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Occurrence and diversity of *Grapevine leafroll-associated virus 1* in Algeria

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Summary. A survey was conducted of central and western Algerian grape cultivars and a germplasm collection to detect the presence of *Grapevine leafroll-associated virus 1* (GLRaV-1), using DAS-ELISA. The virus was found in 26 of 484 sample (5.4%). No infection was found in the germplasm collection. Analysis of the sequence of a coat protein gene region revealed that of the 17 recognised phylogenetic groups, the Algerian isolates belong to Groups I, II and XVI. This is the first study of genetic diversity of GLRaV-1 in Algeria.

Keywords. GLRaV-1, RT-PCR, genetic variability, HSP70h, CP.

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INTRODUCTION

Grapevine leafroll disease (GLD) is caused by a complex of virus species from the family *Closteroviridae*, and this disease occurs in all grape-growing regions of the world (Almeida *et al.,* 2013). It is an economically important virus disease (Alabi *et al.,* 2016). The associated viruses are referred as the grapevine leafroll-associated viruses, including GLRaV-1, -2, -3, -4; -7 and -13. GLRaV-1, GLRaV-3, GLRaV-4 and its strains and GLRaV-13 belong to the genus *Ampelovirus,* GLRaV-2 belongs to the genus *Closterovirus*, and GLRaV-7 belongs to a newly proposed genus *Velarivirus* (Martelli *et al*., 2012, Ito and Nakaune 2016).

The GLRaV-1 genome size is nearly 19.5 kb (Fazeli *et al*., 2000), from which partial sequence information was characterized (AF195822). The full-length genomic sequence of two isolates of GLRaV-1 has been determined, at ~18,730 nucleotides (nt) (isolate WA-CH) and ~18,945 nt (isolate WA-PN). Isolates WA-CH and WA-PN encode ten open reading frames, and their overall genome organization is similar to previously reported GLRaV-1 isolates (Donda *et al.,* 2017).

Grapevine leafroll-associated viruses are known to spread by grafting and by mealybug transmission. Several species of mealybugs have been shown

to transmit GLRaV-3 (Almeida *et al.,* 2013). GLRaV-1 is restricted to the phloem tissues of host plants and is frequently present with other viruses. Transmission of GLRa-1 by mealybugs follows the "semi-persistent and non circulative" mode (Tsai *et al.,* 2008), and several mealybug and soft scale species have been reported to transmit GLRaV-1 (Naidu, 2017).

Little *et al.* (2001) identified ten genes associated with GLRaV-1, several of which are hypervariable. Genetic diversity of GLRaV-1 has been less investigated compared to other leafroll disease associated viruses. Based on the HSP70h gene region, two phylogenetic groups were designed (Kominek *et al.,* 2005). Based on the heat shock 70 protein homolog (HSP70h) gene region, three phylogenetic groups were identified (Alabi *et al.,* 2011). Based on the coat protein (CP) gene, Esteves *et al.,* (2013) described eight groups. More recently, eignt groups were designated for the CP gene region and seven groups for the HSP70h gene region (Fan *et al .,* 2015).

Here we describe a survey of GLRaV-1 in grapevines in Algeria, and the chararterization of the genetic relationship of Algerian virus isolates with other isolates, in a partial fragment of the CP gene.

MATERIALS AND METHODS

In autumn 2012, 484 grapevine samples were collected from the major grapevine growing areas in the western and central regions of Algeria. Dormant canes from individual vines were randomly collected in commercial vineyards, including from eight introduced cultivars (355 samples) and two local cultivars (100 samples), as well as 18 cultivars (29 samples) from the autochthonous grapevine germplasm collection at the Institut Technique de l'Arboriculture Fruitière et de la Vigne (ITAF). All samples were tested by DAS-ELISA (Clark and Adams, 1977) for the presence of GLRaV-1, using a commercial kit (Agritest). Extracts were obtained by macerating phloem tissues of dormant canes in the grinding buffer described by the manufacturer (1 g per 10 mL). Absorbance was recorded at 405 nm using an automatic microplate reader (Labsystems Multiskan Ascent). Samples with absorbance readings equal or exceeding three times those of negative controls were considered as positive.

