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Genetic diversity of GRSPaV-associated virus in Algeria

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Summary. Grapevine rupestris stem pitting-associated virus (GRSPaV; *Foveavirus rupestris*, *Betaflexiviridae*) is the most widespread grapevine virus in most viticulture regions, and this virus has high genetic variability. Genetic diversity of GRSPaV in Algeria was examined, based on the complete capsid protein (CP) gene. Similarity proportions between the CP sequences varied from 76% to 99%. The complete coding sequence of Algerian GRSPaV isolate ALG99 (8,646 nt) was determined using high-throughput sequencing and bioinformatic analyses. Algerian GRSPaV isolates clustered into four groups, with most sequences in groups III and IV. This research is the first to determine the genetic variability of GRSPaV in Algeria.

Keywords. GRSPaV, high throughput sequencing, RT-PCR.

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV; *Foveavirus rupestris*, *Betaflexiviridae*) (Martelli and Jelkmann, 1998; Petrovic *et al.*, 2003) is widespread in many wine-producing countries (Martelli *et al.*, 1997; Meng and Gonsalves, 2007; Morelli et *al.*, 2011). GRSPaV is known to be associated with rupestris stem pitting and vein necrosis in grapevine plants (Martelli, 2014), and the virus induces typical stem pitting symptoms on the indicator *Vitis rupestris* 'St. George' (Goszczynski and Jooste, 2003). However, definitive proof for the etiological role of this virus in stem pitting or vein necroses is lacking (Meng and Rowhani, 2017). No insect vectors have been related to the spread of GRSPaV, and its dissemination is explained by the exchange of infected material (Meng and Gonsalves, 2003).

GRSPaV particles are flexuous filaments particles of length approx. 723 nm. Each particle contains a positive-sense RNA molecule with a total of 8,725 nucleotides (nt) (Meng *et al*., 1998; Zhang *et al*., 1998; Petrovic *et al*., 2003). The GRSPaV genome contains five open reading frames (ORFs), and is believed to be capped at the 5' end and have a polyA tail at the 3' end (Meng and Gonsalves, 2007). ORF1 encodes a polyprotein with domains including a methyl transferase, a helicase, and an RNA-dependent RNA polymerase (RdRP) (Meng *et al*., 2013). ORF 2 encodes triple gene block protein 1 (TGBp1), ORF 3 triple gene block protein 2 (TGBp2), and ORF 4 encodes triple gene block protein 3 (TGBp3), which are involved in intra- and inter-cellular movement of the virus (Meng and Gonsalves, 2007; Rebelo *et al*., 2008). The product of ORF5 is the coat protein (CP), which is essential for virion formation (Nolasco *et al*., 2000; Minafra *et al*., 2000; Haviv *et al*., 2006; Meng *et al*., 2006; Meng and Li, 2010).

Considerable knowledge has been developed on this virus in the past two decades. There is compelling evidence that GRSPaV consists of a range of genetic variants that differ in their genome sequences. To date, the genomes of 15 isolates have been completely of partially sequenced, and eight clusters of viral variants have been identified through phylogenetic analyses (Meng and Rowhani, 2017). A comparison of the full-length genome sequences of four Slovakian GRSPaV isolates and 14 sequences retrieved from GenBank showed four main phylogenetic lineages within populations of the virus. Additionally, genetic recombination and mutations may have shaped GRSPaV evolutionary history contributing to its genetic diversity (Glasa *et al*., 2017).

No genetic diversity study of GRSPaV has been conducted in Algeria. Therefore, main objective of the present study was to examine the variability of GRSPaV in this country.

MATERIALS AND METHODS

Plant material

Ten infected grapevine accessions, including eight of table grape cultivars and two cultivars of wine grapes, were selected for this study (Table 1).

Table 1. List and identifiers of GRSPaV strains sequenced and analysed in the present study.

