complete genomic sequences of GLRaV-2 have been deposited at GenBank. Phylogenetic studies have suggested that the virus occurs as divergent molecular variants, segregating either into five (Bertazzon *et al*., 2010) or six distinct lineages (Jarugula *et al*., 2010) based on the HSP70h and/or capsid protein genes. Divergent variants were reported to have different pathological properties in reference to the respective phylogenetic groupings, ranging from asymptomatic (Poojari *et al*., 2013) to symptomatic by inducing leafroll, graft union-incompatibility on sensitive rootstocks, young vine decline and rootstock stem lesion disease (Greif *et al*., 1995; Bertazzon *et al*., 2010; Alkowni *et al*., 2011).

Routine detection of GLRaV-2 has mainly been performed by serological assays targeting the capsid protein (reviewed by Bertazzon and Angelini, 2004). For some of the phylogenetic groups, RT-PCR and real-time RT-PCR assays have been developed (Beuve *et al*., 2007), as well as molecular typification assays based on PCR-RFLP (Bertazzon and Angelini, 2004; Jarugula *et al*., 2010). Despite the various types of host damage associated with GLRaV-2, this virus is not part of the grapevine certification scheme implemented in European Union countries. Although GLRaV-2 is known to occur in grapevine growing European countries, little information is available on its incidence and variability in the vineyards. No mandatory certification is implemented for this virus with no known vector, and for which the main transmission route is likely to be through vegetative propagation of infected material. Furthermore, epidemiological and evolutionary processes shaping GLRaV-2 genetic variability are poorly documented. This means that vital information on effective diseases management measures is lacking. Detailed assessment of the incidence and of the genetic structure of field isolates is urgently required, to provide a foundation for evaluating the role of GLRaV-2 in the epidemiology of GLRD. For this purpose nucleotide sequences of the capsid protein gene of GLRaV-2 were obtained from Portuguese field isolates of the virus. A phylogenetic analysis, including complete homologous sequences available from other grapevine growing regions, revealed the existence of co-infections by divergent variants in some of the isolates. This information was used to develop a reverse-transcription (RT) followed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay to allow routine typing of GLRaV-2 variants detected to date in Portugal.

Materials and methods

Plant material and virus sources

Each of the 19 GLRaV-2 isolates analysed in this study were obtained from different field-grown grapevines (*Vitis vinifera*; Table 1).

Nine of the isolates were obtained from the National Collection of Grapevine Varieties (CAN PRT051) at INIAV, Dois Portos, Portugal. This collection consists of grafted vines on certified rootstock material (SO4, clone 73). It was established 30 years ago (Teixeira *et al*., 2009b) on nematode-free soil, and is routinely tested for several viruses, including GLRaVs, either by DAS-ELISA or RT-PCR. The Collection is also maintained free of mealybugs through insecticide applications. Several clonal plants represent each variety accession at CAN. Virus isolates from each variety were usually obtained from the respective clonal plant number 3, with the exception of isolates VD (5) and TB (1), which were obtained from clonal plant number 5 and clonal plant number 1 of the respective variety. Isolate Sr3 was obtained from a grapevine in a production vineyard at INIAV, Dois Portos, planted with the Síria variety grafted on cer-

Table 1. Details of *Grapevine leafroll-associated virus 2* isolates analysed in this study.

Location	Grapevine variety Rootstock/ Vineyard location	Isolate ID	GLRaV-2 Clade	GenBank Accession Number	Other GLRaVs detected[a]
Dois Portos (INIAV) CAN PRT051	Pinheira Branca, white grape SO4 CAN- vineyard 5	PB(3)	93/955	KM012056 to KM012060	GLRaV-3
	VinhãoDouro, red grape SO4 CAN- vineyard 5	VD(5)	93/955	KM012061 to KM012064	None
	Tempranilla Blanca, white grape SO4 CAN – vineyard 5	TB(1)	PN1, H4	PN1 – KM012065; H4 – KM012066 to KM012067	None
		TB(3)	H4	KM012068 to KM012070	GLRaV-1, -3, -5
	Tinta Carvalha, red grape SO4 CAN- vineyard 5	TC(3)	PN1	KM012071	GLRaV-1
	Brancelho, red grape SO4 CAN – vineyard 5	Br(3)	PN1	KM012072 to KM012075	GLRaV-1, -3
	Touriga Nacional, red grape SO4 CAN – vineyard 5	TN(3)	PN2	KM012076	GLRaV-3
	Mortágua, red grape SO4 CAN- vineyard 5	Mt(3)	PN3	KM012077 to KM012082	None
	São Saul, red grape SO4 CAN – vineyard 5	SS(3)	PN1	KM012083 to KM012084	GLRaV-1, -3
Dois Portos (INIAV)	Síria, white grape 1103 Paulsen CAN – vineyard 10	Sr(3)	PN1	KM012085	GLRaV-3
Merceana	Unknown, table grape SO4 Merceana- private vineyard	UvaMesa	PN1, PN2	PN1 – KM012086; PN2 – KM012087	None

(Continued).

Table 1. (Continued).

Location	Grapevine variety Rootstock/ Vineyard location	Isolate ID	GLRaV-2 Clade	GenBank Accession Number	Other GLRaVs detected[a]
Algarve	Unknown, white grape Unknown rootstock Mata Lobos – private vineyard I	MLI(2)	PN2, H4	PN2 – KM012088 to KM012089; H4 – KM012090	GLRaV-5
	Unknown, white grape Unknown rootstock Mata Lobos – private vineyard II	MLII(1)	PN1	KM012091 to KM012092	GLRaV-3
	Unknown, red grape Unknown rootstock Cerro do Guilhim – private vineyard	CG(3)	PN1	KM012093 to KM012094	GLRaV-3
	Unknown, white grape Unknown rootstock	G(1)	PN1	KM012095 to KM012100	GLRaV-3
	Gambelas – private vineyard	G(5)	PN1	KM012101 to KM012106	GLRaV-3
	Unknown, red grape Unknown rootstock	MA(2)	PN1	KM012107 to KM012108	None
	Moncarapacho– private vineyard	MA(3)	93/955	KM012109 to KM012110	GLRaV-3
	Unknown, red grape Unknown rootstock Bordeira – private vineyard	Vv(1)	PN1	KM012111 to KM012114	GLRaV-3

[a] Detection by first stand cDNA synthesis followed by PCR assays with virus specific primers (Esteves *et al.* ,2009a, 2009b; Teixeira Santos *et al.*, 2009a; Esteves *et al.* 2012; Esteves *et al.* ,2013)

tified 1103 Paulsen rootstock. Isolate UvaMesa was obtained from a commercial vineyard at Merceana, 10 km from Dois Portos.