All positive samples were analyzed by RT-PCR using total nucleic acids extracted from 0.2 g of cortical scrapings, as described by Foissac *et al.* (2001). The negative and positive controls were provided from the virus collection of the INRAT, Tunis. Reverse transcription was performed using MMLV reverse transcriptase (200 units μL^{-1}), and PCR was carried out using the primer pairs HSP70-417F (5'- GAGCGACTTGCGACTTATCGA-3') and HSP70-737R (5'-GGTAAACGGGTGTTCTTCAATTCT-3'), designed by Osman and Rowhani (2006), and the primer GLRaV-1-CP/F (5'-CGCGCTTGCAGAGTTTAAGTGGTT-3') and GLRaV-1 CP/R (5'-TCCGTGCTGCATTGCAACTTTCTC-3') designed by Alabi *et al.* (2011), to amplify fragments of 320 bp for the HSP70h gene region and of 734 bp for the CP gene region. Some virus isolates were randomly chosen for sequencing of the CP and HSP70h gene regions (Supplementary data 1).

Mean genetic distances of the analysed 104 sequences of each group, as well as between the groups, were calculated using MEGA7 with best fit alignment (Kumar *et al.*, 2016). The Dna SP software v.5.10.01 (Librado and Rozas 2009) was used to estimate the mean values of non-synonymous to synonymous substitution ratios (dN/dS) for the different coding regions of GLRaV-1. The ratio of non-synonymous to synonymous substitutions per site (dN/dS) was also determined.The genetic diversity π and the Tajima'D value were calculated using the MEGA7 software package.

Multiple alignments of nucleotide sequences from this study and corresponding sequences available in GenBank (Supplementary data 1) were performed using ClustalW implemented in MEGA7. Using the best fit models, a phylogenetic tree was constructed for the CP gene region using the best fit alignment (585 nt) with the neighbor-joinning method with 1,000 bootstraps (Figure 1)

A search for recombination events was conducted for the CP gene region with the genetic recombination detection method (GARD) (Kosakovsky Pond *et al.*, 2006) implemented in the Datamonkey web server (https://www.datamonkey.org/).

No analyses were performed for the HSP70h gene region in this study due to the small length of the obtained sequences.

RESULTS

Results revealed that GLRaV-1 was present in 26 (5.4%) of the 484 samples tested. For the variety Dattier de Beyrouth, six of 71 samples were infected, seven of 100 for Gros noir and eight of 70 for Muscat (Table 1). No infected samples were found in the autochtonous grapevine germplasm collection at ITAF. According to previous results for the presence of GLRaV-3 in the same samples, mixed infections of GLRaV-1 and GLRaV-3 were detected in 15 samples, and infection of only GLRaV-1 was detected in 11 samples.

In order to determine the diversity of GLRaV-1 isolates from Algeria, a comparison was performed at the