Cultivar type	Accession number	Strain	Grapevine Cultivar
Table grape	OU862947.1	DZ-GRSPaV-N9	Dattier
	OU862948.1	DZ-GRSPaV -N10	Cardinal
	OU862949.1	DZ- GRSPaV -N11	Cardinal
	OU862950.1	DZ-GRSPaV -N13	Cardinal
	OU862951.1	DZ-GRSPaV-N14	Dattier
	OU862952.1	DZ-GRSPaV-N15	Gros noir
	OU862953.1	DZ-GRSPaV-N16	Dattier
	PP976048.1	ALG99	Local
Wine grape	OU862954.1	DZ-GRSPaV-N17	Valensi
	OU862955.1	DZ-GRSPaV-N18	Carignan

Nucleic acid extractions

Total nucleic acids (TNA) were extracted from phloem tissues of the different grapevine accessions, and were purified using the procedure of Foissac *et al*. (2001). Selected samples were tested by reverse transcription polymerase chain reaction (RT-PCR) for GRSPaV, using the specific primers RSP52 and PSR53 (Rowhani *et al*. 2000) to amplify approx. 905 nts of the entire CP genes as well as 62 bp of the upstream CP and 63 bp of the downstream CP.

Reverse transcription and amplification

TNA (10 µL) of each sample was mixed with 1 µL random primers (1 µg μL^{-1}) and 1.5 µL sterile water, and was then denatured at 95°C for 5 min. Reverse transcription was carried out for 1 h at 39°C in a solution of 8 U μL^{-1} Moloney murine leukaemia virus reverse transcriptase (M-MLV RT; Invitrogen), 5 µL RT buffer (1×), 8 mM DTT, and 200 µM dNTPs, in a final volume of 25 µL with sterile distilled water. A volume of of the synthesized cDNA (2.5 µL) was used for PCR amplification, using a mixture of 1× Taq DNA polymerase buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 50 µM of each primer, and 0.05 U μL^{-1} of Taq DNA polymerase (Invitrogen Corporation), and adjusted to a final volume of 25 µL with sterile distilled water. PCRs each consisted of one cycle at 94°C for 5 min; followed by 35 cycles each of denaturation at 94°C for 35 s, annealing at 52°C for 45 s, and elongation at 72°C for 50 s; and a final extension step at 72°C for 7 min. The PCR products were analysed by electrophoresis in 1.2% agarose gel in 1× TBE buffer.

Sequencing and sequence analysis

The PCR products of ten GRSPaV isolates were purified using the ExoSAP-IT purification kit (Affym-

Table 2. Phylogenetic group GRSPaV reference sequences.

Accession number	Strain	Host plant	Origin	Phylogenetic group	Reference
KR054735.1	LSL	*Vitis vinifera* cv. Riesling	China	VII	(Hu *et al.*, 2015)
FJ943356.1	ORPN24b	*V. vinifera*	USA	VI	(Alabi *et al.*, 2010)
LT855243.1	AV99	Autochtones accession AV99	Tunisia	VIII	(Selmi *et al.*, 2020)
FR691076.1	MG	*V. vinifera* cv. Moscato Giallo	Italy	II	(Morelli *et al.*, 2011)
AY368590.1	Strain_Syrah	*V. vinifera* cv. Syrah	USA	IV	(Lima *et al.*, 2006)
AY881627.1	BS	Hybrid Bertille Seyve 5563	USA	III	(Meng *et al.*, 2005)
AF057136.1	CG1	*V. rupestris* cv. St. George	USA	I	(Meng *et al.*, 2006)
AY368172.2	PN	*V. vinifera* cv. Pinot noir	USA	V	(Lima *et al.* 2009)

etrix). Direct sequencing was carried out in both directions using the same primers as those used for RT-PCR (above), on a 3734xl automated analyzer sequencer (Applied Biosystems). The obtained sequences were submitted to the European Nucleotide Archive (ENA) (Table 1). Comparison of sequences of the CP gene of the Algerian GRSPaV isolates obtained in the present study with those of the different phylogenetic groups available in GenBank (Table 2) was conducted using MEGA X version 10.2.2 software.

