

# Rt-PCR Analysis and Evolutionary Relationship of Some Hungarian *Grapevine leafroll associated virus 1 and 3* Isolates\*

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## ABSTRACT

Hungarian isolates of *Grapevine leafroll associated virus 1 and 3* (GLRaV-1, GLRaV-3) were tested using serological (DAS-ELISA) and molecular (RT-PCR) methods. Five hundred bp long PCR products of the part of HSP70 gene of one serologically positive GLRaV-1 and four GLRaV-3 isolates were sequenced. These sequences were applied for phylogenetic analysis and compared to foreign virus isolates of NCBI GenBank. Phylogenetic analysis of GLRaV-1 HSP70 gene supported the earlier results that it could be divided into two clusters: E and A. The Hungarian isolate 6.4.1 belonged to the group E. This isolate showed the highest homology with the AY754914 isolate from the Czech Republic. GLRaV-3 sequence data could cluster five groups. Hungarian 2.2; 3.5 and 4.2 isolates were estimated belonging to the group II. The 1.4 isolate from the same vineyard as 2.2 varied in sequence data so it belonged to the other, IV. variant group with two South African, two Austrian and a Syrah isolate. According to the phylogenetic analysis, two variant groups occurred in Hungary. These isolates related with each other, but showed higher similarity of foreign countries. In some cases, they were similar to isolates of the neighbour countries such as Slovakia and Austria. It could be supposed that mainly the exchange of virus infected propagation materials caused the dissemination of GLRaV isolates.

**Keywords:** Grapevine; Virus; GLRaV-1; GLRaV-3; HSP70; RT-PCR; Hungary

## 1. Introduction

Grapevine leafroll symptoms may be induced by a complex of viruses, the majority of which belong to the *Closteroviridae* family.

Members of GLRaV-1-4 have been distinguished in Hungary by DAS-ELISA [1]. GLRaV-1 and -3 spread via propagation material and grafting [2] and are transmitted by the scale insect [3], and by mealybugs [4-11]. GLRaV-1 and -3 belong to the genus *Ampelovirus*. The GLRaV-1 genome contains 9 open reading frames encoding replication complex consisting of the methyltransferase, helicase, RNA depending RNA polymerase domains, a 70 kDa heat shock protein homologue, a HSP90-like protein, the coat protein, two minor copies of coat

protein and other proteins of unknown function [12]. RNA genome of GLRaV-3 is positively single-stranded, and contains 13 open reading frames [13]. It is most closely related to GLRaV-1. Both viruses encode a 70 kDa heat shock protein homologue with conserved amino acid sequence motives [14,15]. This region is used for phylogenetic analysis in order to assess evolutionary relationship among members of ampeloviruses [16].

## 2. Materials and Methods

### 2.1. Plant Material

Forty-eight grapevine leaf samples of cultivars Kékfrankos, Juhfark, Pinot noir, Kéknyelű, Cabernet sauvignon, Olaszrizling and Tempranillo originated from different geographical areas: North-West, Middle-West, South-West and Middle-East part of Hungary (Kőszeg, Balatonboglár, Badacsonytomaj, Cserszegtomaj, Kecskemét

\*The nucleotide sequence data reported in this work have been submitted in the Genbank database and assigned the accession numbers HE794021, HE794022, HE794023, HE794024 and HE794025.

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and Pécs), showing typical leafroll symptoms, were collected in autumn, 2010.

## 2.2. Das Elisa

Collected leaf samples were tested for GLRaV-1 and -3 by DAS-ELISA method [17] with antiserum of Bioreba AG (Switzerland).

## 2.3. RT-PCR Detection

Total RNA was extracted and purified from grapevine leaf tissues by SPEKTRUM Plant Total RNA Kit (Sigma-Aldrich Chemie GmbH, Germany). The primer pairs were designed based on the nucleic sequence of isolates GLRaV-3 NY1 (GenBank accession number AF037268) and GLRaV-1 according to the sequence data of GenBank Acc. No. AF 195822. The specific primers: 1FHSP70 5'-CAGGGCTCGTTTGTACTGG-3', 1RHSP70 5'-TCGGACAGCGTTTAAGTT CC-3' [4] and in the case of GLRaV-3: LC1F 5'-CGCTAGGGCTGTGGAA-GTATT-3', LC2R 5'-GTTGTCCCGGTACCAGAT-AT-3' [13] An 540 and an 546 bp fragments encompass from the central part of HSP70 gene. The cDNA synthesis was carried out by reverse transcription by M-MuLV enzyme. PCR conditions in a PCR Applied Biosystems GeneAmp PCR System were as follows: denaturation 94°C/1 min, followed 40 cycles of 94°C/75 s, 52°C/30 s and 72°C/1 min. The final elongation step was at 72°C/10 min. Aliquots of PCR products were run on 1.5% agarose gel. PCR products were gel-purified using Roche High Pure Purification Kit, cloned into pGEM-T Easy (Promega) cloning vector [18] and sequenced by BAY-GEN (Hungary).

