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Research Papers

Occurrence and genetic diversity of grapevine leafroll-associated virus 4 in Algeria

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Summary. Grapevine leafroll-associated virus 4 (GLRaV-4, *Ampelovirus tetravitis*) has considerable genetic diversity. A survey was conducted in central, western, and southern Algeria to investigate the distribution and the genetic diversity of GLRaV-4 in commercial and autochthonous grape cultivars. DAS-ELISA detected an overall grapevine infection rate of 18.2%, and infections in a subset of samples were confirmed by RT-PCR. Analysis of the P23 protein gene sequence revealed six known phylogenetic groups, with Algerian isolates clustering in strains -4, -5, -6, -9, and -Pr of GLRaV-4. In addition to this virus, three viruses and two viroids were identified using high throughput sequencing: grapevine Pinot gris virus (GPGV, *Trichovirus pinovitis*), grapevine leafroll-associated virus 2 (GLRaV-2, *Closterovirus vitis*), grapevine fanleaf virus (GFLV, *Nepovirus foliumflabelli*), hop stunt viroid (HSVd, *Hostuviroid impedihumuli*) and grapevine yellow speckle viroid 1 (GYSVd-1, *Apscaviroid alphaflavivitis*). This study was the first research on genetic diversity of GLRaV-4 in Algeria.

Keywords. GLRaV-4, RT-PCR, genetic diversity, P23, high throughput sequencing.

INTRODUCTION

Grapevine leafroll disease (GLD), one of the most economically important virus disease affecting grapevines, results from infection by one or more grapevine leafroll-associated viruses (GLRaVs), a group of genetically distinct species in the family *Closteroviridae* (Naidu *et al.*, 2015; Atallah *et al.*, 2012). Among these viruses, grapevine leafroll-associated virus 4 (GLRaV-4, *Ampelovirus tetravitis*) is a recognized species within *Ampelovirus*, which includes several genetically diverse variants known as GLRaV-4 Strains -4, -5,

-6, -9, -Car, -Pr, and -Ob (Martelli *et al*., 2012; Aboughanem-Sabanadzovic *et al*., 2017). GLRaV-4 and its strains are identified by positive RNA molecules of genome size between 13.6 and 13.8 kb, along with six open reading frames (ORFs), flanked by short 5′ or 3′ untranslated regions (UTRs). The virus sequence encodes six proteins (in the 5′ to 3′ direction): the replication-associated polyprotein (expressed via +1 ribosomal frameshift of two partially overlapping ORFs), a small hydrophobic protein (p5), heat shock protein 70 homologue (HSP70h), heat shock protein 90 homologue (HSP90h or p60), virus coat protein (CP), and a protein of unknown function with a molecular mass of 23K (p23). GLRaVs are known to spread through infected host planting material, and through grafting practices (Abou Ghanem-Sabanadzovic *et al*., 2012; Aboughanem-Sabanadzovic *et al*., 2017; Adiputra *et al*., 2019).

Members of *Ampelovirus* responsible for grapevine leafroll are primarily transmitted by specific hemipteran insect vectors, mainly mealybugs and soft scales (Herrbach *et al*., 2017). Additionally, several species of mealybugs and soft-scale insects have been identified as vectors of GLRaV-4, semi-persistently transmitting the virus within and between neighbouring vineyards (Naidu *et al*., 2015; Aboughanem-Sabanadzovic *et al*., 2017). In contrast to the severe symptoms typically associated with GLRaV-1, GLRaV-2, and GLRaV-3, infections by GLRaV-4 strains generally induce milder symptoms (Maree *et al*., 2013; Reynard *et al*., 2015; Abou Ghanem-Sabanadzovic *et al*., 2017).

High-throughput sequencing (HTS) technologies and bioinformatics have revolutionized identification of viral pathogens without knowledge of their primary structures (Maliogka *et al*., 2018). HTS has been instrumental for identifying and characterizing new grapevine virus species, the study of unknown diseases, identification of candidate disease-associated agents, and improvements to existing diagnostic assays for some viruses (Saldarelli *et al*., 2017). HTS has been recently applied to identify and characterize grapevine viruses in Algeria (Bachir *et al*., 2024; Laidoudi *et al*., 2025; Mahdid *et al*., 2025).

Few studies have focused on occurrence of grapevine leafroll disease in Algeria. While GLRaV-1, -2, and -3 have been investigated (Lehad *et al*., 2015a; 2015b; 2019), the present study focused on GLRaV-4 in this country. The main objective of this research was to determine occurrence and genetic diversity of the grapevine virus GLRaV-4 associated with grapevine leafroll disease in Algeria.

