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RESEARCH PAPERS

First detection of *Grapevine rupestris stem pitting-associated virus* and *Grapevine rupestris vein feathering virus*, and new phylogenetic groups for *Grapevine fleck virus* and *Hop stunt viroid* isolates, revealed from grapevine field surveys in Spain

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Summary. Evaluation of the prevalence of virus and viroid infections was conducted in a grapevine field collection in Valencia, Spain. Samples of autochthonous and traditional grapevine cultivars were collected during November 2011 and tested for the presence of fourteen viruses and five viroids, using RT-PCR. The prevalent viruses were *Grapevine rupestris stem pitting-associated virus* (GRSPaV: 49% infected samples) and *Grapevine leafroll-associated virus 2* (GLRaV-2: 15% of samples). GLRaV-1, GLRaV-3, GLRaV-4 (variants 4 and 5), *Grapevine fanleaf virus*, *Grapevine fleck virus* (GFkV), *Grapevine rupestris vein feathering virus* (GRVFV) and *Grapevine virus A* were also detected. *Hop stunt viroid* (HSVd: 92% of plants infected) and *Grapevine yellow speckle viroid 1* (6% of plants) were also detected. Mixed infections with two, and up to six different viruses and/or viroids were common. Only five samples (4%) were free from 19 pathogens tested. This is the first report of GLRaV-4 (variants 4 and 5) in the Valencia region of Spain, and the first record of GRSPaV and GRVFV in this country. Phylogenetic analyses performed with the sequences of these viruses showed that the Spanish isolates of GLRaV-4, GFkV and HSVd belong to new phylogenetic groups.

Key words: detection, reverse transcription-polymerase chain reaction, sequencing, phylogenetic analysis.

Introduction

The wine industry in Spain is very important, particularly due to the designation of origin classification given to local varieties. In the Valencia region, different red and white wines are produced, the local cultivars Bobal, Tempranillo, Garnacha, and the international cultivars Cabernet Sauvignon, Chardonnay, Pinot noir and Merlot. Cultivated grapevine area is 74,000 ha, so Valencia is one of the largest wine producing Spanish regions (MAGRAMA, 2013).

Viral diseases, including leafroll, infectious degeneration and rugose wood complex, are very important limiting factors for grape production. Previous reports have indicated the presence of *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 1, 2, 3, 4, 5, 9* (GLRaV-1, -2, -3, -4, -5, -9), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV), *Arabis mosaic virus* (ArMV), *Citrus exocortis viroid* (CEVd), *Grapevine yellow speckle viroid 1 and 2* (GYSVd-1, 2) and *Hop stunt viroid* (HSVd) in different regions of Spain (Flores *et al.*, 1985; Duran-Vila *et al.*, 1990; Zabalgogeazcoa *et al.*, 1997; Duque *et al.*, 2004; Velasco *et al.*, 2004; Abelleira *et al.*, 2010; Bertolini *et al.*, 2010; Cretazzo *et al.*, 2010; Padilla *et al.*, 2010a; 2010b; 2013). However, there is little informa-

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tion on the phytosanitary condition of the Valencian vineyards, although a few studies have reported the presence of GFLV, GFkV, GLRaV-1, 2, 3 (Bertolini *et al.*, 2010) and CEVd (Flores *et al.*, 1985).

In the present study, samples were collected in autumn of 2011 from vineyards of the 'Escuela de Viticultura y Enología' of Requena, that contain both autochthonous and international grapevine varieties. Vegetative material is frequently collected from this collection for multiplication by local producers. Varieties and rootstocks, used for wine and table grape production, were sampled and analyzed using specific RT-PCR protocols. Amplicons produced from positive plants were sequenced, aligned and characterized in order to obtain information about molecular clustering of the detected viruses and viroids. This study also provided relevant information useful for preservation of the local cultivars to be submitted to sanitation programs.

Materials and methods

Plant material

In November 2011, 127 grapevine samples, corresponding to 13 rootstocks and 48 varieties, including 15 Spanish autochthonous cultivars (Table 1), were collected from the vineyard collection of the 'Escuela de Viticultura y Enología' of Requena (Valencia, Spain). Samples consisted of mature canes kept up to 2 weeks after collection under controlled conditions of temperature (4ºC) and humidity (100%), before being processed for pathogen detection.