Total RNA extraction and Illumina platform sequencing

The infected leaf sample ALG99 was placed in an Eppendorf tube and immersed in RNA, and the resulting solution was sent to DNA-link Company, Republic of Korea. The RNeasy® Plant Mini Kit (QIAGEN) was used to extract RNA from plant samples according to manufacturer's instructions. A TruSeq total RNA library prep kit for total RNA sequencing was used by QIAGEN to prepare an Illumina-NGS library. Total RNA sequences were generated using NovaSeq6000, 2x101PE, (Platform: Novaseq6000; Application: WTS/mRNA), after RNA quality was checked with an Agilent 2100 Expert Bioanalyzer. The raw reads were then trimmed using Trimmomatic-0.39 and BBduk v 37.22 in Geneious Prime® 2024.0.5 (Kearse *et al.*, 2012; http://www.geneious.com).

Map to reference

Geneious RNA was used to map the RNASeq data (Sensitivity: Medium-Low) to the reference sequence. The RNA clean reads were mapped against suspected virus genomes using Geneious Prime® 2024.0.5 (Kearse *et al.*, 2012), and then consensus sequence was then extracted. All 5,040 plant virus sequences from GenBank were also concatenated into one representative sequence (76,145,671 nt), and this was mapped against the whole RNA reads. The outcomes were shown in a report that had a number of assembled reads, total used reads, coverage and pairwise similarity.

Phylogenetic analyses

To construct the phylogenetic tree, multiple alignments were constructed with sequences from retrieved from GenBank and originating from different world regions (www.cbi.nlm.nih.gov). Alignments of 662 nt for the CP gene were obtained using the Clustalw program implemented in MEGAX10 software (Kumar *et al.*, 2018). The nucleotide sequences of the GRSPaV strains were trimmed to obtain the best fits. Percent identity values were calculated using p-distance methods. A phylogenetic tree was constructed using the Neighbor Joining (NJ) method with 2-parameter Kimura (1,000 bootstrap).

RESULTS

Sequence analysis

The nucleotide-level comparison of the sequenced GRSPaV strains revealed sequence similarities ranging from 76 to 99%. Among the strains, DZ-GRSPaV-N18 had the highest similarity (99%) with the reference strain CG1. In contrast, the least similarity (76%), was observed between the reference sequences GRSPaV-

MG and GRSPaV-PN, and between GRSPaV-PN and GRSPaV-LSL.

The similarity proportions among the Algerian GRSPaV strains was considerable. Nucleotide comparison between the ten Algerian GRSPaV isolates showed similarities ranging from 79 to 97% (Table 1). Isolates DZ-GRSPaV-N9 and DZ-GRSPaV-N10 had the greatest similarity (97%), as did isolates DZ-GRSPaV-N13 and DZ-GRSPaV-N17. Isolates DZ-GRSPaV-N14 , DZ-GRSPaV-N16, DZ-GRSPaV-N18, and ALG99 showed similarities from 81 to 93%. Isolate ALG99 has 93% similarity with isolates DZ-GRSPaV-N15 and DZ-GRSPaV-N18, indicating genetic diversity among these isolates. Overall, these results highlight the considerable genetic diversity present among the ten Algerian GRSPaV strains.

The isolates DZ-GRSPaV-N14, DZ-GRSPaV-N9, DZ-GRSPaV-N15, and DZ-GRSPaV-N10 exhibited respective similarities of, respectively, 93, 94, 92, and 95% with the reference strain Syrah, suggesting low genetic variation and close phylogenetic relationship with each other. Compared with other reference strains, similarity percentages were 87 to 91% with GRSPaV-CG1, 81 to 82% with GRSPaV-MG, 79 to 81% with BS, 80 to 84% with GRSPaV-PN, and approx. 83 to 85% with ORPN24b, GRSPaV-LSL, and AV99. This indicates that although DZ-GRSPaV-N14, DZ-GRSPaV-N9, DZ-GRSPaV-N15, and DZ-GRSPaV-N10 has strong resemblance to Strain Syrah, they presented only moderate similarities with other reference strains, suggesting genetic diversity among these strains.