MATERIALS AND METHODS

Plant material

During the winter of 2021/2022, 444 grapevine samples were collected from the major grapevine growing regions of central, western, and southern Algeria (Figure 1). Cuttings from different grapevine cultivars were randomly collected from commercial cultivars (421 samples), and from 23 cultivars (23 samples) from the autochthonous grapevine collection at the Institut Technique de l'Arboriculture Fruitière et de la Vigne (ITAFV) (Supplementary Table 1). Additionally, an analysis of 122 grapevine samples (bark scrapings from mature canes) was included, that were randomly collected in 2012 and were provided by the plant protection laboratory of INRAT from vineyards in the wine-growing regions of Algeria (Supplementary Table 2).

DAS-ELISA

Preliminary detection of GLRaV-4 presence in the 444 collected grapevine samples was carried out using DAS-ELISA (Clark and Adams, 1977), employing a commercial kit (GLRaV-4 strains Complete kit 480, Bioreba AG). An extraction was carried out from phloem tissue of each field sample (bark scrapings from mature canes) following the kit manufacturer's instructions, using the provided PBS grinding buffer. The absorbance was measured at wavelength 405 nm using a microplate spectrophotometer (SunriseTM absorbance microplate reader, Tecan Trading AG). Two positive controls and two negative controls supplied by Bioreba AG were used on each plate. In addition, one control well containing only the buffer without antibodies was included per plate. To reduce the risk of false-positive results, a sample was considered positive only if its absorbance value was equal to or exceeded three times the mean absorbance of the negative controls.

Extraction of TNA from the phloem tissue

Total nucleic acids (TNA) were extracted from 0.2 g of phloem tissue from each mature grapevine cane sample, using the CTAB-based protocol of Gambino *et al.* (2008), with minor modifications. This involved grinding the material with liquid nitrogen, extraction with CTAB buffer at 65°C, followed by extraction with a chloroform/isoamyl alcohol mixture, precipitation with isopropanol, an ethanol wash, and then resuspending the TNA in sterile ultrapure water before storing at -20°C. The quality of the TNA was assessed through