Total nucleic acids (TNA) extraction and RT-PCR analyses

For each sample, approx. 150 mg of phloem scrapings were ground in extraction buffer (guanidine thiocyanate 4.0 M, sodium acetate 0.2 M, EDTA 25 mM, potassium acetate 1 M, PVP 40 kdal 2.5% w/v and 3 mM β-mercaptoethanol) and processed according to the silica capture method (Malinovski, 1997; Rott and Jelkmann, 2001). Purified TNA was eluted in 150 µL RNase-free water. Ten microliters of TNA were denatured at 95ºC for 5 min, using random DNA hexanucleotides for priming (Roche), and reverse transcribed with *Moloney murine leukaemia virus* reverse transcriptase (M-MLV RT; Promega) at 42ºC for 60 min. Complementary DNA (cDNA) was stored at -20ºC until use. DNA amplification was performed in 30 µL reaction volume, using 3 µL of cDNA as template and 27 µL amplification mixture containing 0.2 mM of each d-NTP, 0.8 µM of each primer, 1.5 mM of $MgCl_2$, 1 U Taq DNA polymerase (Invitrogen, Sao Paulo, Brazil), 3 µL of supplied 10 × buffer and deionized sterile water. Target-specific primers are reported in Table 2, and amplification conditions were followed according authors' information. GLRaV-1 detection was performed using specific primers designed in this study, LR1-For (CGTTTGAAAATCCTATGCGTCA) and LR1-Rev (CATTACTTTTCCGCCCGA) amplifying 235 bp of a partial coat protein (CP) gene region. PCR conditions were 94ºC for 2 min, followed by 35 cycles of 94ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s, and a final extension of 72ºC for 7 min. Four additional primers were also designed: forward LRsF (GGYATGAACAARTTCAATGC), used in combination with 1:1 mixture of reverse primers LRsR1 (GCRGTCGGCTCGTTCAC) plus LRsR2 (GCTGTTGGTTCATTCAC) for detection of the GLRaV-4 variants 4, 5, 6 and 9, and with LR6R reverse primer (CAACAGCCTGAACCATCAC) for specific detection of variant 6. The expected amplified product was 312 bp for multi-detection of the GLRaV-4 variants, and 295 bp for specific GLRaV-6 detection. PCR cycling was 94ºC for 2 min, followed by 35 cycles of 94ºC for 30 s, 50ºC for 45 s, 72ºC for 45 s and a final extension of 72ºC for 7 min for both analyses.

Cloning, sequencing and phylogenetic analyses

Arbitrarily selected amplicons (Table 3) were purified and cloned in *Escherichia coli* DH5α strain, using the pGEM-T Easy system vector (PROMEGA). Putative recombinant clones were analyzed by colony-PCR using primers to vector sequences flanking the polylinker. Amplicons obtained from three colonies per cloned fragment were sequenced in both directions by Macrogen USA Corp.

Reference sequences from GenBank were used in alignment with CLUSTAL-W program inside BioEdit (Thompson *et al.*, 1997; Hall, 1999). Phylogenetic trees were constructed using the neighbour joining method, with 1,000 bootstrap replicates as statistic support of node separation in MEGA 4.0 environment (Tamura *et al.*, 2007), on the basis of partial gene sequences obtained from amplification. In the *Closteroviridae* family, two trees were constructed because of the different target genes used for detection.

Table 1. Number and rates of infected plants for each grapevine cultivar tested in Requena.

Cultivar	Infected / analyzed plants	Infection rate (%)	Cultivar	Infected / analyzed plants	Infection rate (%)
Asirtiko	1/1	100	Macabeo[a]	2/2	100
Barbera	1/1	100	Monastrell[a]	2/2	100
Bronx	1/1	100	Petit verdot	2/2	100
Cardinal	1/1	100	Pinot gris	2/2	100
Fiano	1/1	100	Riesling	2/2	100
Malvasia	1/1	100	Sauvignon blanc	2/2	100
Malvasia Candía	1/1	100	Sylvaner	2/2	100
María	1/1	100	Verdejo[a]	2/2	100
Marsanne	1/1	100	Vermentino[a]	1/2	50
Marselan	1/1	100	Victoria	2/2	100
Maturana[a]	1/1	100	Xarel·lo[a]	2/2	100
Merlot	1/1	100	Chenin blanc	3/3	100
Merseguera[a]	1/1	100	Crujidera[a]	3/3	100
Nebbiolo	1/1	100	Viognier	3/3	100
Parellada[a]	1/1	100	Chardonnay	4/4	100
Red Globe	1/1	100	Moscatel[a]	4/4	100
Rousanne	1/1	100	Cabernet Sauvignon	5/5	100
Sagrantino	1/1	100	Pinot noir	5/5	100
Semillon	1/1	100	Bobal[a]	6/6	100
Superior	1/1	100	Graciano[a]	6/6	100
Thompson Seedless	1/1	100	Syrah	6/6	100
Tintorera[a]	1/1	100	Garnacha[a]	9/9	100
Cabernet Franc	2/2	100	Tempranillo[a]	10/10	100
Gewürtraminer	2/2	100	Rootstocks	9/13	69
Italia	2/2	100	Overall infection[b]	122/127	96

[a] Spanish autochthonous grapevine cultivars.
[b] Total number of positive samples for at least one virus or viroid against all analyzed samples.

For viroid analyses, the phylogenetic trees were constructed using the complete sequence.

Results

All tested plants of the surveyed cultivars were infected by at least one virus or viroid, except cv. Vermentino (one infected out of two tested plants) and 101-14 (two plants), 110-R and 1103P rootstocks (nine infected out of 13 tested) (Table 1). HSVd was the most widespread pathogen, being present in 92% of the analyzed samples. Without considering HSVd presence, the incidence of infections with at least one other virus or viroid was 69%. The other viruses

Table 2. Primer pairs used in this study.

