Phylogenetic analysis of two Iranian grapevine virus A isolates using coat protein gene sequence

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Abstract

Symptomatic grapevine samples were collected from vineyards in Zanjan province to detect grapevine virus A. Total RNA was extracted from symptomatic leaf samples and subjected to cDNA synthesis using random hexamer primers. Then, a DNA fragment around 800 bp including the complete coat protein (CP) gene was amplified from nine out of 57 samples by polymerase chain reaction (PCR) using specific primers. The infection rate of GAV in vineyards was around 4%, 6%, 2%, and 6% in Zanjan, Abhar, Tarom, and Khoramdareh, respectively. Two DNA fragments corresponding to samples Abhar (p25) and Zanjan (p26), were purified and sequenced. The CP-nucleotide sequence identity between two Iranian isolates was 97.3%. However, sequence identity with previously reported isolates were 76 to 95% and 82 to 98% at the nt and amino acid levels, respectively. CP-based phylogenetic trees showed three main groups (I, II, III) in which p25 (MG977013) and p26 (MG977013) isolates were placed in the group I together with isolates from different geographical regions including Palestine (Israel), Italy, Czech Republic, Jordan, USA and South Africa. To our knowledge, this is the first report of detection and phylogenetic analysis of GVA isolates from Iranian vineyards based on the complete CP gene. Positive selection value was observed on codon 25 indicating the role of this position probably in virus survival and flexibility against evolutionary forces.

Key words: Coat protein, Phylogenetic analysis, RT-PCR, Sequencing, *Vitivirus*.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an economically important fruit crop in Iran and around the world (Mullins *et al.*, 1992; Zeinali *et al.*, 2012). Nowadays, the growing areas of grapevine are more than ten million hectares throughout the world; hence making it one of the most widely grown fruit crops worldwide (Meng *et al.*, 2017). Viral diseases constitute a major prevention to the development and highly profitable production of viticulture (Meng *et al.,* 2017). Several viruses have been reported that infect grapevines (*Vitis* spp.) such as grapevine fanleaf virus (GFLV), Arabis mosaic virus (ArMV), grapevine leafroll-associated virus 1, -2, -3, -4, -5, -6, -7, -8 and -9, grapevine virus A (GVA) and grapevine virus B (GVB) (Alkowni *et al.*, 2004; King *et al.*, 2011; Murphy *et al.*, 2012; Hu *et al.*, 2014; Naidu *et al.*, 2014; Meng *et al.,* 2017). The most common symptoms of these viruses are fan leaf degeneration (Andret-Link *et al.*, 2004), leafroll (Alkowni *et al.,* 2004; Naidu *et al.,* 2014), rugose wood (Bonavia *et al.*, 1996) and fleck (Boscia *et al.*, 1995). Rugose wood complex occurs by most viruses in *Vitivirus* genus (Garau *et al.*, 1994; Nickel *et al.*, 2002; Meng *et al.,* 2017), causing four different disorders including rupestris stem pitting, Corky bark, Kober stem grooving and LN33 stem grooving (Garau *et al.,* 1994; Murphy *et al.,* 2012). Rugose wood complex diseases cause serious damages to grapevine in most grapevine growing regions of the world (Bonavia *et al.,* 1996; Nickel *et al.,* 2002; Basso *et al.*, 2017). The damages in infected grape vines include less vigorous growth,

delayed bud opening, and decline and death within a few years (Wang *et al.*, 2003). Kober stem grooving (Goszczynski and Jooste, 2003b; Goszczynski *et al.*, 2008), is a part of the rugose wood complex that is closely associated with grapevine virus A (GVA).

GVA the type species of the genus *Vitivirus* (family *Betaflexiviridae*) (King *et al.,* 2011; Murphy *et al.,* 2012) has a filamentous flexuous particle, with 800 nm in length, and 11–12 nm in diameter (King *et al.,* 2011). The positive-sense-single stranded RNA (+ssRNA) genome is organized into five open reading frames (ORFs1-5). ORF1 encodes replication proteins (194 kDa) (Minafra *et al.*, 1997; Murolo *et al.*, 2008). ORF2, which is partially overlapped by ORF1 and ORF3, encodes a polypeptide of 19 kDa that has no homology to any known proteins but has some similarity to the polypeptide encoded by ORF2 of GVB (Saldarelli *et al.*, 1996). ORF3 encodes a putative movement protein (31 kDa), while ORF4 encodes the coat protein (21.5 kDa), and ORF5 (10 kDa) encodes a suppressor of RNA silencing and has a role in pathogenicity known as nucleic acid binding protein (Minafra *et al.,* 1997; Galiakparov *et al.*, 2003). GVA isolates could be biologically separated according to the four kinds of symptoms on mechanically inoculated *Nicotiana benthamiana* (Galiakparov *et al.,* 2003; Goszczynski and Jooste, 2003a; Goszczynski *et al.,* 2008). These four kinds of symptoms include mild vein clearing; vein clearing with interveinal chlorosis; vein clearing, interveinal chlorosis and strong curling of top leaves; and extensive patchy necrosis. On the other hand, molecular analysis of the genomic nucleotide sequence of the 3' region revealed that GVA isolates can be divided into three groups (I, II, and III), which mild isolates are classed in group III and share only 78.0 to 79.6% nt sequence identity with other isolates (Goszczynski and Jooste, 2003a). The study of variability is one of the most important aspects of plant virology because strains vary in the severity of the disease caused in the field, and this variation may be highly relevant to the development of control strategies. Variability is also important for understanding how viruses have evolved and are evolving (García-Arenal *et al.*, 2001).