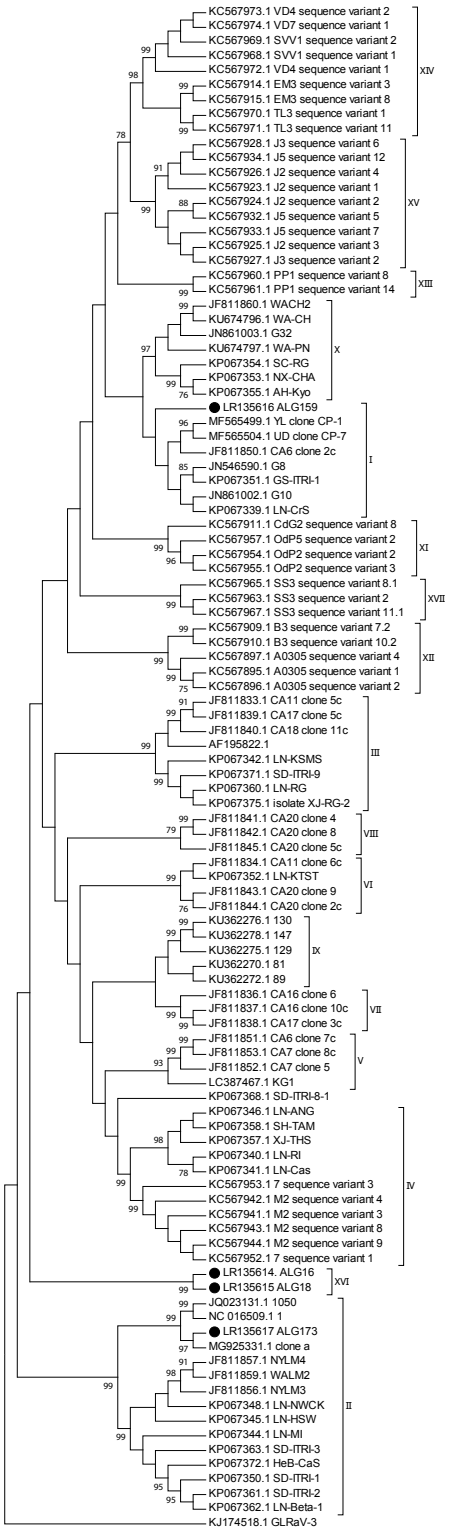

Figure 1. Phylogenetic tree of *Grapevine leafroll associated virus 1*(GLRaV-1) isolates, constructed with sequences of a 585 nt fragment of the viral coat protein gene. Percentages of bootstrap support (≥ 75%) from 1,000 replicates are indicated on relevant tree branches.

Table 1. Rates of virus infections in the surveyed grapevine varieties.

Variety	Number of samples	Number of samples infected
Dattier	71	6
Gros noir	100	7
Cardinal	52	1
Alicante	30	1
King Rubi	9	0
Muscat	70	8
Mersguira	10	0
Çinsaux	40	1
Carignon	31	1
Valensi	42	1
Autochthones	29	0
Total	484	26

nucleotide level of HSP70h and CP gene regions. For HSP70h, proportional nucleotide similarity of Algerian sequences revealed an average of 93%. Isolates ALG5, ALG128, ALG132, ALG270 and ALG420 were closely similar with 95 to 97% similarity. Isolates ALG16 and ALG18 showed 100% similarity between them. A BLASTn search showed that isolates ALG16 and ALG18 had 95% similarity to isolate WA-CH (KU674796.1), while isolates ALG420, ALG132, ALG128, ALG270 and ALG5 had 91-94% similarity between them.

For the sequence of the CP gene region, a comparison at the nucleotide level showed that ALG16 and ALG18 were identical, ALG159 showed less than 87% similarity and ALG173 less than 85% similarity compared to the three Algerian sequences. BLASTn search showed that ALG16 and ALG18 had 92-93% similarity with other sequences available in GenBank. (isolate A0305 variant 4 (KC567897) was very similar).

For the CP gene region, analyses performed with DNA sp 0.6 and MEGA7 showed that the number of non-synonymous sites was 443.91 sites out of 585, and the synonymous site was 141.09 sites out of 585. The mean dN/dS was 0.095 calculated with the Single-Likelihood Ancestor Counting (SLAC) algorithm implemented in the datamonkey server. The genetic diversity analyses gave a value of $\pi = 0.103$ and the the D Tajima's test was -0.92. The dN/dS and the Tajima's D test showed that the CP gene was under negative selection.

Tajima's D and Fu and Li's D and F statistics were calculated for groups I- and II-containing Algerian sequences. This gave a negative value for Tajima's D and positive value for Fu and Li's D and F statistics. A value of genetic divertsity π for the two groups was estimated at 0.03282 for Group I and 0.04688 for Group II (Table 2).

Table 2. Representative parameter estimates for the Algerian GLRaV-1 phylogroups.