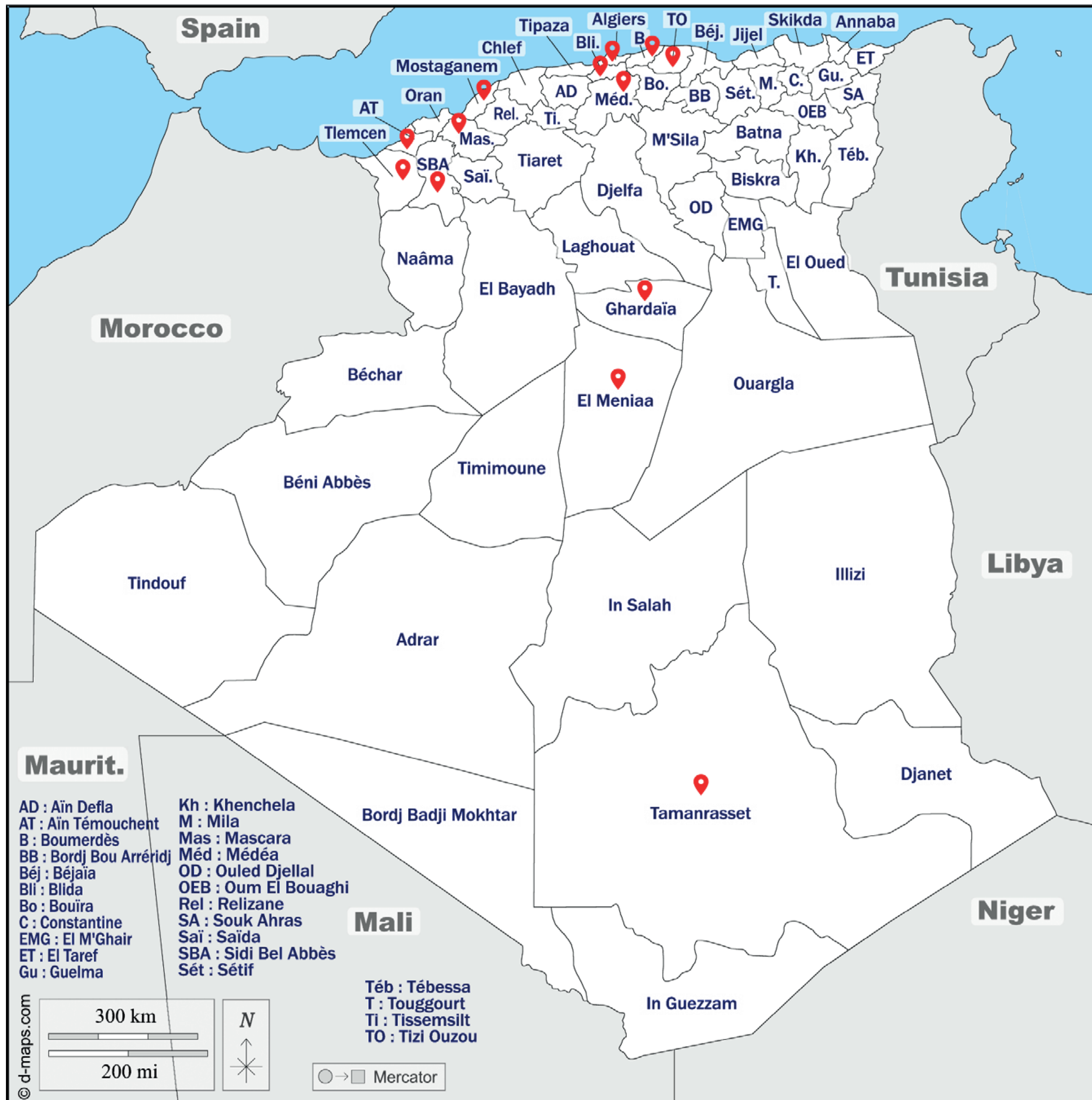

Figure 1. Map of the primary sampling regions in Algeria for this study. The symbols indicate the locations where samples were collected.

agarose gel electrophoresis, while their concentrations were determined using a spectrophotometer (NanoDrop® ND-100 spectrophotometer, Thermo Scientific).

Reverse transcription-polymerase chain reaction (RT-PCR)

A two-step RT-PCR protocol was followed for synthesis of cDNA. In the first step of reverse transcription, the random primer (1 μg μL^{-1}; Invitrogen™) was added with 1.5 μL of sterile water to 10 μL of TNA, and the reaction was incubated at 95°C for 5 min. Then, the components necessary for reverse transcription, including 0.5 μL dNTPs (10mM), 4μL RT 5× Buffer, DTT (0.1M), and M-MLV Reverse Transcriptase (200 U μL^{-1}) (Invitrogen™), were introduced for the second step. The reaction was incubated at 39°C for 1 h to allow cDNA

synthesis, followed by enzyme inactivation at 70°C for 10 min. The total reaction volume was 20 µL.

The PCRs was carried out using the primers LRAmp-F and LRAmp-R, designed by N. Abou Ghanem-Sabanadzovic *et al.* (2012), to amplify 485 bp fragments for the p23 protein (ORF6) gene region. The reactions were each carried out with 2.5 µL of denatured cDNA, 0.5 µL of each primer (20 µM), and DreamTaq DNA Polymerase (5U μL^{-1}) (Thermo Scientific™) in a total volume of 25 µL. They included initial denaturation at 94°C for 5 min, followed by 40 cycles, each of denaturation at 94°C for 30 sec, annealing at 50°C for 35 sec, then extension at 72°C for 45 sec, and a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis on 1.2% TBE agarose gel, and visualized using ethidium bromide staining. RT-PCR was carried out specifically for the ELISA-positive samples. Additionally, 122 samples were used that were from plant material collected during another sampling in 2012 from grapevine growing regions in Algeria. Phloem tissues were used for RNA extractions, and the samples were stored as cDNA at −80°C at the plant protection laboratory of INRAT. Quality control of the cDNA was carried out using a positive control plant sample infected by GLRaV-4, which showed a positive reaction and confirmed good conservation of the cDNA.

Some virus isolates were selected for sequencing of the p23 protein gene regions using the Sanger method, and the obtained sequences were submitted in the NCBI database. A total of 14 positive samples were selected for sequencing based on their geographic and grapevine cultivar origins, to assess the genetic diversity of their partial p23 gene. The PCR products were processed for Sanger sequencing by Carthagenomics (Tunisia).

High throughput sequencing

Total RNA extraction and Illumina-HTS

To complement the DAS-ELISA and RT-PCR tests, a single grapevine sample from the autochthonous cultivar 'Cherchalli', collected in Medea and confirmed positive for GLRaV-4, was selected for HTS to detect the potential infections by other viruses and viroids. A 0.5 × 0.5 cm piece of leaf tissue from this GLRaV-4–infected plant was placed in an Eppendorf tube and immersed in RNALater solution, and then sent to JS-Link Company (South Korea). The RNA was extracted from plant sample using the RNeasy® Plant Mini Kit (QIAGEN). To obtain the cDNA, reverse transcription was carried out using M-MLV Reverse Transcriptase, following the manufacturer's instructions (JS-Link Company). To prepare the Illumina-HTS library, the Company used the TruSeq total DNA library prep kit. The sequences of total DNA were obtained using NovaSeq6000, 2×101PE (Platform: NovaSeq6000; Application: WTS/mRNA), after DNA quality was determined using an Agilent 2100 Expert Bioanalyzer (Agilent). Trimmomatic-0.39 and BBduk v 37.22 were used to trim raw reads in Geneious Prime® 2024.0.5 (Minimum quality = 6) Both ends were trimmed and minimum read length was 10 (Adapter/Quality Trimming Version 38.84; Brian Bushnell) (Kearse *et al.* 2012; www.geneious.com).