GVA has been detected from several countries in the Middle East including Syria (Mslmanieh *et al.*, 2006), Jordan (Osman and Rowhani, 2008; Osman *et al.*, 2013), Egypt (Fattouh *et al.*, 2014), Afghanistan (Digiaro *et al.*, 1999), Lebanon (Haidar *et al.*, 1996), Palestine (Alkowni *et al.*, 1998; Alkowni *et al.,* 2004) Turkey (Koklu *et al.*, 1998) and in other countries such as Italy (Ioannou, 1993), Spain (Zabalgogeazcoa *et al.*, 1997), USA (Goszczynski and Habili, 2012) and, Portugal (Digiaro *et al.,* 1999). Also, GVA has been isolated from some vineyards in Iran (Rakhshandehroo *et al.*, 2005; Roomi *et al.*, 2006) based on serological methods. In addition, there are some submitted sequences for GVA that are related to a distinct genomic regions of RdRp (Goszczynski *et al.,* 2012). The present study aims to detect GVA isolates from Zanjan province, one of the major grapevine production areas in Iran and characterize their coat protein (CP) gene sequences to find the phylogenetic relationships with other GVA isolates.

MATERIALS AND METHODS

Plant material

Samples (leaves and green shoots) showing symptoms (Figures 1A to 1F) characteristic of virus infections like leaf deformation, stem pitting and discoloration of bark were collected from 20 vineyards of Abhar, Khoramdareh, Mahneshan, Soltaniyeh, Tarom and Zanjan, in Zanjan province during spring through fall in 2015 and 2016. These samples were collected from different varieties of grapevine. A part of each sample was stored at −80 °C before being used for RNA extraction and the rest was kept in a plastic bag in a refrigerator to other processing.

RT-PCR

Total RNA was extracted according to Rowhani *et al.* protocol (1993) with minor modifications from the infected samples and the resulted pellet was suspended in 30 µl RNase-free sterile distilled water and stored at −80 °C. The reverse transcription reaction was carried out in a final volume of 10 µl using two step Hyperscript master mix (Genall, South Korea) following manufacturer's instruction. PCR was performed using the PCR master mix (Ampliqon, Denmark) in 12.5 µl final volume by a pair of specific primers designed by primer premium 6 software corresponding to the coat protein gene and a part of nucleic acid binding protein of GVA, GVA-H557F (5- GACAAATGGCTCACTACG -3) and GVA-C7273R (5- CATCGTCTGAGGTTTCTACTAT -3) to amplify an expected DNA fragment around 800 bp encompassing complete CP gene. The initial denaturation was performed at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 50 s. A final polymerization step at 72 °C for 7 min was also applied in both cases. Healthy grapevine leaves without any symptoms were used as negative controls in PCR. The PCR products were run on a 1% agarose gel in 1×TBE buffer.