Phylogroup	π	Tajima's D	Fu & Li's D	Fu & Li's F
I	0.03282	-1.041	1.29777	1.47497
II	0.04688	-0.056	0.26019	0.13310

No recombination event was found in the CP gene region, using the GARD algorithm implementated in the Datamonkey database (https://www.datamonkey.org).

A phylogenetic tree with four Algerian sequences and 104 other sequences downloaded from GenBank was constructed for the CP gene region using the neighbor joining method with MEGA7 software (Kumar *et al.,* 2016). Results confirmed the high diversity of the GLRaV-1 population. The tree revealed that the GLRaV-1 population clustered into 17 groups. Eight clusters were previously reported by Fan *et al.* (2015). The Algerian sequences obtained in this study clustered into three groups. The sequences of ALG16 and ALG18 clustered in a new group (Group XVI), the sequence ALG159 was in G I and ALG173 was in G II.

The within group proportional similarity ranged from 95% to 100%, indicating a close relationship between isolates from the same group, except for the Group VIII which showed 88% similarity. The between group distances ranged from 78% to 96%, showing divergence present between groups especially for some groups which had less than 80% similarity (Groups II and XVI). The tree revealed the absence of correlation between the geographical origins and phylogentic distribution for some groups. Group I was constituted by sequences from France, China and Poland, and Group II with sequences from China, the United States of America and Canada. However, some groups contained only isolates from one country. Group VII only contained sequences from the United States, Group IX contained sequences only from Turkey, and Groups XI, XII XIII, XIV and XV contained sequences only from Portugal (Supplementary data 1).

DISCUSSION

This study describes the occurrence of GLRaV-1 in different regions of Algeria, and the genetic diversity of population of this virus from other countries. There have been few studies of the diversity of this virus, which appears to be very variable.

Our results have demonstrated shown the presence of GLRaV-1 in Algeria. GLRaV-1 is less prevalent in Algeria than GLRaV-2 and GLRaV-3, which were reported in a previous study (Lehad *et al.* 2015). Mixed infections of this virus with GLRaV-3 may explain its parallel transmission by common vectors. Vector transmission of GLRaV-1 has been reported in Algeria (Bisaad *et al.,* 2017). *Parthenolecanium corni* described as vector of this virus was also observed in some vineyard in central Algeria (unpublished data). Other mealybug species, such as *Phenacoccus aceris*, were described as vectors of GLRaV-1 (Alliaume *et al.,* 2015). More research is required on the epidemiology of this virus in Algeria. It is important to determine the efficiency of mealybug transmission of GLRaV-1 compared with other viruses, in order to further understand the epidemiology of grapevine viruses in this country.

Nucleotide comparisons were carried out, and and results for HSP70 showed that the Algerian GLRaV-1 sequences had close genetic relationships ranging from 89% to 100% similarity, when CP gene region sequences were compared. However, some sequences shared less than 85% similarity. This indicates that the the HSP70h gene region was more conserved than the CP gene region. The HSP70h gene region was reported to be highly conserved in the family of *Closteroviridae* (Tian *et al*., 1996).

The genetic diversity of the Algerian sequences compared to the sequences in NCBI showed an important genetic diversity value for the CP gene region, confirmed by others (Alabi *et al.,* 2011; Esteves *et al.,* 2013). This may be explained by the natural pressure enforced on this protein. The CP gene plays an important role in plant/virus interactions. The Tajima's D test and the mean dN/dS results showed that the CP gene evolves under negative selection. Similar results were obtained for the CP and Rdrp gene of *Grapevine virus A* (Alabi *et al.,* 2014).

The Tajima's D test gave a negative value for the CP gene region. This may indicate that the gene evolved under a low frequency of polymorphism suggesting population size expansion (e.g. after a bottleneck or a selective sweep) and/or purifying selection.

Previously, two groups were reported for HSP70h gene region (Komínek *et al*., 2005). Alabi *et al.* (2011) reported three clusters, and Fan *et al.* (2015) revealed the presence of eight groups based on the CP gene region and seven based on the HSP70h region.

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