Virus	Target gene	Size (bp)	Reference
GLRaV-2	CP	514	Bertazzon and Angelini, 2004
GLRaV-3	HSP70	546	Boscia *et al.*, 2001
GLRaV-4			
variant 4	HSP70	321	Pei *et al.*, 2010
variant 5	CP	690	Good and Monis, 2001
variant 9	HSP70	393	Jarugula *et al.*, 2008
GLRaV-7	HSP70	507	Engel *et al.*, 2008
GVA	CP	432	Minafra and Hadidi, 1992
GVB	CP	155	Boscia *et al.*, 2001
GVD	CP	574	Osman and Rowhani, 2008
GFLV	CP	312	MacKenzie *et al.*, 1997
GFkV	RdRp	353	Shi *et al.*, 2000
GRVFV	RdRp	328	Al Rwahnih *et al.*, 2009
GRSPaV	CP	334	Osman and Rowhani, 2006
ArMV	CP	440	Nassuth *et al.*, 2000
GVCV	RdRp	530	Zhang *et al.*, 2011
CEVd	Complete genome	369	Eiras *et al.*, 2006
HSVd	Complete genome	300	Astruc *et al.*, 1996
AGVd	Complete genome	369	Elleuch *et al.*, 2002
GYSVd-1	Complete genome	220	Eiras *et al.*, 2006
GYSVd-2	Complete genome	363	Eiras *et al.*, 2006

and viroids detected included: GFLV, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 (variants 4 and 5), GVA, GFKV, GRSPaV, GRVFV, and GYSVd-1. HSVd, GRSPaV and GLRaV-2 showed the highest prevalence levels, followed by GFkV, GLRaV-3, GFLV, GRVFV, GYSVd-1, GLRaV-1, GLRaV-4 variant 5, GVA and GLRaV-4 variant 4 (Table 4).

In the case of GLRaV-4 variant 5, all isolates were detected using LRsF/LRsR1-LRsR2 universal primers (LRs) and primers previously described by Good and Monis (2001). Sequence analysis of the corresponding amplicons confirmed previous detection data and also showed the presence of a new variant of GLRaV-4 (named "Req") in the sample E26. This was detected using LRs universal primers, sharing 88% of nucleotide identity (94% of aminoacid identity) with a GLRaV-10 isolate from the Mavro variety from Cyprus (GenBank Acc. number FM244689). Single and mixed infections (two to six viruses and/or viroids) were present (Table 5). The prevalent mixed infections were GRSPaV + HSVd (20%) followed by GLRaV-2 + HSVd (5%). The most common mixed infections with three pathogens were GFkV + GRSPaV + HSVd (6%), followed by GLRaV-3 + GRSPaV + HSVd (4%).

Virus and viroid origin of amplicons was confirmed using BLAST tools, selecting one sequence per isolate, because no differences were observed among the three clones of the same sample. Selected sequences were deposited in GenBank (Table 3). Three sequences of GLRaV-1 had nucleotide identities of 94.9 to 99.6% with reference isolates, nine se-

Table 3. Spanish isolates used for phylogenetic analyses.

Virus or viroid	Isolate	Cultivar	Origin of plants	Other viruses and viroids in mixed infection	Symptomatology	Accession number
GLRaV-1	E41 LR1	Verdejo	Spain	GVA, GRSPaV, HSVd	Asymptomatic	KU884968
	E42 LR1	Moscato	Spain	GFkV, GRSPaV, HSVd	Asymptomatic	KU884969
	E115 LR1	Chenin blanc	Spain	GFLV, GRSPaV, HSVd	Asymptomatic	KU884970
GLRaV-2	E2 LR2	Crujidera	Spain	GRSPaV, GRVFV, HSVd	Asymptomatic	KJ466285
	E4 LR2	Bobal	Spain	GRSPaV, HSVd	Asymptomatic	KJ466289
	E24 LR2	Victoria	Spain	GLRaV-4 variant 5, GRSPaV, HSVd	Asymptomatic	KJ466286
	E26 LR2	María	Spain	GLRaV-4 variant Req, GFkV, GRSPaV, HSVd	Mosaic, yellowing of leaves	KJ466288
	E40 LR2	Chardonnay	Italy	GRSPaV, GYSVd-1, HSVd	Asymptomatic	KJ466290
	E52 LR2	Syrah	Italy	GLRaV-3, GFkV, GRSPaV, HSVd	Asymptomatic	KJ466291
	E54 LR2	C. Sauvignon	Italy	HSVd	Asymptomatic	KJ466292
	E57 LR2	S. blanc	Italy	GRSPaV, HSVd	Asymptomatic	KJ466293
	E112 LR2	Macabeo	Spain	GFLV, GRSPaV, HSVd	Mosaic	KJ466287
GLRaV-3	E37 LR3	Garnacha	Spain	GLRaV-2, GFkV, GRSPaV,GYSVd-1, HSVd	Leafrolling	KJ466294
	E39 LR3	Sagrantino	Italy	GFLV, GRSPaV, HSVd	Reddening of leaves, leafrolling	KJ466295
	E63 LR3	Graciano	Spain	GRSPaV, HSVd	Asymptomatic	KJ466296
	E81 LR3	Fiano	Italy	GFLV, HSVd	Leafrolling, mosaic	KJ466297
	E82 LR3	Vermentino	Italy	HSVd	Leafrolling	KJ466298
	E93 LR3	Chenin blanc	Italy	GRSPaV, HSVd	Asymptomatic	KJ466299
GLRaV-4						
variant 4	E28 LR4	Superior	Spain	GLRaV-4 variant 5, HSVd	Asymptomatic	KJ466300
variant 5	E22 LR5	Maturana	Spain	GRSPaV, HSVd	Leafrolling	KJ466301
	E23 LR5	Moscatel	Spain	HSVd	Yellowing of leaves	KJ466302
	E24 LR5	Victoria	Spain	GLRaV-2, GRSPaV, HSVd	Asymptomatic	KJ466303
	E28 LR5	Superior	Spain	GLRaV-4 variant 4, HSVd	Asymptomatic	KJ466304
variant Req	E26 LR-Req	María	Spain	GLRaV-2, GFkV, GRSPaV, HSVd	Mosaic, yellowing of leaves	KJ466305
GVA	E32 GVA	Italia	Spain	GYSVd-1, HSVd	Reddening of leaves	KJ466321
	E41 GVA	Verdejo	Spain	GRSPaV, HSVd	Asymptomatic	KJ466322
	E83 GVA	Malvasia Candía	Italy	GLRaV-2, HSVd	Asymptomatic	KJ466323

(Continued)

Table 3. (Continued).