The remaining eight isolates were collected from field-grown grapevines at various locations in the Algarve (Portugal), in private small-scale vineyards. These sampling sites are located, on average, 350 km from the CAN site. Isolates collected at Gambelas (G1 and G5) and Moncarapacho (MA2 and MA3) were obtained from clonal plants of a different unknown grapevine variety at each site. The maximum distance between sampling sites in the Algarve was 17 km (Gambelas–Moncarapacho), and the minimum was 5 km (Cerro do Guilhim-Mata-Lobos).

The presence of GLRaV-1, -3 and GLRaV-4 strain-5 in mixed infections with GLRaV-2 was detected by PCR using virus-specific primers (Esteves *et al*., 2009a, 2009b; Teixeira *et al*., 2009a) with each cDNA. Further details of each isolate are provided in Table 1.

RNA extraction

Total plant RNA was extracted with an E.Z.N.A.™ Plant RNA Kit (Omega Bio-Tek, USA), with the alterations to the manufacturer's protocol as reported previously (MacKenzie *et al*., 1997). Based on routine testing of the CAN plants (Teixeira *et al*., 2009b), and to ensure that the procedures produced uniform and comparable samples, the starting material for each isolate was obtained by pooling leaf veins and petioles from at least six fully expanded leaves from different branches of each plant for RNA extraction. Sampling took place in 2012, from the grapevine growth stages of pea-sized-berry to the onset of the véraison. Samples composed of 700 mg fresh leaf veins and petioles were ground in liquid nitrogen and extraction buffer (4 M guanidine thiocyanate, 0.2 M sodium acetate, pH 5.0, 25 mM EDTA, 2.5% PVP-40 [w/v] and 1% 2-mercaptoethanol [v/v]) was added at 1:3 (w/v). One mL of the homogenate was collected, and 100 µL of 20% sarcosyl was added. Samples were incubated at 70°C for 10 minutes with occasional shaking and then centrifuged at room temperature at 10 000 × g for 10 min. The kit manufacturer's instructions were followed from this point on. RNA extraction was conducted in duplicate for each sample.

cDNA synthesis and PCR

Synthesis of cDNA using an *iScript* cDNA First Strand Synthesis Kit™ (Bio-Rad, USA) was achieved according to the manufacturer's protocol using 5 µL of total RNA.

PCR reactions were performed in a final volume of 50 µL with Pfu DNA polymerase (Thermo Fisher Scientific Inc., USA), using 3 µL of the first-strand reaction mixture. A primer pair was designed in this work (CP2-1: 5'-TCT AGG GAG GTA CTA AGC ACG-3' and CP2-2: 5'-GCT CAA CAC TAG CAT CA/T /CGA C/TT-3') based on GenBank accessions of complete genomes of previously reported variants from different phylogenetic lineages: AF039204, FJ43623, JQ771955, JX669644 and Y14131 for PN, NC007448 for 93/955, DQ286725 for BD, AF314061, JX513891 and EF012720 for RG and EF012721 for PV20. A 779 bp fragment was amplified, between nucleotides 14493 and 15271 of sequence NC007448, comprising the 3' end of P25 (CPm, ORF 5) and intergenic region (84 nt), the complete coding sequence of the CP (597 nt, ORF 6) and the 5' end of P19 (98 nt, ORF 7). PCR amplifications included an initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Electrophoretic analysis of PCR products were done using 1.5% agarose gels stained with GreenSafe (NZYTech, Portugal). Fragments were visualized under UV light.

The above procedures were conducted separately for the two RNA extracts obtained for each sample. Amplicons of the CP gene obtained from the two extractions were combined before cloning.

Cloning and sequencing

Amplicons of the CP gene obtained from each isolate were ligated using a CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific Inc., USA) and used to transform *E. coli* XL1Blue (Agilent Technologies Inc., USA) competent cells. The PCR products of at least 16 positive clones per isolate were subsequently analysed by SSCP (single-strand conformation polymorphism) (Orita *et al*., 1989) to identify different DNA conformational patterns. For each SSCP pattern detected, at least two recombinant clones (purified plasmid with insert) were obtained using the NZY-Miniprep kit (NZYTech, Portugal), and the insert size was verified by PCR amplification with the respective primer pairs, prior to commercial sequencing (STAB VIDA, Portugal).

The different sequence variants obtained per isolate were deposited at the GenBank database, and their accession numbers are listed in Table 1.

Sequence data analysis

The sequences obtained for each isolate under study were initially aligned using BioEdit Sequence Alignment Editor (BioEdit) (Hall, 1999) and visually screened in order to exclude repeated sequences within isolate. All homologous sequences were retrieved from GenBank. Sequences were processed with BioEdit, aligned with ClustalW (Thompson *et al*., 1994), and the alignments corrected by hand. Incomplete sequences were trimmed from the alignment except those that had been published previously as reference for phylogenetic groups. A final dataset with 107 sequences was obtained, which included 59 new complete sequences from this work.

The graphic views of the nucleotide sequences alignment and of the corresponding deduced AA sequences alignment, as well as the Hopp and Woods (Hopp and Woods, 1981) hydrophilicity profile for the CP, were obtained using BioEdit. Phylogenetic trees were constructed using MEGA5 (Tamura *et al*., 2011). Phylogeny was inferred using the Minimum Evolution method implemented in MEGA5. Bootstrap values were estimated with 1000 replicates, and the tree with the highest log likelihood is presented.