## 2.4. Phylogenetic Analysis

Phylogenetic studies were performed using alignments of the HSP70h genes in both cases from several virus isolates. Constructions of the evolutionary models were performed using the CLC Sequence viewer 6.5.1. (CLC bio, Denmark). The phylogenetic trees were also obtained with CLC Sequence Viewer 6.5.1. using UPGMA method and 1000 bootstrap iterations as a confidence test. In order to assess the relationship of the four Hungarian GLRaV-3 isolates and one Hungarian GLRaV-1 isolate, their HSP70h gene sequences were used in a phylogenetic analysis in which the HSP70h sequences of isolates from elsewhere in the world were included.

## 3. Results

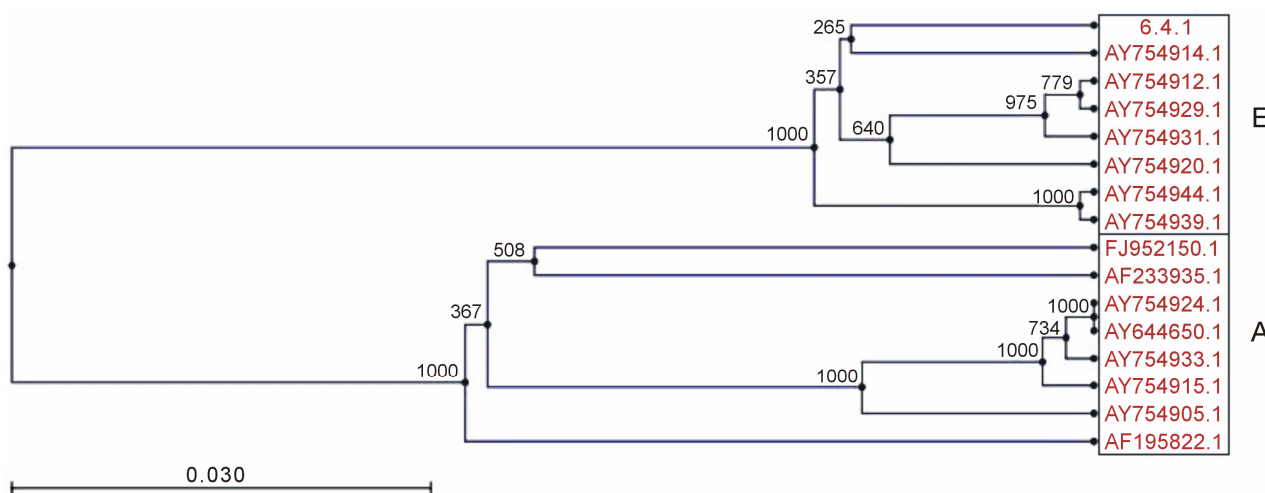
DAS-ELISA and RT-PCR gave positive results for GLRaV-3 in four samples (1.4; 2.2; 3.5; 4.2) and one sample (6.4.1) for GLRaV-1 (Table 1). Results of sequence analysis showed high homologies of Hungarian GLRaV-3 virus isolates. However, this isolates showed some differences. Alignment trees have been constructed from the same items of sequence data from Gene bank of 500 nt part of HSP70 genes using UPGMA methods compared of isolates from elsewhere in the world.

This phylogenetic analysis of GLRaV-1 HSP70 gene is corresponded to the results of Kominek and his co-worker's [4]. According to their results this cluster can be separated into two groups, an "E" and an "A". Our results suggest that Hungarian isolate from Badacsonytomaj (6.4.1) belonged to the group "E" (Figure 1). This isolate showed the highest homology to the (AY754914.1)

Table 1. Occurrence of *Grapevine leafroll-associated virus* 1 (GLRaV-1) and 3 (GLRaV-3) isolates in Hungary.