Virus or viroid	Isolate	Cultivar	Origin of plants	Other viruses and viroids in mixed infection	Symptomatology	Accession number
GRSPaV	E2 RSP	Crujidera	Spain	GLRaV-2, GRVFV, HSVd	Asymptomatic	KJ466309
	E4 RSP	Bobal	Spain	GLRaV-2, HSVd	Asymptomatic	KJ466311
	E37 RSP	Garnacha	Spain	GLRaV-2,GLRaV-3,GFkV,GYSVd-1 HSVd	Leafrolling	KJ466310
	E46 RSP	Syrah	Italy	GLRaV-3, HSVd	Reddening of leaves, leafrolling	KJ466312
	E57 RSP	S. blanc	Italy	GLRaV-2, HSVd	Asymptomatic	KJ466313
	E78 RSP	Tempranillo	Spain	HSVd	Asymptomatic	KJ466314
	E98 RSP	Pinot noir	Italy	HSVd	Asymptomatic	KJ466315
	E118 RSP	Gewürztraminer	Spain	HSVd	Asymptomatic	KJ466306
	E120 RSP	Riesling	Spain	GFLV, HSVd	Asymptomatic	KJ466307
	E127 RSP	Syrah	Italy	HSVd	Reddening of leaves, stem pitting	KJ466308
GFLV	E39 GFLV	Sagrantino	Italy	GLRaV-3, GRSPaV, HSVd	Mosaic, reddening of leaves, leafrolling	KJ466284
	E109 GFLV	Garnacha	Spain	HSVd	Asymptomatic	KJ466279
	E110 GFLV	C. Sauvignon	Spain	HSVd	Mosaic	KJ466280
	E114 GFLV	Parellada	Spain	GRSPaV, GYSVd-1, HSVd	Asymptomatic	KJ466281
	E115 GFLV	Chenin blanc	Spain	GRSPaV, HSVd	Mosaic	KJ466282
	E122 GFLV	C. Franc	Spain	HSVd	Mosaic	KJ466283
GFkV	E3 GFkV	Crujidera	Spain	HSVd	Asymptomatic	KJ466275
	E26 GFkV	María	Spain	GLRaV-2, GLRaV-4 variant Req, GRSPaV, HSVd	Mosaic, yellowing of leaves	KJ466274
	E30 GFkV	Cardinal	Spain	GRSPaV, HSVd	Asymptomatic	KJ466276
	E52 GFkV	Syrah	Italy	GLRaV-2, GLRaV-3, GRSPaV, HSVd	Asymptomatic	KJ466277
	E95 GFkV	Bobal	Spain	GRSPaV, HSVd	Asymptomatic	KJ466278
GRVFV	E1 GRVFV	Crujidera	Spain	HSVd	Asymptomatic	KJ466316
	E36 GRVFV	Garnacha	Spain	HSVd	Asymptomatic	KJ466317
	E45 GRVFV	C.Sauvignon	Italy	GFkV, GRSPaV, HSVd	Asymptomatic	KJ466318
	E66 GRVFV	Graciano	Spain	HSVd	Asymptomatic	KJ466319
	E75 GRVFV	Tempranillo	Spain	HSVd	Asymptomatic	KJ466320
GYSVd-1	E27 YS1	Bronx	Spain	GRSPaV, HSVd	Reddening of leaves, leafrolling	KJ466324
	E40 YS1	Chardonnay	Italy	GLRaV-2, GRSPaV, HSVd	Asymptomatic	KJ466326

(Continued)

Table 3. (Continued).

Virus or viroid	Isolate	Cultivar	Origin of plants	Other viruses and viroids in mixed infection	Symptomatology	Accession number
	E114 YS1	Parellada	Spain	GFLV, GRSPaV, HSVd	Asymptomatic	KJ466325
HSVd	E3 HSVd	Crujidera	Spain	GFkV	Asymptomatic	KJ466329
	E4 HSVd	Bobal	Spain	GLRaV-2, GRSPaV	Asymptomatic	KJ466327
	E5 HSVd	Bobal	Spain	GRSPaV, GYSVd-1	Leafrolling	KJ466330
	E32 HSVd	Italia	Spain	GVA, GYSVd-1	Reddening of leaves	KJ466331
	E64 HSVd	Graciano	Spain	No	Asymptomatic	KJ466332
	E65 HSVd	Graciano	Spain	No	Asymptomatic	KJ466328
	E77 HSVd	Tempranillo	Spain	GRVFV	Asymptomatic	KJ466333

quences of GLRaV-2 had 71.5 to 99.8% identities, and four of GLRaV-4 variant 5, corresponding to a partial sequence of CP gene, had 91.7 to 95.5% identities with reference isolates. The partial sequences of the Heat Shock Protein 70 (HSP70) gene of six GLRaV-3 had of 92.4 to 99.4% identities with the reference strain, and one of the GLRaV-4 variant 4 isolates had 99.4% identity with the reference strain. GLRaV-4 variant Req (E26) showed the highest nucleotide identity level (88.7%) with the unique sequence available in GenBank of variant 10 (GenBank Acc. number NC011702). Six GFLV isolates on a partial sequence of CP gene showed nucleotide identities with reference strains ranging among 84.5 to 92.6%.