RT-PCR based RFLP typing assay

The alignment of the nucleotide sequences was used to detect unique recognition sites of restriction enzymes for each phylogenetic group inferred, using BioEdit. CP-specific amplicons from the different isolates analysed in this work, obtained previously by RT followed by PCR, were then subjected to the typing assay using FastDigest (Thermo Fisher Scientific, Inc., USA) restriction enzymes PdmI and BamHI in double digestion reaction, MseI, EcoRI and SspI in single digestion reaction, according to the manufacturer's instructions. Ten microliters of each amplicon were used per restriction reaction. The DNA fragments were resolved on 2% agarose gels stained with ethidium bromide and visualized under UV light.

Estimates of evolutionary divergence and selection pressure

Estimates of average evolutionary divergence were made using MEGA5 with specific substitution models. The rates of synonymous and non-synonymous substitutions per site were estimated by the algorithms for single likelihood counting (SLAC), fixed-effects likelihood (FEL), internal fixed-effects likelihood (IFEL) and random-effects likelihood (REL), using the HKY85 nucleotide substitution bias model (Hasegawa *et al*., 1985) from the Datamonkey webserver (Kosakovsky Pond and Frost, 2005) of the HyPhy package (Kosakovsky *et al*., 2005). Mean dN/dS values were calculated.

Evolutionary analyses included Tajima's D test of neutrality (Tajima, 1989) and the test of natural selection, G-test statistics, of the McDonald-Kreitman test (McDonald and Kreitman, 1991), both performed with DnaSP software v. 5.10.01 (Librado and Rozas, 2009). Tajima's D test was used to estimate the correlation between the number of segregating sites and the average nucleotide diversity. The McDonald-Kreitman test was used to determine the ratio of the number of polymorphic non-synonymous to synonymous differences to the ratio of the number of fixed non-synonymous to synonymous differences between GLRaV-2 isolates.

Recombination analyses

Detection of evidence of putative recombination events in the CP gene sequences was performed with the genetic algorithms for recombination detection (GARD) available on the Datamonkey webserver (Kosakovsky Pond *et al*., 2006) and also using the RDP v.3 alpha44 software (Heath *et al*., 2006) and associated programs package.

Results

Diversity of the capsid protein gene sequence variants within GLRaV-2 isolates

Primers CP2-1 and CP2-2 amplified a DNA fragment of approximately 779 bp, encompassing the entire sequence of the CP gene (597 nt) and including, respectively, 84 and 98 bp upstream and downstream of the CP. These primers were degenerate at variable sites, based on the alignment of available sequences, in order to allow amplification of all variant types known to date. The amplicons obtained were specific to GLRaV-2, based on cloning and sequencing as described below.

A total of 19 GLRaV-2 isolates were analysed in this study. Co-infections with other leafroll-associat-

ed viruses were detected in the majority of the isolates, the most common of which was with GLRaV-3 (Table 1).

Assessment of the molecular variability of GLRaV-2 present in each isolate was based on the CP gene. Cloning of the amplicons, followed by SSCP analysis and sequencing, revealed the presence of divergent sequence variants within the isolates, with the exception of isolates TC(3), TN(3), Mt(3) and Sr(3) from CAN (Table 1), for which only one sequence type was detected.

GLRaV-2 variants isolated from Portugal comprise five phylogroups

The phylogenetic relationships of GLRaV-2 isolates were assessed among themselves and with corresponding sequences from other grapevine growing regions. This analysis, using the minimum evolution (ME) method, included 59 sequences from this work and 48 available at GenBank obtained from other geographic regions. Global sequences of GLRaV-2 CP gene segregated into six major phylogenetic groups (Figure 1). At least one reference isolate from the phylogenetic lineages proposed previously (Meng *et al*., 2005; Fuchs *et al*., 2009; Bertazzon *et al*., 2010; Jarugula *et al*., 2010) could be found in each of the six lineages, and hence the standardized nomenclature was maintained. However, two of the major phylogroups were each composed of three subgroups: PN1, PN2 and PN3 in the case of the PN lineage and San, CS and CNP for the H4 lineage. Stability of the phylogenetic tree topology was supported by bootstrap values >75% at each node. In keeping with the nomenclature used for the PN lineage, we opted to name PN3 the new subgroup with the sequences from the Portuguese isolate Mt (3), although this designation had been used previously for the 93/955 type variants, found clustering in the PN lineage. (Bertazzon *et al*., 2010). However, in view of the evidence presented, no confusion should subsist. In turn we suggest the designation of CNP for the H4 new subgroup, based on the initials of the variants reported previously (Can, Ner and Pol).

Sequences obtained from the Portuguese isolates were thus placed in subgroups PN1, PN2 and PN3 of the PN lineage, in group 93/955 and in subgroup CNP of the H4 lineage. A great number of sequences (35/59) were found in the PN1 group, followed by the 93/955 group. Three of the isolates harboured a mixture of sequences: from groups PN1 and H4 [TB (1)], from groups PN1 and PN2 (UvaMesa), and from groups PN2 and H4 [ML1 (2)].

Genetic analysis of global phylogroups

Mean distance between the sequences retrieved from the Portuguese isolates was 0.056 ± 0.006 (data not shown on table), and a value of 0.101 ± 0.022 was estimated between sequences from global isolates. The population genetic parameters for each major phylogroup considered are listed in Table 2A. Haplotype diversity (Hd) values for CP sequences were close to 1.000. Values of within group evolutionary divergence over sequence pairs (d) ranged from 0.005 for PN1 to 0.052 for H4. Nucleotide polymorphisms in GLRaV-2 CP groups were evaluated using Tajima's D (Tajima, 1989) statistical test to assess the influence of demographic forces on each population. A negative D statistic can indicate a selective sweep and allelic fixation driven by natural selection. The significantly negative values of Tajima's D found for lineages PN (subgroups PN1 and PN2) and 93/955 discount the neutral hypothesis but suggest the occurrence of demographic expansion of GLRaV-2 populations in those groups. A positive D statistic, although not significant (P>0.1), was found in the case of the H4 and subgroup San. A positive D statistic is usually interpreted as indicating balancing selection but can also be obtained when variants from more than one population are sampled. The latter is probably the case here, as sequences in phylogroup San carry evidence of further population subdivision (Figure 1) and sequence H4 (AY697863) was retrieved from *V. rupestris* St. George.