Isolates DZ-GRSPaV-N11, DZ-GRSPaV-N13 and DZ-GRSPaV-N17 showed similarities from 91 to 96%. Isolate DZ-GRSPaV-N17 and DZ-GRSPaV-N13 exhibited close similarity (96%) with the reference strain BS, indicating very low genetic variation. In contrast, DZ-GRSPaV-N11 had 91% similarity with BS, indicating strong resemblance but slightly greater variation. Compared to other reference strains, these strains had similarity identity percentages of 82 to 84% with GRSPaV-CG1, 84 to 86% with GRSPaV-MG, and 77 to 78% with Strain Syrah.

DZ-GRSPaV-N16 had 94% similarity with GRSPaV-MG, indicating a close relationship and low genetic variation between these two isolates. Compared to other reference strains, DZ-GRSPaV-N16 had 91% similarity with GRSPaV-CG1, 83% with BS, 85% with Strain Syrah, and approx. 83% with other strains (GRSPaV-PN, ORPN24b, GRSPaV-LSL, and AV99). These values suggest that DZ-GRSPaV-N16 shares a strong resemblance with GRSPaV-MG and GRSPaV-CG1, but moderate similarities with other strains, indicating genetic diversity from these other strains.

Isolate DZ-GRSPaV-N18 had close similarity (99%) with GRSPaV-CG1, indicating minimal genetic variation between these two strains. Compared to other reference strains, DZ-GRSPaV-N18 was similar to DZ-GRSPaV-N16, 91% similarity with GRSPaV-MG and approx. 83 to 84% for the other virus strains. This indicates that DZ-GRSPaV-N18 shared strong similarity with GRSPaV-CG1 and GRSPaV-MG, but moderate similarities with other strains, confirming genetic diversity among these strains.

The strain ALG99 had 93% similarity with DZ-GRSPaV-N11 and 94% with DZ-GRSPaV-N13, indicating strong genetic resemblance between these two strains. Compared to reference strains, ALG99 had 81% similarity with BS, 78% with GRSPaV-CG1, and 78% with GRSPaV-MG, indicating greater divergence from these strains. ALG99 was also 83% similar to GRSPaV-PN, 86% to ORPN24b, 77% to GRSPaV-LSL, and 83% similar to AV99. These results indicate that while ALG99 had moderate similarity with ORPN24b and AV99, and ALG99 had more pronounced genetic divergence from GRSPaV-PN and especially from GRSPaV-LSL. This suggests considerable genetic diversity among these virus strains.

Phylogenetic analyses

The phylogenetic analysis of the CP gene sequences of Algerian isolates of GRSPaV, compared to sequences from other countries, revealed nine distinct groups (Figure 1). Among these, four contained Algerian sequences: Group I with one sequence (DZ-GRSPaV-N18), Group II with one sequence (DZ-GRSPaV-N16), Group III with four sequences (DZ-GRSPaV-N17, DZ-GRSPaV-N13, DZ-GRSPaV-N11, and ALG99), and Group IV with four sequences (DZ-GRSPaV-N9, DZ-GRSPaV-N10, DZ-GRSPaV-N14, and DZ-GRSPaV-N15). This distribution showed that the Algerian sequences were primarily in Groups III and IV, which also exhibited intra-group diversity as well as varied genetic similarities with GRSPaV sequences from other countries.

The intra-group similarity proportions for the CP gene of the GRSPaV isolates indicated high genetic homogeneity within each group. The intra-group similarity was from 96 to 99%, while the inter-group proportions were from 81 to 90%.