Map to reference

Geneious Prime® 2024.0.5 was used to map the DNA-Seq data to the reference sequences. The mapping was carried out with parameters set to "Medium-Low Sensitivity", using the Geneious DNA mapper. The DNA clean reads were mapped to a package of all the 5040 plant virus sequences available in GenBank (downloaded 24 August, 2024). These sequences were concatenated into a single representative sequence (76,145,671 nt), to ensure comprehensive detection of all virus and viroid elements present in the sample data. The results were displayed in a report that included the total number of reads used and the number of assembled reads (Khaffajah *et al.*, 2022).

Phylogenetic analyses

Blastn search was used for sequence comparisons, and multiple nucleotide sequence alignments were carried out using the ClustalW algorithm implemented in the Molecular Evolutionary Genetics Analysis software (MEGA 11.0.10) (Tamura *et al.* 2021). These alignments included sequences obtained in the present study and additional sequences retrieved from the GenBank database (www.cbi.nlm.nih.gov), some of which correspond to reference sequences from previously characterized strains of GLRaV-4 (Table 1). The p-distance method was used to calculate percent similarity values. A phylogenetic tree was then constructed based on nucleotide identity distances, using the neighbor-joining (NJ) method (Kimura 2-parameter model) with 1000 bootstrap replicates and the best-fit alignment (318 nt).

RESULTS

The DAS-ELISA test showed that GLRaV-4 was detected in 81 (18.2%) of the 444 samples assessed.

Table 1. Strain reference sequences of GLRaV-4 selected for phylogenetic analysis of the P23 gene.

Accession number	Isolates	Strain	Origin	Reference
FJ467503.1	LR106	Strain 4	USA	Abou Ghanem-Sabanadzovic *et al.* 2012
MF669482.1	WALA-9	Strain 9	USA	Adiputra *et al.* 2019
MF669481.1	WASB-5	Strain 5	USA	Adiputra *et al.* 2019
FJ467504.1	Estellat	Strain 6	USA	Abou Ghanem-Sabanadzovic *et al.* 2012
AM182328.4	Pr	Strain Pr	Greece	Maliogka *et al.* 2008
KP313764.1	Ob	Strain Ob	Switzerland	Reynard *et al.* 2015

GLRaV-4 was detected in different grapevine varieties. Among the varieties assessed, Ahmar Bouamar had an infection rate of 26.8%. Ladhari and Valensi each had rates of 40%, while Alicante had 30% infection, and Muscat had 20% GLRaV-4 infection. The 23 autochthonous grapevine accessions collected from ITAF had a combined infection rate of 39.1% (Table 2). The greatest highest infection rate (36.8% grapevines infected) was detected in the southern region of Algeria, particularly in Ghardaïa (36.4% infected). The central region followed, with an overall infection rate of 18.1%, particularly in Boumerdès and Médéa (approx. 23% positive samples. Lowest GLRaV-4 prevalence (16.7%) was detected in the western region (Table 3). These results highlighted variations in GLRaV-4 incidence among different grapevine varieties and viticultural regions.

Table 2. Infection rates of GLRaV-4 in surveyed grapevine cultivars, detected using DAS-ELISA.

Cultivar	Number of samples	Number of infected samples	Infection rate (%)
Ahmar Bouamar	41	11	26.8
Dattier	46	2	4.3
Michele Palieri	10	0	0.0
Muscat Italia	28	2	7.1
Cinsault	70	8	11.4
Gros noir	29	4	13.8
Red globe	20	3	15.0
Sabel	22	4	18.2
Cardinal	35	4	11.4
Ladhari	20	8	40.0
Carignan	20	4	20.0
Alicante	10	3	30.0
Merseguerra	10	0	0.0
Muscat	20	4	20.0
Valensi	20	8	40.0
Autochthonous	23	9	39.1
Unknown	20	7	35.0
Total	444	81	18.2

Some DAS-ELISA positive samples were further analyzed by RT-PCR using primers targeting the P23 protein (ORF6) gene, to assess genetic variability of GLRaV-4. Among the 81 positive samples (obtained in 2021/2022) assessed using DAS-ELISA for the presence of the virus, 59 were selected for further analyses by RT-PCR. Of these 29 were confirmed as positive for GLRaV-4. The PCR analyses of the 122 samples from the 2012 sampling showed that 16 samples were positive. Combining these results with those from the 2021/2022 samples collected, a total of 45 GLRaV-4 positive samples were detected. Among the positive samples, 14 were Sanger-sequenced, and two using HTS, selected from different grapevine cultivars and viticultural regions in the central, eastern, western, and southern parts of Algeria (Table 4).