Intergroup sequence divergence (Table 2B) between PN, 93/955 and H4 lineages were 0.081 to 0.122, and between BD, RG and PV20 were 0.221 to 0.286. High levels of divergence were also found when comparing PN, 93/955 and H4 with BD, RG and PV20 (0.232 to 0.274).

To assess if natural selection acting on the protein coding sequences could be responsible for the divergence between isolates, a McDonald-Kreitman (MK) test (McDonald and Kreitman, 1991) was performed. The MK test compares the levels of diversity at neutral sites and potentially functional sites with the respective levels of divergence, to evaluate whether neutral evolution can be rejected at the functional sites. Because only synonymous substitutions could be found between groups PN1, PN2 and PN3, the G

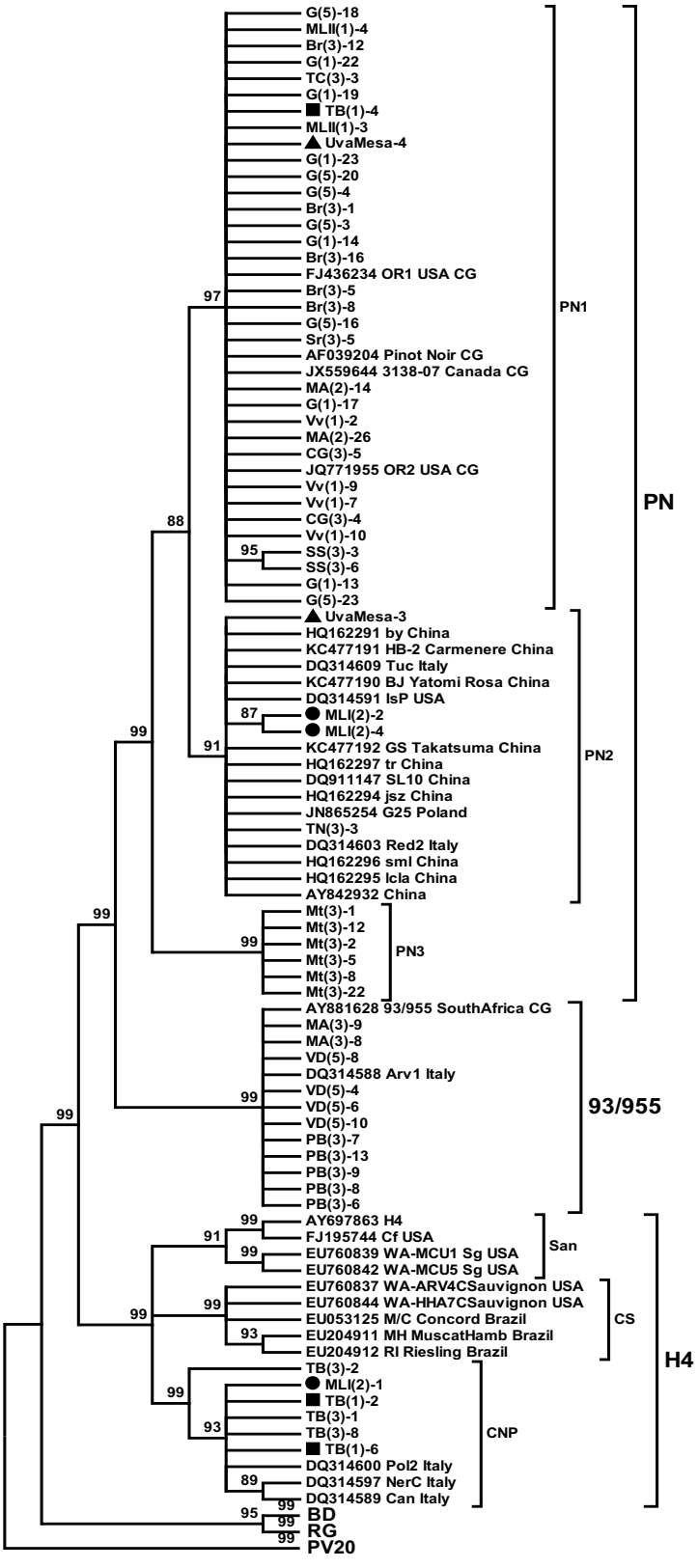


Figure 1. Evolutionary relationships among global sequence variants of the capsid protein gene of *Grapevine leafroll-associated virus 2* (GLRaV-2). The evolutionary history was inferred using the Minimum Evolution method, and the optimal tree with the sum of branch length = 1.30850251 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 107 nucleotide sequences. Capsid gene sequences retrieved from complete genome sequences are indicated with the suffix CG. Groups BD, RG and PV20 were collapsed for clarity. Matching black symbols indicate divergent sequence variants obtained from the same isolate.

Table 2. (A) Genetic distance within phylogroups and (B) differentiation between phylogroups of *Grapevine leafroll-associated virus 2*, and summary of test statistics examined for demographic trends.

(A) Genetic distance and test of neutrality within groups[a]

Phylogroup[b]	# variants	d[c]	S[c]	Hd[c]	Tajima's D[d]	*P* value
PN	61	0.014±0.002	59	0.912	-1.86639	$P<0.05$
PN1	37	0.005±0.001	35	0.858	-2.62468	$P<0.001$
PN2	18	0.008±0.001	19	0.848	-2.07886	$P<0.01$
PN3	6	0.006±0.001	11	1.000	-1.44477	$0.1>P>0.05$
93/955	13	0.007±0.002	20	0.990	-1.87631	$P<0.05$
H4	18	0.052±0.005	56	0.971	0.15168	$P>0.1$
San	4	0.048±0.008	27	0.833	1.94266	$P>0.1$
CS	5	0.002±0.001	1	1.000	ND	
CNP	9	0.008±0.002	14	1.000	-1.60365	$0.1>P>0.05$
BD	2	0.008±0.004	5	1.000	ND	
RG	11	0.037±0.004	68	1.000	-1.77595	$P<0.05$
PV20	2	0.051±0.008	30	1.000	ND	
All	107	0.099±0.005	202	0.957	-1.03801	$P>0.1$

[a] (A) Average evolutionary divergence analysis was conducted in MEGA5 using gene-specific substitution models (Tamura *et al.*, 2011), and codon positions included were 1st-2nd-3rd noncoding. Positions containing gaps and missing data were treated as a complete deletion.