The Groups I and VII isolates had the greatest intra-group similarity (99%), indicating low genetic variation within these groups. Group VI isolates had 97% similarity, also close intra-group relatedness. The isolates in Groups II, III, IV, and VIII had lower intra-group similarity percentages (95% for Group II, 94% for Groups III and IV, 96% for Group VIII). These results indicate moderate to

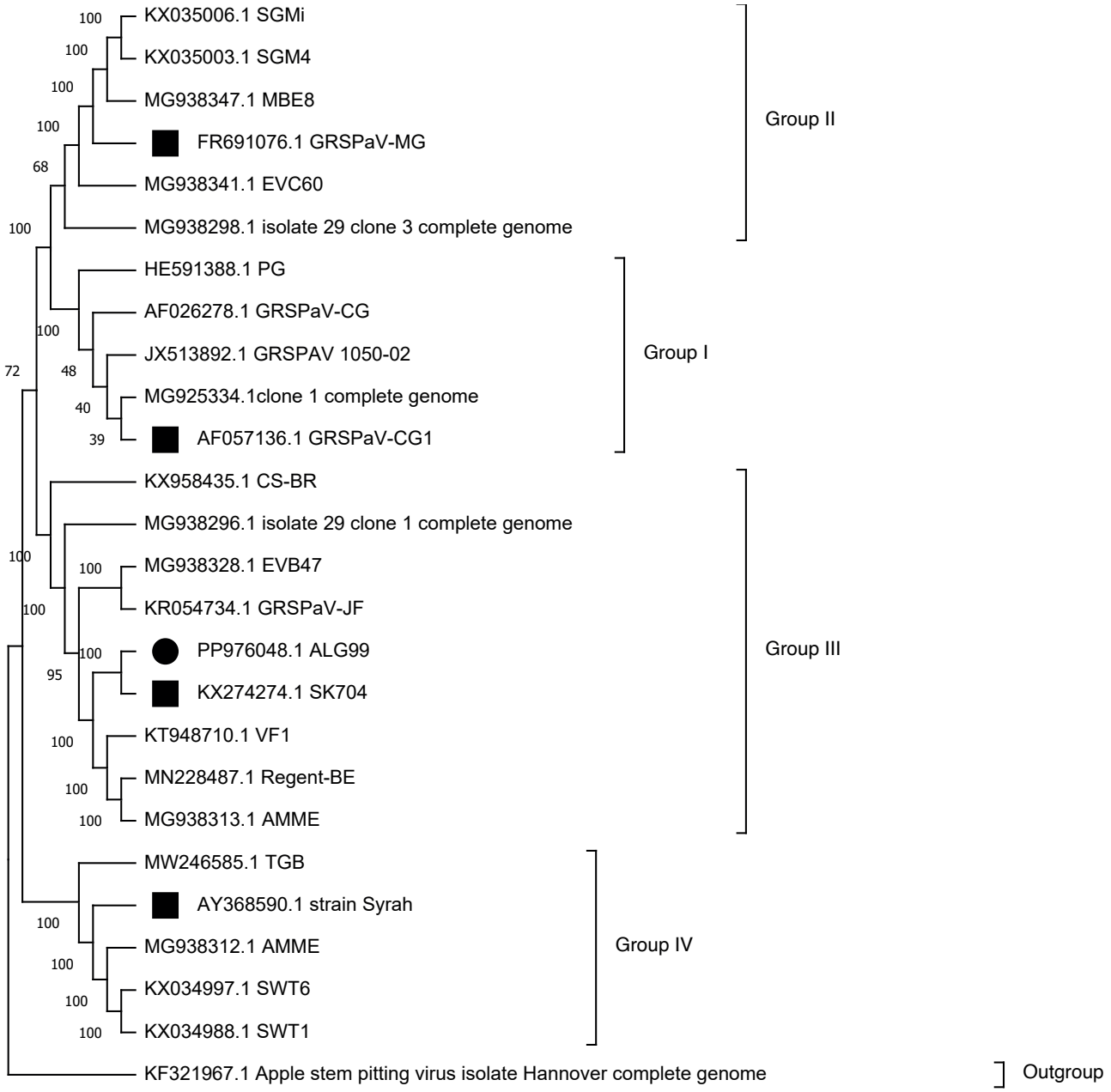

Figure 1. Phylogenetic tree of GRSPaV strains, constructed with sequences of a 662nt fragment of the viral capsid protein (CP) gene, obtained in this study and retrieved from GenBank, with percentage bootstrap support (≥50%) with 1,000 bootstrap replicates indicated on each tree branch. The CP gene of apple stem pitting virus strain KF321967.1 was used as the outgroup. ● indicates Algerian sequences, and ■ indicates the reference sequence for the group.

low genetic variation within these groups. These results indicate that some GRSPaV groups consisted of genetically similar strains, while others had greater genetic diversity.