Number of collected samples	Cultivar	Origin	Number of infected samples by ELISA	Selected samples for molecular analysis
7	Kékfrankos	Kőszeg	2 GLRaV-3	1.4, 2.2
2	Pinot noir	Balatonboglár	1 GLRaV-3	-
3	Juhfark		1 GLRaV-1	6.4.1
2	Kéknyelű	Badacsonytomaj	-	-
10	Olaszrizling		2 GLRaV-3	-
1	Pinot noir		1 GLRaV-1	3.5
3	Cabernet sauvignon	Kecskemét	1 GLRaV-3	-
7	Unknown		2 GLRaV-1	-
3	Olaszrizling	Cserszegtomaj	1 GLRaV-3	4.2
3	Tempranillo		1 GLRaV-1	-
1	Pinot noir	Pécs	-	-
6	Unknown		-	-
48				

Abbreviations: *Grapevine leafroll-associated virus* 1 (GLRaV-1) *Grapevine leafroll-associated* 3 (GLRaV-3).



**Figure 1. Phylogenetic tree reconstructed from 500 nt long fragment of HSP70 gene from GLRaV-1 isolates. Abbreviations:** AY754914.1 (Czech Republic), AY754912.1 (Czech Republic) AY754929.1 (Czech Republic), AY754931.1 (Czech Republic), AY754920.1 (Czech Republic), AY754944.1 (Slovakia), AY754939.1 (Slovakia), FJ952150.1 (Iran), AF233935.1- (USA), AY754924.1 (Czech Republic), AY644650.1 (Czech Republic), AY754933.1 (Czech Republic), AY754915.1 (Czech Republic), AY754905.1 (Czech Republic), AF195822.1 (Australia), Hungarian isolate: 6.4.1 (Badacsonytomaj).

isolate from Czech Republic. The “E” group holds the isolates (AY754912.1), (AY754929.1), (AY754931.1), (AY754944.1) from Czech Republic; and the (AY754944.1) and (AY754939.1) isolates from Slovakia. The group “A” includes the Iranian (FJ952150.1), an Australian (AF195822.1), an American (AF233935.1) and five (AY754924.1), (AY754915.1), (AY754905.1), (AY754933.1) and (AY644650.1) isolates from Czech Republic. Phylogenetic analysis showed that the GLRaV-1 Hungarian isolate belonged to the European “E” group.

**Figure 2** represents the relationship of 500 bp fragments of HSP70 gene of GLRaV-3 from twenty five isolates from different countries, included four Hungarian ones. These sequences clustered into five groups similar in Fuchs’ study [19].

Only one virus isolate (ef508151) from New-Zealand belongs to the first group.

The largest second group contained fifteen isolates as Asian isolates: one (aj748524) from Israel; two Chinese (aj748514) and (dq780887); and one (aj748517) from Syria. Other members of this group were from America (North and South part), two from USA (dq780891) and (af037268) and one (eu344893) from Chile. South Africa was represented by one isolate (gq352631). Some sequence data of GLRaV from Europe showed high homology with the second group, as one (aj748521) Italian; two Austrian (aj748512) and (aj748511); one Tunisian (aj748522) and three Hungarian (3.5 from Badacsonytomaj; 2.2 from Kőszeg and 4.2 from Cserszegtomaj). Only one isolate from China (dq780889) represented the third group.

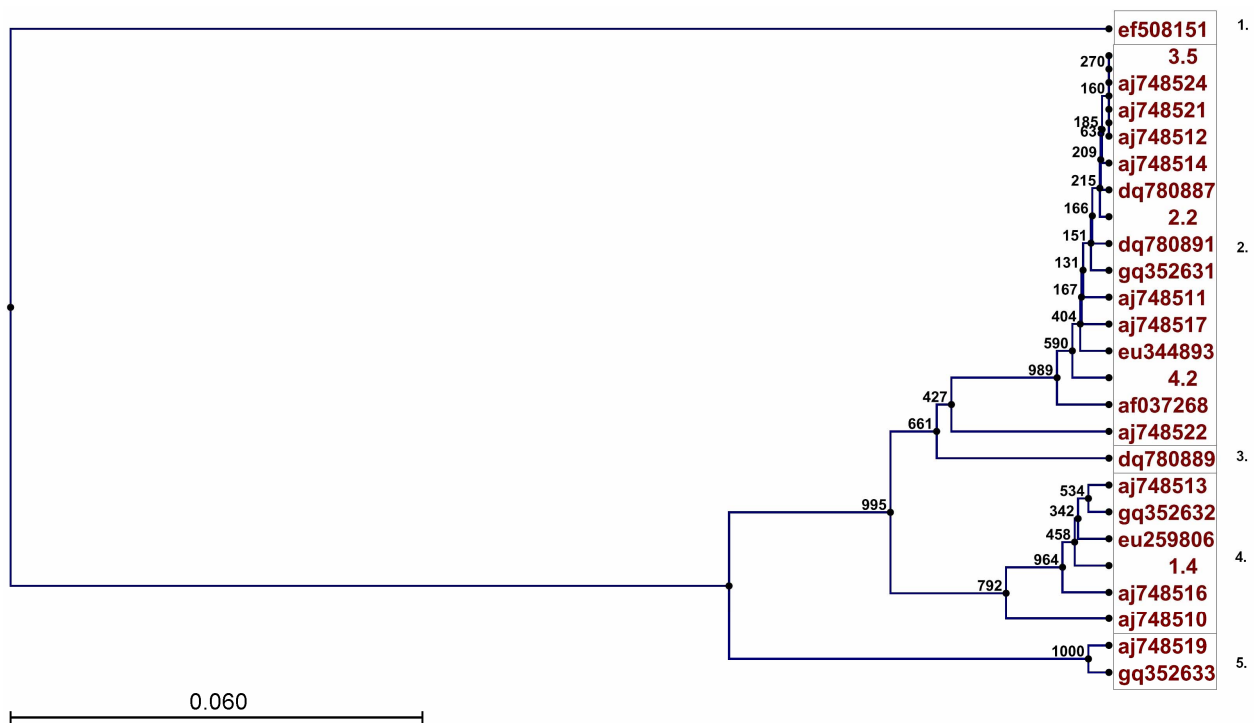
Six isolates, (aj748513) and (aj748510) from Austria;