[b] Phylogroups are as defined in Figure 2.

[c] d: Average evolutionary divergence; S: number of segregating sites; Hd: Haplotype diversity.
Results are based on the pairwise alignment of 107 CP gene sequences. Standard error estimates were obtained by a bootstrap procedure (1000 replicates).

[d] Tajima's D statistic as a measure of the departure from neutrality for all mutations in the genomic region (Tajima, 1989) was performed using the DnaSP software version 5.10.11 (Librado and Rozas, 2009). The D test is based on the differences between the number of segregating sites and the average number of nucleotide differences. The DnaSP software provides a measure of significance of the D value. Values for neutrality tests were not significant for phylogroups H4 and RG and when all sequences were considered. ND = not determined due to lack of sufficient data.

statistics associated with the MK test could not be determined. However, the Fisher's exact test (data not shown) revealed significant values between PN1 and PN3 (0.0016) and between PN2 and PN3 (0.0152). The G-test statistics obtained for all the other possible comparisons between phylogroups showed significant differences between the ratios of synonymous to replacement substitutions with good statistical support between PN and lineages BD and RG, and also between BD, RG or H4 and PV20 (G-test statistics and respective significance levels are shown in Table 2(B) upper diagonal). The three subgroups in PN and also phylogroup 93/955 all showed significant differences in relation to subgroups CS and CNP in lineage H4. These and the other significant comparisons found in Table 2(B) suggest that natural selection, instead of random processes like isolation or drift, are likely to be driving the evolutionary divergence between the groups concerned. However, the majority of the comparisons gave no significant G-test statistics. These results indicate that the ratio of fixed differences to polymorphisms is not significantly different between synonymous and non-synonymous changes, and, consequently, that random processes together with natural selection are likely to be driving the evolutionary divergence of those phylogroups. Such would be the cases of comparisons involving subgroup San, except with PV20.

Table 2. (A) Genetic distance within phylogroups and (B) differentiation between phylogroups of *Grapevine leafroll-associated virus 2*, and summary of test statistics examined for demographic trends.

(B) Differentiation between GLRaV-2 phylogroups and test of natural selection[a]

Phylogroup[b]	PN				93/955	H4				BD	RG	PV20
		PN1	PN2	PN3			San	CS	CNP			
PN	...	...	...	...	3.092ns	2.816ns	2.261ns	6.575*	8.536**	3.855*	4.363*	0.141ns
PN1	...	...	NP	NP	6.544*	3.564ns	2.871ns	9.644**	12.330***	5.539*	4.453*	0.026ns
PN2	...	0.023±0.005	...	NP	4.703*	1.258ns	0.389ns	2.550ns	10.230**	3.908*	1.197ns	0.729ns
PN3	...	0.035±0.007	0.032±0.006	...	7.485**	1.528ns	0.678ns	8.497**	11.857***	7.735**	1.026ns	0.174ns
93/955	0.081±0.010	0.081± 0.010	0.081±0.010	0.078±0.010	...	1.277ns	0.019ns	4.984*	9.499**	3.704ns	0.230ns	0.166ns
H4	0.117±0.011	0.117±0.011	0.114±0.011	0.123±0.011	0.122±0.010	...	...	...	...	0.271ns	0.390ns	4.269*
San	0.123±0.012	0.124±0.012	0.120±0.012	0.133±0.012	0.129±0.013	...	...	1.135ns	0.145ns	0.522ns	0.011ns	7.104**
CS	0.112±0.012	0.113±0.012	0.106±0.012	0.116±0.012	0.109±0.012	...	0.067±0.009	...	6.741**	0.371ns	0.238ns	6.151*
CNP	0.112±0.012	0.112±0.012	0.115±0.012	0.122±0.012	0.121±0.012	...	0.087±0.010	0.043±0.009	...	2.763ns	0.850ns	0.732ns
BD	0.251±0.016	0.252±0.016	0.249±0.016	0.245±0.016	0.232±0.016	0.241±0.015	0.238±0.015	0.231±0.016	0.244±0.016	...	3.989*	4.815*
RG	0.240±0.016	0.241±0.017	0.235±0.017	0.226±0.016	0.237±0.016	0.233±0.016	0.236±0.017	0.232±0.017	0.233±0.016	0.221±0.016	...	7.309**
PV20	0.274±0.017	0.273±0.018	0.273±0.017	0.280±0.017	0.271±0.017	0.265±0.017	0.278±0.017	0.260±0.017	0.260±0.017	0.286±0.017	0.282±0.017	...

[a] (B) Lower diagonal: values of comparisons between groups; upper diagonal: G-value of G statistics of the McDonald-Kreitman test. The G statistics of the McDonald-Kreitman test (MKT) was used to test for evidence that divergence in GLRaV-2 lineages is driven by natural selection: *** $P<0.001$; **$0.001<P<0.01$; * $0.01<P<0.05$; NP: the G test could not be performed because non-synonymous substitutions between groups =0; ns: not significant. MKT was performed using DnaSP software version 5.10.01 (Librado and Rozas, 2009).

[b] Phylogroups are as defined in Figure 2.

Selection constraints on the CP gene

The CP gene sequence dataset was analysed with the aid of the RDP v.3 alpha44 software and Datamonkey web interface (Kosakovsy Pond and Frost, 2005; Kosakovsy Pond *et al*., 2005; Kosakovsy Pond *et al*., 2006). No evidence of recombination was detected and the tree constructed by the web interface was used to compute mean dN/dS values in order to analyse the types of evolutionary forces at play. Overall values obtained (Table 3) considering all sequences in the alignment were less than for the sequences obtained in this work. The greater value was obtained for phylogroup 93/955. Nevertheless, the values found indicated that the CP gene is under purifying selection in each of the phylogroups considered.