Phylogenetic analysis revealed that Pairwise P23 nucleotide identities ranged from 71 to 100% for the

Table 3. Distribution of GLRaV-4 infections across major grapevine sampling regions and wilayas in Algeria.

Region	Wilaya	Number of samples	Number of infected samples	Infection rate (%)
Central	Medea	99	23	23.2
	Boumerdes	43	10	23.3
	Blida	40	1	2.5
	Tizi Ouzou	31	5	16.1
	Algiers	2	0	0.0
	Total	215	39	18.1
Western	Mostaganem	60	8	13.3
	Aïn Temouchent	70	11	15.7
	Tlemcen	20	3	15.0
	Sidi Bel Abbès	10	0	0.0
	Mascara	50	13	26.0
	Total	210	35	16.7
Southern	El Menia	4	1	25.0
	Ghardaia	11	4	36.4
	Tamanrasset	4	2	50.0
	Total	19	7	36.8

Table 4. GenBank accession numbers of Algerian GLRaV-4 isolates sequenced for the P23 protein gene.

Isolate	Cultivar	Origin	Sequencing method	Accession number
ALG85	Cinsault	Aïn Temouchent	Sanger	PQ406674
ALG107	Cherchali	Medea	HTS	PP983228
ALG108	Cherchali	Medea	HTS	PP983229
ALG122	Muscat	Aïn Temouchent	Sanger	PQ633193
ALG130	Muscat	Aïn Temouchent	Sanger	PQ619712
ALG157	Valensi	Mascara	Sanger	PQ633194
ALG212	Unknown	Ghardaia	Sanger	PQ633195
ALG253	Gros noir	Boumerdes	Sanger	PQ518714
ALG265	Sabel	Boumerdes	Sanger	PQ633197
ALG378	Unknown	Tamenrasset	Sanger	PQ619713
ALG383	Ahmar de Machetras	Medea	Sanger	PQ633196
ALG388	Ghanez	Medea	Sanger	PQ800099
ALG424	Ahmar Bouamar	Medea	Sanger	PQ807038
ALG20-12	Gros noir	Mascara	Sanger	PQ816343
ALG26-12	Gros noir	Mascara	Sanger	PQ816344
ALG373-12	Cargnan	Mascara	Sanger	PQ821896

Algerian isolates, that indicate a close relationship between Algerian isolates, except for the isolate ALG253 which showed less than 76% identity to the other Algerian sequences, indicating a highly divergent variant (Supplementary Table 3).

The Blast analysis revealed that the Algerian isolates share high sequence identity with NCBI isolates reported from several countries worldwide. As a result, we observed strong similarity with isolates from India, the USA, France, and other geographically distant regions (Table 5).

The phylogenetic tree, based on the P23 protein sequences of Algerian GLRaV-4 isolates and related international sequences available in GenBank, revealed six distinct phylogenetic groups (Figure 2). Among these, the Algerian sequences obtained in the present study clustered into five groups, each corresponding to a specific strain of GLRaV-4, as indicated by reference sequences from previous studies (Table 1). Distribution of the Algerian isolates across these strains was: Strain 4, isolates ALG383, ALG20-12, and ALG378; Strain 5, isolates ALG373-12, ALG157, and ALG85; Strain 6, isolates ALG107, ALG108, ALG265, ALG424, and ALG26-12; Strain 9, isolates ALG122, ALG130, ALG212, and ALG388; and Strain Pr, isolate ALG253. This clustering highlights the genetic diversity of GLRaV-4 within the Algerian isolates and provides valuable insights into their relationships with reference strains.

More than 95% nucleotide similarity was shown within the GLRaV-4 strain groups 4, 5, 6, and 9, except for Strain Ob, which had 90% similarity (Supplementary Table 4). In contrast, the between-group similarity percentages ranged from 62% to 90%, highlighting significant genetic diversity among isolates from different strains, especially Strain Pr and Strain Ob, with similarities of less than 75%.

Table 5. Pairwise nucleotide similarities between Algerian isolates of GLRaV-4 and reference sequences available in NCBI.