The between group similarity analysis showed that the GRSPaV isolates in Groups I and II had 90.47% similarity, indicating close phylogenetic relationship. Similarly, Groups III and V, with 87.97% similarity, had strong resemblance. In contrast, most of the groups had moderate similarity proportions (80 to 85%) with other groups, such as Groups II and IV (84.52%), IV and VI (84.49%), III and VI (85.52%), and VII and IV (84.34%). Isolates in Group IX had similarities ranging from 41.53 to 43.00% with other Group isolates, indicating that the Group IX isolates were genetically distinct from the other groups.

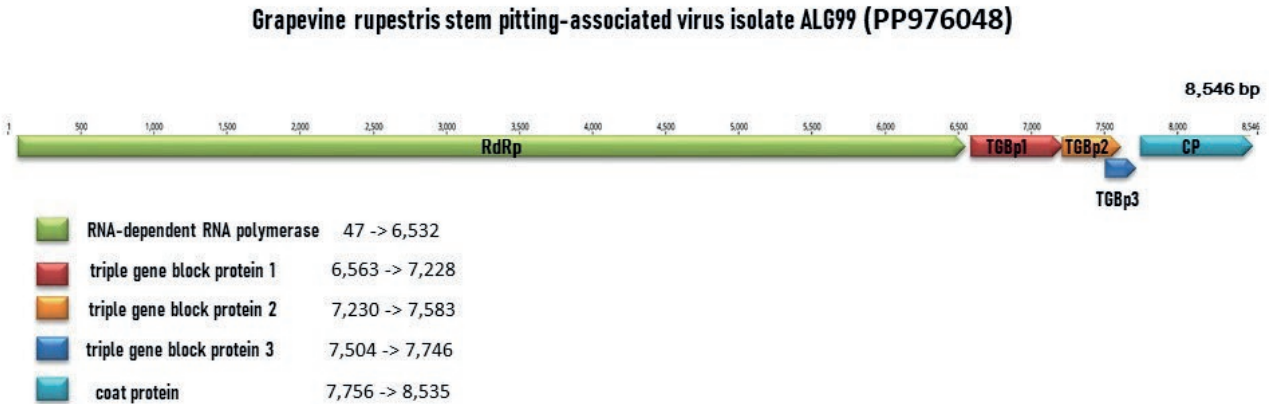

Figure 2. Genome organization of isolate ALG99 of grapevine rupestris stem pitting-associated virus. There are five open reading frames (ORFs) in the genome. ORF1 encodes RNA-dependent RNA polymerase protein (47 to 6,532 nt). ORF 2 represents triple gene block protein 1 (TGBp1) (6,563 to 7,228 nt). ORF3 encodes triple gene block protein 2 (TGBp2) (7,320 to 7,583 nt). ORF4 encodes triple gene block protein 3 (TGBp3) (7,504 to 7,746 nt). The last ORF represents the coat protein (7,756 to 8,535 nt).

Total RNAseq data generated by the Illumina platform included 47,034,642 short reads of 101 bases. In Geneious software, all RNA reads were paired and mapped against suspected virus and viroid genomes. The assembled reads were 8,484 against the sequence of GRSPaV and produced a consensus sequence of 8,546 bp. The coverage was 96.5% and the GC content was 43.2%. The sequence covering the whole coding regions was deposited in GenBank as accession number PP976048. The genome of GRSPaV isolate ALG99 had five open reading frames. ORF1 codes a RdRP with 6,486 nt and 2,161 residues. ORF2 represents triple gene block protein 1 (TGBp1), with 666 nt and 222 residues. ORF3 encodes triple gene block protein 2 (TGBp2), with 354 nt and 118 residues. ORF4 encodes triple gene block protein 3 (TGBp3), with 243 nt and 81 residues. The last ORF represents the coat protein gene, with 780 nt and 260 residues. The nucleotide alignment with the reference virus genome GRSPaV isolate 12G4102D (MZ484760) was 97.1%, while the amino acid alignments for each ORF were 98.4% for ORF1, 98.2% for ORF2, 99.1% for ORF3, 98.8% for ORF4 and 99.2% for the coat protein (Figure 2).