Examination of the alignments of nucleotide and deduced amino acid sequences of the CP (data not shown) revealed that variants in each major phylogroup share a characteristic pattern of nucleotides or residues substitutions. However, these are mainly silent substitutions between phylogroups PN1, PN2 and PN3. For groups 93/955 and H4, non-synonymous substitutions accumulate mainly in the N-terminal half of the protein, whereas for the remaining groups those are concentrated between AA70 and AA130. The hidrophylicity profiles of GLRaV-2 CP variants (Figure 2) were not altered by the AA substitutions.

RT-PCR-based RFLP protocol for isolate typing

Following an *in silico* analysis of overall GLRaV-2 CP gene sequences alignment with the aid of BioEdit, five restriction enzymes were used to obtain RFLP profiles of the 19 isolates analysed in this study. A molecular typing assay based on RT followed by PCR-based RFLP was implemented for discrimination of variants from phylogroups PN1, PN2, PN3, 93/955 and H4, documented in the Portuguese isolates. Although the variants found in lineage H4 were all in subgroup CNP, it was not possible to set up an assay suitable to discriminate between these and variants in the other two subgroups.

The DNA fragments amplified with the primer pair designed in this work were each submitted to double digestion with PdmI and BamHI and to single digestions with MseI, EcoRI and SspI, and resolved by agarose gel electrophoresis. The restriction profiles observed confirmed the expected phylogroup-specific pattern of the fragments obtained, as shown in Figure 3. This figure also shows the typification results for isolates TB (1), UvaMesa and MLI (2), harbouring sequence variants from more than one phylogroup. For TB (1) the complete digestion using PdmI and BamHI reveals the 504 and 587 bp fragments identifying the presence respectively of PN1 and PN2. For UvaMesa and MLI

Table 3. Estimates of selection pressures acting on the CP gene of *Grapevine leafroll-associated virus 2*.

Population	Mean dN/dS				Selection pressure[c]			
	Log(L)	Mean	Positive N	(%)	Negative N	(%)	Neutral N	(%)
All[a]	-4184.26	0.133	1	0.2	120	20.2	473	79.6
All[b]	-1888.43	0.206	1	0.2	38	6.4	555	93.4
PN	-1587.74	0.328	1	0.2	23	3.9	570	95.9
93/955	-971.4	0.354	0	0	3	0.5	591	99.5
H4	-1276.78	0.104	0	0	29	5.6	565	94.4
RG	-1225.4	0.169	4	0.7	10	1.7	580	97.6

Mean dN/dS values <1 indicate negative or purifying selection, dn/dS values = 1 suggest neutral selection, and dN/dS values >1 indicate positive selection for each gene-specific dataset.

[a] Dataset represented by 107 sequences with 198 codons.

[b] Dataset represented by 59 sequences from this work with 198 codons.

[c] Positively or negatively selected sites are identified by at least one of the three selection pressure detection methods: single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL) and internal fixed effects likelihood (IFEL). SLAC is a counting method, while the other two are likelihood methods (Kosakovsky Pond and Frost, 2005).