Algerian isolate	Strain	Reference isolate	Origin	Similarity (%)
ALG20-12 ALG378 ALG383	Strain 4	GDR-I (KT933072)	India	90–99.7
		LR106 (FJ467503)	USA	
ALG85 ALG157 ALG373-12	Strain 5	WASB-5 (MF669481)	USA	96–98
ALG26-12 ALG107 ALG108 ALG265 ALG424	Strain 6	WIL24N5 (OP752662)	Australia	94–97
		Estellat (FJ467504)	USA	
ALG122 ALG130 ALG212 ALG388	Strain 9	WALA-9 (MF669482)	USA	95–98
		LC12_N5 (OP752665)	Australia	
ALG253	Strain Pr	Pr (AM182328)	Greece	97.6

The between-group identity analyses showed that Strain 9 and Strain 5 had close phylogenetic relationship (90%). Strain 6 had 89% similarity with Strain 9 and 88% with Strain 5, indicating strong genetic connections between these strains. Strain 4 had moderate similar-

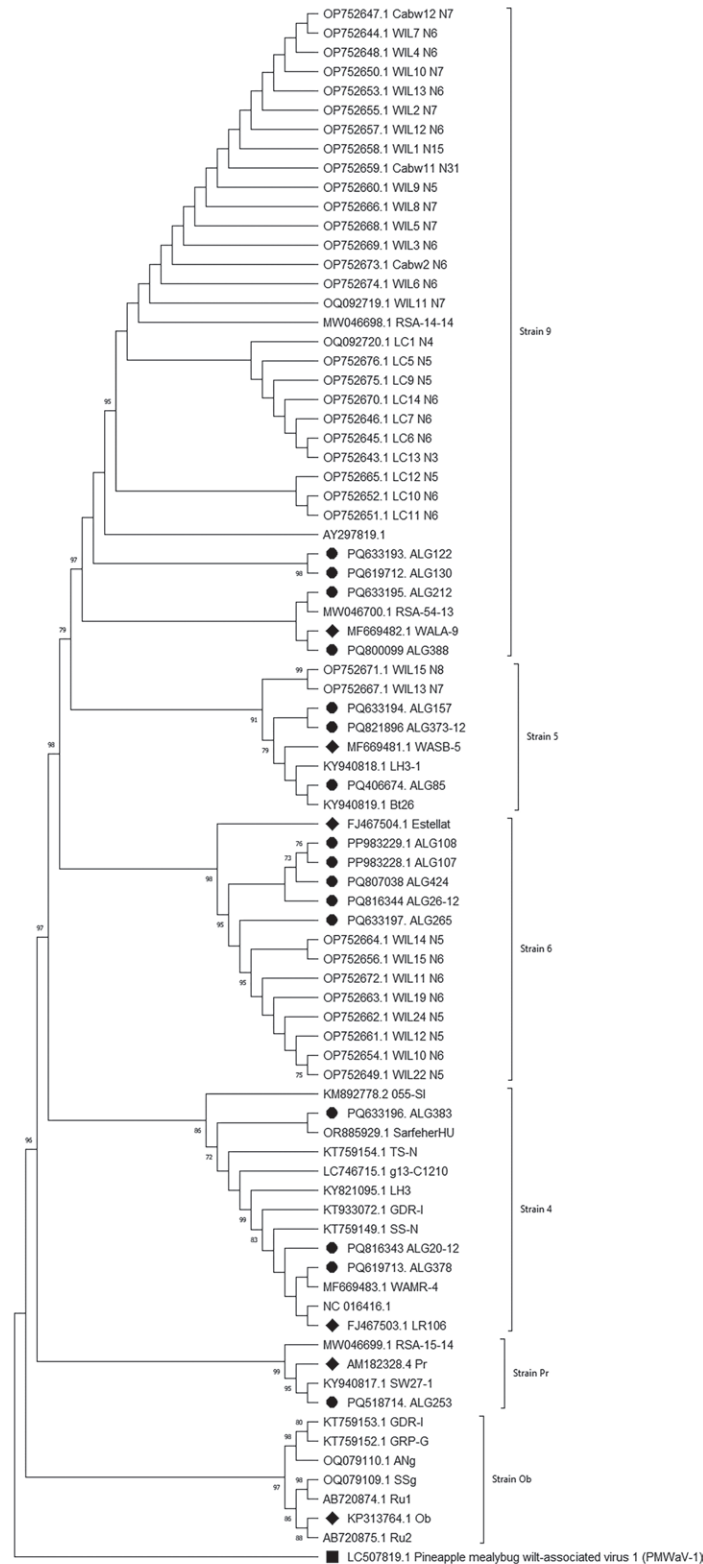

Figure 2. Phylogenetic tree of grapevine leafroll associated virus 4 (GLRaV-4, *Ampelovirus tetravitis*) isolates obtained in the present study and retrieved from GenBank. The tree was constructed with sequences of the 318 nt fragment of the P23 gene, with percentage of bootstrap support (≥70%) with 1,000 bootstrap replicates. Pineapple mealybug wilt-associated virus (LC507819: PMWaV-1, *Ampelovirus unananas*) was used as the outgroup. ● indicates Algerian sequences, and ♦ indicates the reference sequence for the group.

ity (83%) with Strain 6 and Strain 5 (83%), and Strain 9 (84%), suggesting a closer genetic relationship within these strains compared to others. Strain Pr had low similarity (maximum of 74%) across all comparisons, indicating divergent genetic composition in this strain. Strain Ob had the lowest similarity (62% to 64%), indicating increased degree of genetic divergence compared to the other strains (Supplementary Table 5).