Aminoacidic sequence identity percentages were greater than those obtained with nucleotide sequences, but phylogenetic trees constructed with protein sequences had the same distribution of those obtained with nucleotide sequences (data not shown). The nucleotide identity comparison of the partial CP gene sequence of three GVA showed 75.0 to 92.2% similarity with reference strains, and for ten GRSPaV isolates was 87.5 to 100%. Amplification of a short region of the RNA-dependent RNA polymerase (RdRp) gene of five GFkV isolates had nucleotide identity with reference isolates of 80.2 to 98.2%, and for five GRVFV isolates this was 78.0 to 83.5%. For isolates where the complete genomes were compared with reference strains, HSVd showed 89.7 to 97.3% nucleotide similarity, and GYSVd-1 93.7 to 97%.

Phylogenetic analyses for GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-4 variant 5 showed their close relationship with previously reported strains. Valencian isolates of GLRaV-1 E41, E42 clustered in phylogenetic group 1, and E115 with reference strain BL4, belonging to phylogenetic group 3, according to the distribution proposed by Esteves *et al*., 2013. GLRaV-2 isolates E4, E24, E26, E40, E52, E54, E57 and E112 clustered with PN reference strain, while the E2 isolate clustered in 93/955 lineage. In the phylogenetic tree constructed using fragments of CP gene, four isolates of GLRaV-4 variant 5 clustered with reference isolate Y217 from France (GenBank Acc. number NC016081) (Figure 1a). The phylogenetic tree using the HSP70-like gene sequences showed a close relationship among isolates of GLRaV-4, -5, -6, -9, and 10 and Spanish isolates of GLRaV-4, variants 4 and Req. The unique reference sequences of GLRaV-4 variants 4 and 10 available from GenBank, are those included in the phylogenetic tree of Figure 1b. Spanish isolate E28 (variant 4) grouped with its corresponding reference isolate, and E26 (variant Req) grouped with variant 10 (Figure 1b).

The GLRaV-3 isolates E39, E81, E82 and E93 clustered with the NY1 strain forming a closely related lineage, while isolates E37 and E63 clustered in the GP18 lineage.

GRSPaV isolates were clustered in three lineages according to the classification described by Alabi *et al*., (2010). Isolate E78 clustered in group GRSPaV I; isolates E2, E4, E37, E57, E98, E118 and E120 clus-

Table 4. Results of virus and viroid testing by RT-PCR.

Tested viruses or viroids	No. of infected/127 tested plants	Infection rate (%)
GLRaV-1	7	5.5
GLRaV-2	19	15.0
GLRaV-3	11	8.7
GLRaV-4		
variant 4	1	0.8
variant 5	4	3.1
variant 6	0	0.0
variant 9	0	0.0
variant Req	1	0.8
GLRaV-7	0	0.0
GVA	4	3.1
GVB	0	0.0
GVD	0	0.0
GFLV	10	7.9
GFkV	13	10.2
GRSPaV	62	48.8
GRVFV	9	7.1
ArMV	0	0.0
GVCV	0	0.0
GYSVd-1	8	6.3
GYSVd-2	0	0.0
CEVd	0	0.0
AGVd	0	0.0
HSVd	117	92.1
Overall infection[a]	122	96.1

[a] Total number of positive samples for at least one virus or viroid against all analyzed samples.

Table 5. Numbers and rates of single and mixed virus and viroid infections detected in grapevines in this study.

No. of viruses and/or viroids in mixed infections	No. of infected plants	Infection rate (%)
1	36	28.3
2	47	37.0
3	25	19.7
4	11	8.7
5	2	1.6
6	1	0.8

tered in the SG1 lineage, and isolate E46 clustered in the BS lineage. Isolate E127 was separated from known groups, out of the BS reference group cluster and more closely related with the Syrah reference isolate from USA. Among GVA isolates, E32, E41, and E83 clustered in group I, according to the classification proposed by Goszczynski and Jooste (2003) (Figure 1c), and very distant from mild isolates of GVA in group III, with values of nucleotide identity near 75.0% (Figure 1c).

GFLV isolates E39, E109, E110, E114, E115, and E122 were grouped in the same cluster associated with reference isolates from France (data not shown).

Viruses belonging to the *Tymoviridae* family, GFkV and GRVFV, clustered, respectively, with reference strains of the *Maculavirus* and *Marafivirus* genera. GFkV isolates E3, E26, E30, and E95 grouped together, separately from reference strains, and isolate E52 clustered with L630 isolate from China (Figure 1d).

Valencian isolates of HSVd grouped in one same cluster, named Valencia-g, separated from the two known groups that included grapevine isolates, sharing the same root with *Prunus* isolates (Figure 2a). GYSVd-1 isolates clustered in two groups: E114 was associated with group III, according to the classification proposed by Szychowski *et al.*, (1998). E27 and E40 isolates grouped together in a separate cluster sharing the same root as group III GYSVd-1 isolates (Figure 2b).