(gq352632) and (eu259806) from South Africa; (aj748516) from Syria and one Hungarian isolate 1.4 from Kőszeg formed the fourth numerous group. 1.4. showed the highest homogeneity to the South African isolates. The two members of the fifth group are from Italy (aj748519) and South Africa (gq352633). Hungarian isolates (2.2; 4.2) showed homology to the isolates of China (dq780887) and Chile (eu344893). It is noteworthy that the Hungarian (3.5); the Israeli (aj748524); the Italian (aj748521) and the Austrian (aj748512) isolates showed no sequence differences with each other.

#### 4. Discussion

HSP70 gene was used as a basic target to initial molecular analysis of the members of *Closteroviridae* and evaluation of their genetic diversity. In the most cases this sequenced part is available in the GenBank. In this manuscript could be used HSP70 sequence data of GLRaV-1 isolates from Australia, Iran, America and some intra-isolate sequence variant from Slovakia and Czech Republic. The isolate of Hungary show the highest homogeneity to the sequence data of isolate “sv12-5” from Czech Republic.

Phylogenetic analysis of partial gene sequence of HSP70 of *Grapevine leafroll-associated virus 3* showed, that four Hungarian isolate grouped into two biggest clades. Sequence identities were found among the GLRaV-3 isolates from four European isolates, including the Hungarian 3.5 isolates. The 2.2, 3.5, 4.2 isolates are related to the NY1 isolate in the group II. The isolate 1.4 originated from the same vineyard in Kőszeg, as the



**Figure 2.** Phylogenetic tree of the genomic region HSP70 GLRaV-3 constructed by UPGMA method. Abbreviations: ef508151 (New-Zealand), aj748524 (Israel), aj748521 (Italy), aj748512 (Austria), aj748514 (China), dq780887 (China), dq780891 (USA), gq352631 (South Africa), aj748511 (Austria), aj748517 (Syria), eu344893 (Chile), af037268 (USA), aj748522 (Tunisia), dq780889 (China), aj748513 (Austria), gq352632 (South Africa), eu259806 (South Africa), aj748516 (Syria), aj748510 (Austria), aj748519 (Italy), gq352633 (South Africa), Hungarian isolates: 3.5 (Badacsonytomaj), 2.2 (Kőszeg), 4.2 (Csereszegtomaj) and 1.4 (Kőszeg).

isolate 2.2, but this sequence data show higher homogeneity to the members of the group IV.

Some authors have recognised five variant groups. Three major, one isolate from New-Zealand and one from China represent two other groups [13,19-22]. Our results confirm these clustering and the Hungarian isolates could insert in these groups.

## 5. Conclusion

According to the phylogenetic analysis, two variant groups of GLRaV-3 occurred in Hungary. There is a lack of correlation of sequence data with geographical origin if the isolates were consistent with the view that the infected propagation material plays the most important role in the spreading. The results are not suitable for the justification of origin and spread of Hungarian virus isolates. Further studies are needed to gain information of the biological significance and role in the transmission of these sequence diversity of grapevine leafroll associated viruses.

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