Total RNAseq data generated by the Illumina platform included 47,039,345 raw reads that were trimmed to obtain 47,034,642 short clean reads of 101 bases. In Geneious software, all RNA reads were paired and mapped against suspected virus and viroid genomes. The P23 protein genes of the GLRaV-4 were reconstructed (isolates ALG107, PP983228 and ALG108, PP983229). Additionally, the complete genomes of hop stunt viroid (HSVd, *Hostuviroid impedihumuli*) isolate ALG305 (PQ059409) and grapevine yellow speckle viroid 1 (GYSVd-1, *Apscaviroid alphaflavivitis*) isolate ALG302 (PQ059408) were obtained. Furthermore, fragmented sequences corresponding to three viruses were also identified: grapevine Pinot gris virus (GPGV, *Trichovirus pinovitis*) isolate ALG400, grapevine leafroll-associated virus 2 (GLRaV-2, *Closterovirus vitis*) isolate ALG301, and grapevine fanleaf virus (GFLV, *Nepovirus foliumflabelli*) isolate ALG226, registered, respectively, under accession numbers PQ059410.1, PQ059407.1, and PQ059404.1.

DISCUSSION

GLRaV-4 has been reported as one of the viruses responsible for grapevine leafroll in many regions, and is often found in mixed infections with other GLRaVs (Aboughanem-Sabanadzovic *et al*., 2017).

The present study is the first to report occurrence and genetic diversity of GLRaV-4 in Algeria, covering the different viticultural regions across the country. Previous surveys in Algerian vineyards reported GLRaV-3 as the most prevalent grapevine leafroll-associated virus, with incidences ranging from 44% to 55.3%, while GLRaV-2 and GLRaV-1 were detected at incidences of, respectively, 15.8% and 5.4% (Lekikot *et al*., 2012; Lehad *et al*., 2015a; 2015b; 2019). Based on the 18.2% incidence of GLRaV-4 detected in the present study, this virus probably ranks between GLRaV-2 and GLRaV-3 in prevalence among grapevine leafroll-associated viruses reported so far in Algeria.

The discrepancy observed in the present study between ELISA and RT-PCR results likely resulted from the high genetic variability of the virus, which limits the ability of the primers designed by Abou Ghanem-Sabanadzovic *et al.* (2012) to detect all variants. In contrast, ELISA uses polyclonal antibodies that recognize a broad range of variants, making it more reliable than RT-PCR for large-scale virus detections.

Nucleotide comparisons showed that the Algerian GLRaV-4 sequences exhibited genetic relationships for the P23 gene, with sequence similarity ranging from 71% to 100%. This high level of genetic diversity reflects the presence of distinct GLRaV-4 strains, as confirmed by Martelli *et al.* (2012). They described GLRaV-4 as a virus with multiple genetically diverse variants, including Strains 4, 5, 6, 9, Pr, and others, which are serologically related and share similar genome structures, sizes, and biological and epidemiological characteristics. The similarities observed between strains, ranging from 62% to 90%, further confirms the significant genetic diversity among GLRaV-4 strains.

The analyses showed strong relationships between some virus isolates obtained from different regions and grapevine varieties. Isolates ALG122, ALG130, ALG212, and ALG388 had high nucleotide similarity (97% to 100%). Isolates ALG26-12, ALG107, ALG108, ALG265, and ALG424 were also similar, with nucleotide similarities between 96% and 100%. Additionally, isolates ALG85, ALG157, and ALG373-12 shared nucleotide similarities from 96% to 98%. In contrast, isolate ALG253 had low similarity compared to other Algerian sequences, suggesting the isolate was more genetically distinct. The results showed no correlations between percentage of nucleotide similarity and either the geographical origin or the grapevine cultivar from which the isolates were obtained. Comparable results were obtained by Orfanidou *et al.* (2021).