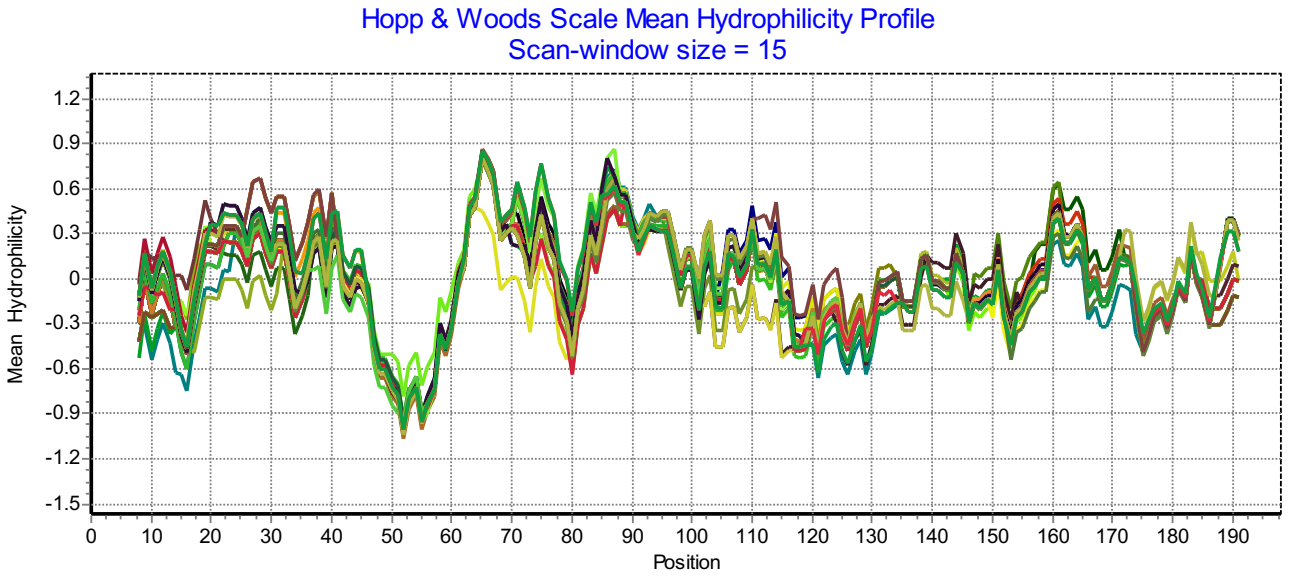


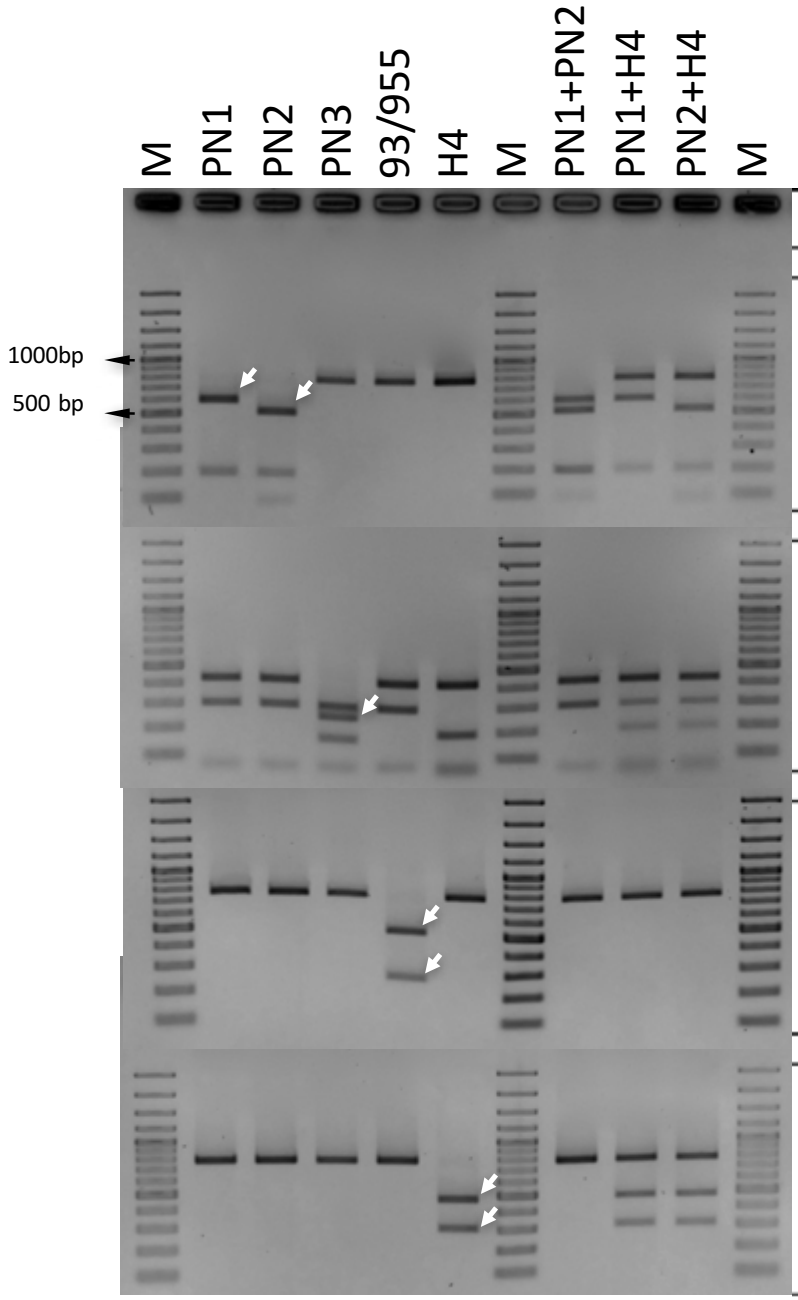
Figure 2. Hydrophilicity profile for the capsid protein of *Grapevine leafroll-associated virus 2* (GLRaV-2). Sequences from phylogroups in Figure 1 are shown. The average antigenicity values are plotted versus position along the amino acid sequence. The x-axis contains 198 increments, each representing an amino acid. The y-axis represents the range of hydrophilicity values (from 3 to -3.4) (Hopp and Woods, 1981).

(2), the presence of PN1 in the first and PN2 in the second is indicated by the 587 and the 504 bp fragments in the double digestion assays, whereas H4 is identified by the 306 and 473 bp fragments obtained by digesting with SspI. For both of these isolates the restriction is complete in the assay with MseI, producing the expected fragments, demonstrating that the presence of variants from another group was not being masked in the other incomplete restriction assays. In any case, these situations could be further confirmed by setting up double restrictions assays, respectively with PdmI + SspI and BamHI + SspI (data not shown).

Although we did not test this assay with isolates known to harbour variants from the BD, RG or PV20 groups, it is foreseeable that these will remain uncut since the available sequences lack any of the restriction sites at the positions indicated, exclusive to the restriction enzymes chosen. Considering that the typification results were congruent with the cloning and sequencing results, and that the primers used to obtain the amplicons were designed to cover variant diversity in the annealing regions, no variants from groups BD, RG and PV20 were detected in the Portuguese isolates.

Discussion

The molecular diversity of field isolates of GLRaV-2 in grapevine cultivars grown in Portugal were assessed relative to virus isolates from other grape growing regions. The comparative results show that the genetic diversity of this virus is great in Portugal, with the sequences we retrieved clustering in three of the major phylogroups, and also mixed infections from different lineages can occur in individual plants. These were found both in the CAN accessions and in plants from private vineyards. This is neither common nor expected for this virus, given the lack of evidence for a natural vector. It is commonly accepted that a bias toward purifying selection, i.e. selection that results in reduced frequency of new variants, purging deleterious variations that arise, should be detected in the CP region of insect-vectored viruses (Chare and Holmes, 2004; Rubio *et al*., 2001), through a low dN/dS value and abundance of negatively selected codon sites. Our analysis showed differences in the dN/dS values between the major phylogroups. However, a moderate to low percentage of negatively selected codon sites was generally detected, hence not revealing signs of selection constraints related to a biological vector. In



Phylogroup	RFLP assay/ Expected fragments (bp)
	PdmI + BamHI
PN1	192;**587**
PN2	83;192;**504**
PN3	779
93/955	779
H4	779
PN1;PN2	83;192;**504**;**587**
PN1;H4	192;587;779
PN2;H4	83;192;504;779
	MseI
PN1	62;295;422
PN2	62;295;422
PN3	62;170;**250**;295
93/955	62;295;422
H4	47;55;62;191;424
PN1;PN2	62;295;422
PN1;H4	47;55;62;191;295;422;424
PN2;H4	47;55;62;191;295;422;424
	EcoRI
PN1	779
PN2	779
PN3	779
93/955	**275**;**504**
H4	779
PN1;PN2	779
PN1;H4	779
PN2;H4	779
	SspI
PN1	779
PN2	779
PN3	779
93/955	779
H4	**306**;**473**
PN1;PN2	779
PN1;H4	306;473;779
PN2;H4	306;473;779