Discussion

Previous studies in the Valencia region (Alicante province) have reported the presence of six viruses in grapevines, with detection rates of 99% for GVA followed by 95% for GLRaV-3, 65% for GFkV, 62% for GLRaV-1, 47% for GFLV and 47% for GVB. The present study had shown large differences in the detection rates of the same viruses. This may be explained by the different method used for detection: RT-PCR assays were used here, while in pre-

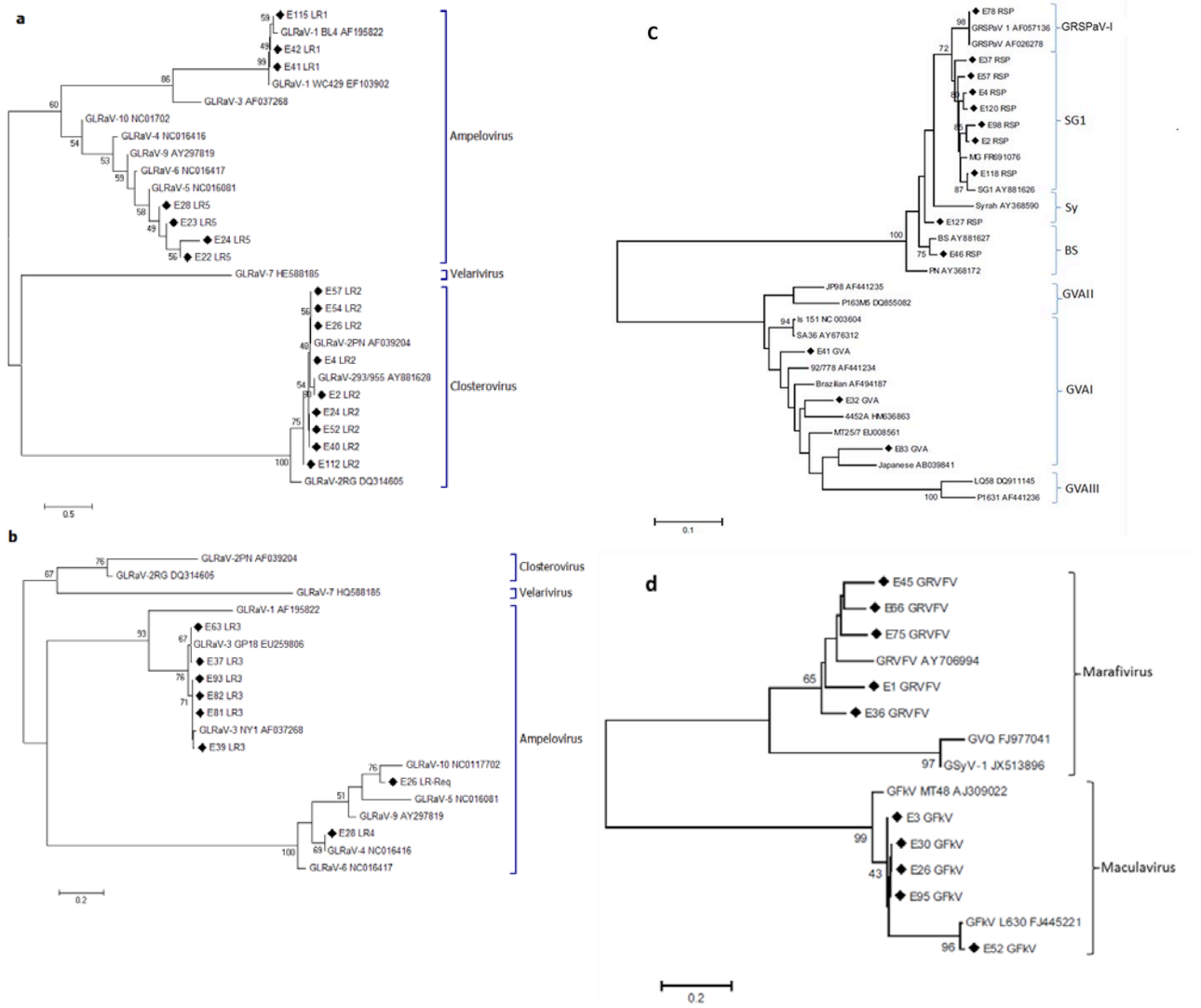

Figure 1. Neighbour-joining phylogenetic analysis of partial nucleotide sequences of viral isolates. **a:** CP gene analysis of *Closteroviridae* family viruses including GLRaV-1 (235 bp), GLRaV-2 (509 bp) and GLRaV-4 variant 5 (LR5) (534 bp) Spanish isolates; **b:** HSP70 gene analysis of *Closteroviridae* family viruses including GLRaV-3 (501 bp), GLRaV-4 variant 4 (321 bp) and variant Req (312 bp) Spanish isolates; **c:** CP gene analysis of *Betaflexiviridae* family viruses including GRSPaV (330 bp) and GVA (396 bp) Spanish isolates; **d:** RdRp gene analysis of *Tymoviridae* family viruses including GFkV (283 bp) and GRVFV (280 bp) Spanish isolates. Numbers at nodes indicate bootstrap values of 1,000 replicates. Information about reference virus isolates used to determine phylogenetic relationships is achievable by the corresponding accession number. Spanish isolates sequenced for this study are marked with ♦ and GenBank accession numbers are listed in Table 3.