Most of the Algerian sequences obtained in the present study, from different grapevine cultivars and regions, clustered with the reference sequences of GLRaV-4 Strain 4, Strain 5, Strain 6, and Strain 9, while only one sequence grouped with GLRaV-4 Strain Pr. No Algerian sequence clustered with GLRaV-4 Strain Ob. Based on the comparison with the reference sequences, isolates ALG20-12, ALG378, and ALG383 were closely related to the reference isolate LR106 (FJ467503), with 92 to 99% similarity, identifying them as GLRaV-4 Strain 4. Isolates ALG85, ALG157, and ALG373-12 were closely related (96 to 98% similar) to the reference isolate WASAB-5 (MF669481), identifying them as GLRaV-4 Strain 5. Isolates ALG26-12, ALG107, ALG108, ALG265, and ALG424 were 94% similar to the reference isolate Estellat (FJ467504), identifying them as GLRaV-4 Strain 6. Isolates ALG122, ALG130, ALG212, and ALG388 had nucleotide similarities of 97% to 98% to the refer-

ence isolate WALA-9 (MF669482), classifying them as GLRaV-4 Strain 9. Isolate ALG253 had 99% similarity to the reference isolate Pr (AM182328), identifying it as GLRaV-4 Strain Pr.

These results are consistent with those reported by other authors. The phylogenetic analysis of Wu *et al.* (2023) showed that Australian isolates were of five major groups, of Strains 4, 5, 6, 9 and Pr, for the complete genome sequence and sequences of the RdRp, HPS70h and CP genes. Argentinian isolates (Talquenca *et al.*, 2023) grouped within GLRaV-4 Strain 5, Strain 6, and Strain 9, based on their coat protein (CP) gene sequences, while no sequence was found to cluster with GLRaV-4 Strain 4, Strain Pr, or Strain Ob. Du *et al.* (2025) built on earlier work that had separated GLRaV-4 into the strain groups 4, 5, 6, 9, Car, Pr and Ob, using CP sequences. Using combined CP and HSP70 phylogenies, they redefined this diversity into seven groups: the distinct strain groups 5, 6, 9, Pr and Car, plus two additional clusters designated as GLRaV-4 Group 1 and Group 2. In this updated framework, Group 1 corresponds to the original GLRaV-4 lineage containing the reference isolate LR106 (NC_016416), while Group 2 corresponds to the former Ob strain lineage. With this updated grouping scheme, the Algerian isolates were found, in the present study, to belong to GLRaV-4 Group 1 and to the previously defined strain groups 5, 6, 9 and Pr.

The P23 gene protein homology analysis in the present study indicated that Strains 5, 6, and 9 are phylogenetically more similar to each other than to Strains 4, Pr, and Ob. The sequences corresponding to GLRaV-4 Strain Ob and GLRaV-4 Strain Pr exhibited the greatest genetic diversity compared to sequences from other groups. This was already observed by Wu *et al.* (2023) in their study of the coat protein (CP) gene of Australian isolates. The Slovenian isolate 055-SI was 87% similar to the reference sequence LR106, and only 61% similar to Strain Ob (Ru1, AB720874.1), for the P23 protein gene (Štrukelj *et al.*, 2016). Indian GLRaV-4 isolates GRP-G and GDR-I also had genetic divergence in their P23 gene compared with other GLRaV-4 isolates (Rai *et al.*, 2017). The phylogenetic tree based on the P23 gene in the present study (Figure 2) is consistent with the taxonomic revision proposed by Martelli *et al.* (2012), which was based on amino acid sequence divergence from RNA-dependent RNA polymerase (RdRp), heat shock protein 70 homologue (HSP70h), and coat protein (CP).

HTS confirmed GLRaV-4 infections and supported mixed infections of this virus with HSVd, GYSVd 1, GPGV, GLRaV-2, and GFLV. These results reflect the previously reported common occurrence of mixed virus infections observed in grapevines (Fajardo *et al.*, 2017).

Limited studies have been conducted specifically on the P23 protein gene within GLRaV-4. Du *et al.* (2025) showed that P23 interacts with HSP70 and CP, playing a key role in virus particle assembly, replication, and grapevine leafroll pathogenesis. Further research is required to provide increased understanding of genetic diversity of the P23 gene across different virus strains. Additionally, observed symptoms caused by GLRaV-4 are closely related to mixed virus infections, and cannot be attributed to any specific virus, viroid or to synergies between these pathogens.

There have been few studies examining GLRaV vectors and transmission in Algeria. However, the mealybug *Planococcus ficus* has been reported to be a vector of GLRaV-4 in other viticultural regions (Tsai *et al.*, 2010), and has been recorded in the Mitidja region of Algeria (Bissaad *et al.*, 2017). Presence of this insect suggests a potential role in the local transmission of GLRaV-4. Information on the distribution of mealybugs and soft scale insects, specifically *P. ficus* and *Phenacoccus aceris*, is important for controlling GLRaV-4 dissemination in viticultural systems. Practical disease management strategies should include the use of certified virus-free planting material (obtained through sanitation and *in vitro* culture protocols), monitoring and control of insect vectors such as mealybugs and soft scale, and removal of infected grapevines to reduce virus spread and protect vineyard productivity.

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