DISCUSSION

GRSPaV *is* the predominant grapevine virus reported in Algeria, with an infection rate of 57.92% (Bachir *et al.*, 2019). The prevalence of this virus in Algerian vineyards is not surprising, as it is a prevalent virus in many grapevine-producing countries (Martelli, 1993; Digiaro *et al.*, 1999; Meng and Gonsalves, 2007; Fiore *et al.*, 2016; Selmi *et al.*, 2017; Morelli *et al.*, 2011). The high incidence in the vineyards can mainly be attributed to dissemination through grapevine propagation material. Natural transmission of GRSPaV has not been described, although the virus has been reported with high incidence from most regions where grapevines are cultivated, and in wild grapevines in the Mediterranean basin (Pacifico *et al.*, 2016; Selmi *et al.*, 2020).

Examination of nucleotide relationships between the GRSPaV isolates for CP genes showed that similarities were from 76 to 99%. The Algerian strain DZ-GRSPaV-N18 exhibited the greatest similarity (99%) with the CG1 strain. The analysis showed that some strains are strongly related. For example, strains DZ-GRSPaV-N09, DZ-GRSPaV-N10, DZ-GRSPaV-N14, and DZ-GRSPaV-N15 had high nucleotide similarity but were different from strains DZ-GRSPaV-N11, DZ-GRSPaV-N13, and DZ-GRSPaV-N17, which had 92 to 97% similarity. The two strains DZ-GRSPaV-N16 and DZ-GRSPaV-N18 had low similarity with each other and with other Algerian strains. These results are consistent with those reported elsewhere. Selmi *et al.* (2020) showed high CP gene variability of Tunisian strains, with nucleotide similarity ranging from 71 to 100%. Hu *et al.* (2015) reported high variability of Chinese strains, with nucleotide similarity from 82 to 98%. Alabi *et al.* (2010) reported significant genetic variability in strains in the United States of America, for CP and RdRP genes of GRSPaV, with nucleotide similarities from 79 to 100%.

A phylogenetic study of the CP gene was carried out for the strains sequenced and for strains downloaded from GenBank. The phylogenetic tree constructed confirmed the results obtained during the nucleotide comparisons of the Algerian strains, and reveals the presence of nine distinct groups. The Algerian GRSPaV sequences

obtained from wine and table grape varieties clustered into four groups: I, II, III, and IV, with the majority (eight) of the sequences clustered in groups III and IV. The Selmi *et al.* (2020) phylogenetic analysis showed that the Tunisian GRSPaV sequences clustered into four main groups: I, II, III, and IV, with group III containing mainly strains from commercial grapevines. However, Selmi *et al.* (2020) showed the presence of two new groups consisting of Tunisian strains from spontaneous and autochthonous cultivated grapevines. This suggests that confined natural habitats could be sources of virus diversity.

Several studies have examined the distribution of GRSPaV variants. Meng *et al.*, (2006) reported that phylogenetic analyses based on the CP gene for strains from table and wine grapes in the United States of America, Canada, and Italy were in Groups I, II, III, and IV. They reported that 68% of GRSPaV were Group I and II variants, and 24% were Group III variants, and only 8% were Group IV variants. Hooker (2017) also reported that more than 50% of the variants in the United States of America clustered in Group IV, and 23% were in Group I.

These virus strain variations may be due to the broad commercialization of *Vitis* plant material infected with these variants. Further studies on the infection rates of autochthones and wild grapevine varieties are required to provide understanding of the distribution and evolution of GRSPaV in different regions of Algeria.

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