vious tests quantitative RT-PCR was used (Bertolini *et al.*, 2009; 2010). Nevertheless, prevalence of viral infections does not differ substantially from the results obtained in the Atacama region in Chile (Fiore *et al.*, 2011). To our knowledge this is the first report of GLRaV-4 variants 4 and 5 in the Valencia region, and the first record in Spain of GRSPaV and GRVFV. HSVd and GYSVd-1 were previously reported in the Valencia region, but there is no information about infection rates (Duran-Vila *et al.*, 1990). The presence of viruses and viroids was determined in grapevine plants with and without symptoms, while specific

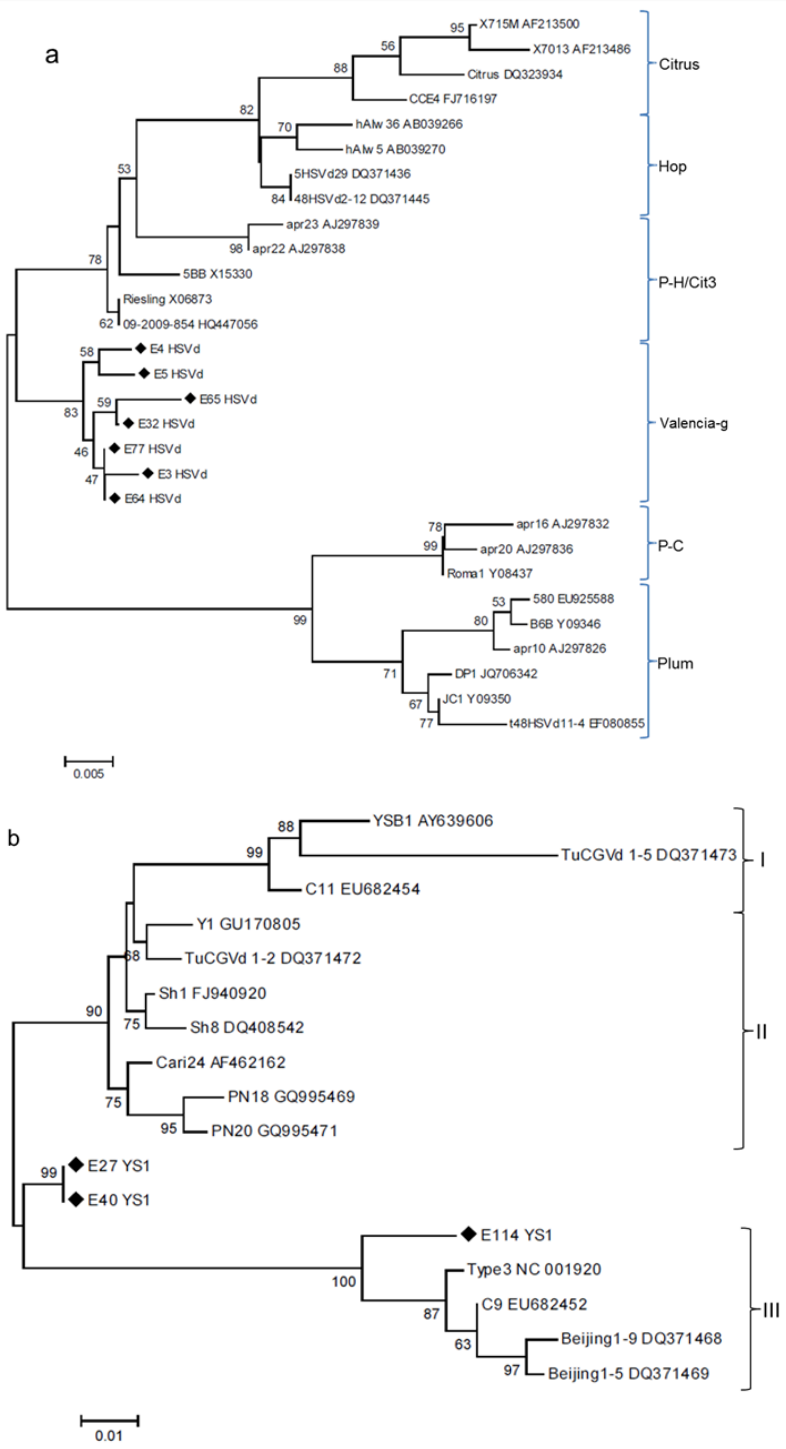

Figure 2. Neighbour-joining phylogenetic analysis of complete nucleotide sequences of viroid isolates. **a:** Phylogenetic analysis of HSVd Spanish isolates (300 bp); **b:** Phylogenetic analysis of GYSVd-1 Spanish isolates (367 bp). Numbers at nodes indicate bootstrap values of 1,000 replicates. Information about reference viroid isolates used to determine phylogenetic relationships is achievable by the corresponding accession number for GYSVd-1, and by Amari *et al.*, 2001 for HSVd. Spanish isolates sequenced for this study are marked with ♦ and GenBank accession numbers are listed in Table 3

disease symptoms related to particular cultivars was not observed.