Figure 3. Reverse-transcription followed by polymerase chain reaction and restriction length polymorphism (RT-PCR-RFLP) analysis of capsid protein gene fragments obtained from isolates of *Grapevine leafroll-associated virus 2* (GLRaV-2), composed of variants from phylogroups PN1, PN2, PN3, 93/933 H4, and mixed infections with PN1+PN2, PN1+H4 and PN2+H4. DNA fragments were digested with enzymes **A** – PdmI and BamHI, **B**- MseI, **C**-EcoRI and **D** – SspI, and resolved on 2% agarose gel. Lane M shows GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bp (Thermo Scientific Inc., USA) for estimating the size of the DNA fragments obtained – the size of the bands corresponding to 500 and 1000 bp fragments of the markers are indicated to the left. The typification fragments for each phylogroup are indicated by arrows on the gel and also in bold in the table on the right.

this scenario, a possible explanation for the existence of GLRaV-2 as quasi-species may relate to the sanitary status of the initial source of scions and rootstocks. Other genes need to be analysed to provide complete understanding of the evolutionary pressures underlying the variability of GLRaV-2.

The number of major phylogroups and respective compositions suggested in our work does not agree with that found previously by Bertazzon *et al*. (2010). They described the phylogeny of GLRaV-2 based on 388 bp fragments of the CP, as composed of five phylogroups, positioning the 93/955 type variants as one of the three subgroups in PN lineage, designed as PN3. The phylogenetic analysis conducted by Jarugula *et al*. (2010), based on the HSP70h and CP genes, suggested the existence of six major lineages, recognizing the 93/955 type as a separate group and the H4 lineage, for which new sequences were available, as composed of the subgroups San and CS. However, these authors did not consider the seventeen CP gene sequences they analysed for the PN lineage, being segregated into subgroups.

In our study, phylogenetic inference was based solely on the CP gene, but the six major phylogroups obtained agree with the findings of Jarugula *et al*. (2010). However, we have considered the subdivision of PN into three groups, one of which (PN3) composed entirely of new sequence variants obtained from a single Portuguese isolate, and also the inclusion of a third subgroup (CNP) in lineage H4, based on existing evidence and sequences obtained in this study. This topology is supported by high bootstrap values (>75%). Examination of the genetic distance between groups revealed that these values are considerably greater than those within each phylogroup (Table 2), which further supports our phylogenetic inference.

As observed with GLRaV-2 previously (Jarugula *et al*., 2010), and usually reported for other grapevine GLRaVs (Alabi *et al*., 2011; Esteves *et al*., 2012; Esteves *et al*., 2013) and for Grapevine fanleaf virus (GFLV) (Vigne *et al*., 2004), clustering patterns of global variants do not seem to be related to geographical origin. This scenario, in which apparently geographically unrelated variants group together, is not strange for a long-lived crop such as grapevine. Worldwide exchange of propagating material between wine-producing countries has occurred repeatedly to the point of overcoming any natural dispersion boundaries, even when geographically distant regions are compared. In the present case, the PN lineage is the one for which more evidence is available and which is more diverse in terms of geographical attribution (Asia, Europe, North and South America). Bertazzon *et al*. (2010) attempted a description of the different pathological properties according to phylogenetic grouping. Based on the data they collected, it could be argued that the absence or attenuation of visual GLD symptoms may have contributed to the worldwide distribution of PN variants, whereas the more conspicuous effects produced by the other types (strong leafroll symptoms produced by 93/955 and H4 variants; graft incompatibility syndrome associated with RG variants) have caused a sustained selection against their distribution.

Given the knowledge that all recognized variant types are capable of inducing different degrees of damage and crop loss, the need for reliable virus detection and robust variant typification assays becomes evident. Bertazon and Angelini (2004) reviewed the efficiency of commercial DAS-ELISA kits available from different sources, and found conflicting results. Other detection assays have included RT-PCR, with a wide array of different primer pairs tested by different authors, and one-step real-time RT-PCR (Beuve *et al*., 2007). Typification based on phylogenetic inference, has relied on PCR-RFLP assays. These have targeted the PN, BD and RG lineages (Angelini *et al*., 2004; Jarugula *et al*., 2010) and also the H4-San and H4-CS subgroups (Angelini *et al*., 2004). Given the variability found in the Portuguese isolates, a RT followed by PCR-RFLP assay was set up to identify the presence of variants from lineages 93/955 and H4 and from subgroups PN1, PN2, PN3 of the PN lineage. Our results showed that even in the mixtures containing divergent variants it was possible to obtain a clear typification of the lineages present. This is also the first RFLP-based assay including the 93/955 lineage. Regarding subgroup PN3, only sequences from the Portuguese isolate Mt (3) are currently available. Our contribution complements the existing typification assays, in agreement with the updated phylogenetic inference of GLRaV-2 variants, and is suitable to be implemented in future certification programmes.

In conclusion, our study has shown that genetically distinct isolates of GLRaV-2 are present in Portugal, both in the CAN cultivars established for more than 30 years, and in field grown grapevines. The information gathered in this study extends the knowl-

edge on the diversity of this virus and provides a background for further analysis of the dynamics of evolution within the different lineages. The update of the global diversity and phylogeny of GLRaV-2 makes a contribution to advance molecular protocols for detection and typification of this virus, as shown by the proposed PCR-RFLP assay.

Acknowledgements

This research and Ms. Filipa Esteves (MSc.) were financed by research projects PTDC/AGR-AAM/65094/2006 and PTDC/AGR-ALI/109869/2009 from Fundação para a Ciência e Tecnologia, Portugal.

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Accepted for publication: December 1, 2015
Published online: May 14, 2016