Sequence analyses confirmed the results of RT-PCR detection in all the cases. The phylogenetic results presented here are based on analyses of a small portion of the virus genomes, and the possibility obtaining different topologies using the full virus genomes cannot be discarded. Regarding the Spanish GLRaV-1 isolates, they belong to the groups 1 (the worldwide prevalent group) and 3, which includes isolates from throughout the world (Esteves *et al.*, 2013). Most of the GLRaV-2 isolates clustered in the PN lineage, as reported for the majority of the worldwide isolates. The exception was the E2 isolate, which was associated with the reference strain from South Africa.

GLRaV-3 isolates were in two lineages from USA and South Africa. In both cases, the relationships with the reference strains were not associated with geographic origin of the plants nor with the symptoms observed (Martelli *et al.*, 2012; Maree *et al.*, 2013). Despite the distribution of GFLV isolates in two monophyletic groups, bootstrap values were very low, generating a polytomy with all GFLV isolates when ArMV was used as the outgroup. This supports previous reports of high stability of the GFLV CP gene sequence, mainly related with the high selective pressure exerted on this gene (Mekuria *et al.*, 2009; Oliver *et al.*, 2010).

GRSPaV distribution showed high heterogeneity, with seven isolates clustering with SG1 group, one isolate with the GRSPaV I type strain group and one isolate with the BS group. Isolate E127, which clustered between Syrah and BS reference strains, was obtained from a declining cv. Syrah plant, and is probably associated with the Syrah reference strain. This is also supported by the topology of the tree, that showed the same lineage distribution obtained when complete CP gene and a partial region of RdRp helicase subunit gene were used (Lima *et al.*, 2006; Alabi *et al.*, 2010). However, this approximation should be further clarified considering larger genomic regions for alignments.

For the GFkV isolates E3, E26, E30 and E95, despite being grouped in the same cluster as isolate L630, the genetic distances greater than 8.0% suggest differentiation of Spanish isolates into a new group, different to those previously reported (Glasa *et al.*, 2011).

GRVFV was mainly detected in local varieties Crujidera, Garnacha, Graciano and Tempranillo, using previously reported primers (Al Rwahnih *et al.*, 2009). Even though phylogenetic distribution associated the isolates with the unique reference of GRVFV, the low percentage of identity of isolates E1 and E36 (near 78%), suggest the presence of variants of the same viral species.

Regarding viroid analyses, both HSVd and GYSVd-1 were previously reported in grapevine in Spain, but no genetic studies had been performed. Considering the high rate of detection of HSVd, similar to that described for this viroid in apricot trees (81%) (Cañizares *et al.*, 1998), and in grapevines growing in Italy in commercial vineyards and germplasm collections (100%) (Gambino *et al.*, 2014), randomly selected isolates were used for phylogenetic analysis. According to phylogenetic classification proposed by Amari *et al.*, (2001), all Valencian isolates obtained in the present study grouped together in a new cluster (Valencia-g). This opens the possibility of a common origin (in this case Valencia region) or a common host (grapevine). Future more extensive studies of different isolates of HSVd of grapevine may give information related to the real situation, and also determine if recombination events among isolates from different hosts has occurred, as previously reported in *Prunus* HSVd isolates (Amari *et al.*, 2001; Pallás *et al.*, 2003; Mandic *et al.*, 2008).

GYSVd-1 isolates have been classified in three major groups by Szychowski *et al.*, (1998). Of these, groups II and III were associated to symptomatic grapevines. One of the viroid isolates (E114), detected in the autochthonous cultivar Parellada, was strictly clustered in group III, although its host plant (also infected with GFLV) did not showed the typical "yellow speckle" symptom of GYSVd-1, or "vein banding", as observed in plants infected by GYSVd and GFLV (Szychowski *et al.*, 1995). This may be explained by the role played by the grapevine variety in symptom development. The other two sequenced isolates grouped together, sharing the same root with type III GYSVd-1 group, but genetically distant. This result suggests the possibility of a geographic differentiation, unlike that proposed by Ward *et al.* (2011) and Jiang *et al.* (2012). No symptoms were observed in the plants infected with E40 and E114 isolates of GYSVd-1. The reddening and leafroll of the leaves observed on the plant infected by isolate E27 were probably induced by a virus not included in this study.

Adverse economic effects have not been associated with viroids in grapevines (Krake *et al.*, 1999).

However, Kawaguchi-Ito *et al.* (2009) demonstrated that cultivated grapevines represent a symptomless reservoir for the transmission of HSVd to other crops (hop in this case). In the Valencian region, vineyards overlap with large extensions of almond crops, which have been shown to host HSVd (Cañizares *et al.*, 1999). In addition, a recent phylogenetic analysis of Chinese HSVd isolates suggests possible cross transmission between grapevine and stone fruit hosts (Zhang *et al.*, 2012). In any case, although apparently single viroid infections do not significantly affect grapevines, mixed infections could trigger synergistic effects having significant economic impacts. Mixed infections of GYSVd with HSVd may alter grape juice pH and reduce vegetative growth, or GYSVd with GFLV may trigger vein banding disease (Szychowski *et al.*, 1995). The high rate of co-infection with viruses and/or viroids in grapevines in Requena, together with the observation that GYSVd-1 and HSVd could be transmitted by grapevine seeds (Wan Chow Wah and Symons, 1999), indicate that propagation protocols should be applied, to avoid the spread of viral and viroid diseases. It is advisable that sanitation by thermotherapy associated with *in vitro* culture of meristems should be used, followed by strict control of the virus- and viroid-free clones produced.

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