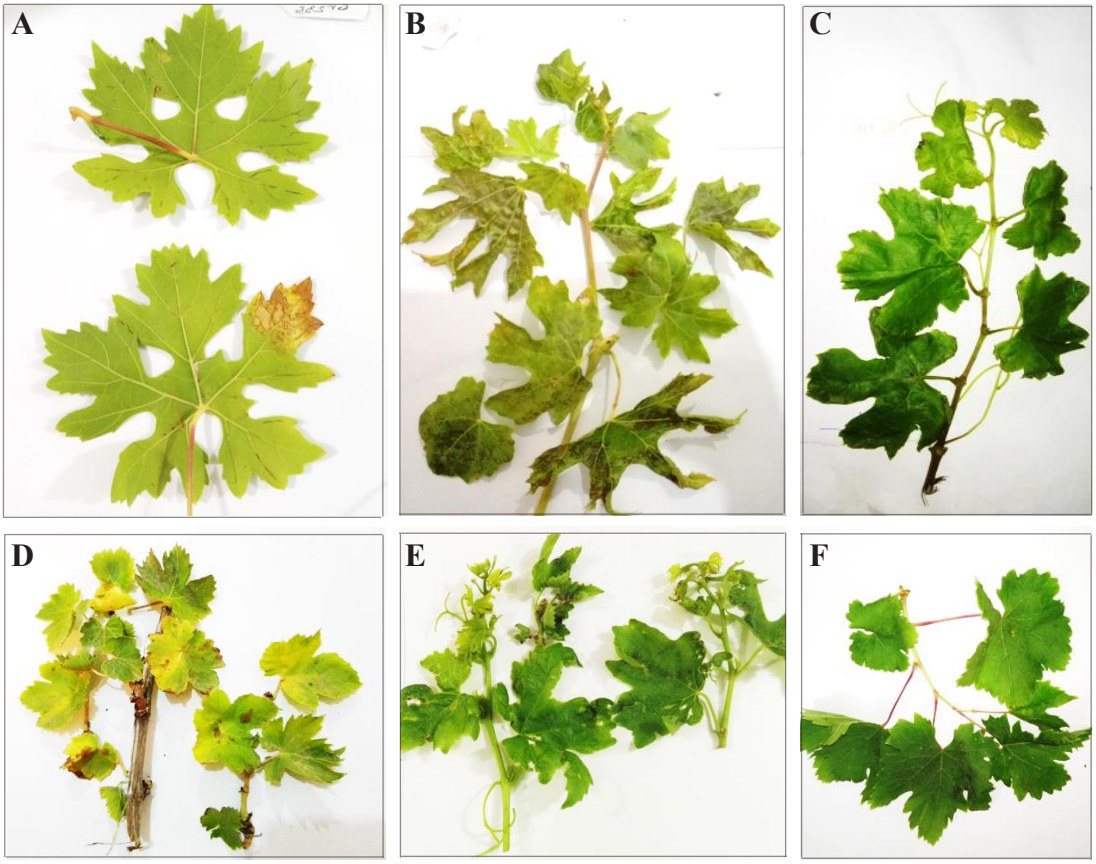

Figure 1. Symptoms of samples recognized as GVA positive **A:** Vein necrosis, **B** and **C:** Leaf deformation, **D:** Rugose wood and yellowing, **E:** Dwarfing and color changing in green shoots, **F:** Rugose wood.

Sequence analysis

The PCR products (isolates from Zanjan and Abhar, named p25 and p26, respectively) were purified and sequenced (Bioneer, South Korea) with GVA-H557F / GVA-C7273R primers. Forward and reverse sequences of each isolate assembled into one contiguous sequence and the primer sequences were removed from each sequence. Complete sequences were compared with the CP gene sequences of other GVA isolates available in the NCBI database using the BLAST network service (Table 1). Since there are no complete CP-sequences of Iranian isolates, previously partial CP-sequences of Iranian isolates were not included in these analyses. Clustal W program in MEGA6 software (Salem *et al.,* 2003) was used to align multiple sequences of the two new Iranian isolates (p25 and p26) and the sequences of 21 known GVA isolates (Table 1). Neighbor-joining phylogenetic tree with 23 corrected nucleotide distance and bootstrap analyses of 1000 replicates were generated within MEGA7 software. Nucleotide identity and similarity were determined using the SIAS software program (http://imed.med.ucm.es/Tools/sias.html). The sequences were deposited in NCBI.

Genetic diversity analysis

DnaSP version 6.10.01 (Rozas *et al.,* 2017) was used to estimate the number of haplotypes (H), haplotype diversity (Hd), number of polymorphic (segregation) sites (S), total number of mutations η (Eta), average number of nucleotide differences (K), average pairwise nucleotide diversity π (Pi), total number of synonymous sites (SS), total number of non-synonymous sites (NS) and the ratio of non-synonymous nucleotide diversity to synonymous nucleotide diversity [Pi(a)/Pi(s)] known as ω=dN/dS (Table 2). Furthermore, HyPhy software package as implemented in Datamonkey server (www.datamonkey.org), were used to identify individual codon positions evolving under natural selection, two different codon-based maximum-likelihood algorithms; single likelihood ancestor counting (SLAC) and fixed effects likelihood (FEL) with significance level set at P-value<0.05.

Neutrality, genetic differentiation and gene flow statistical tests

To investigate the neutral selection hypothesis operating by Tajima's D (Tajima, 1989), Fu and Li's D* and F* (Fu and Li, 1993) statistical tests of population

Table 1. GVA strains/isolates, accession numbers, and origin with new Iranian isolates used in the present research.

Isolates	Host	Country of region	Accession number	Data of collection
CBSM119_2c	*Vitis vinifera*	USA	KF013767	2011
LVCH92-04_4c2	*Vitis vinifera*	USA	KF013816	2011
SLWZF1-11c	*Vitis vinifera*	USA	KF013798	2011
VHLM1_5c	*Vitis vinifera*	USA	KF013806	2011
BVPN1_21c	*Vitis vinifera*	USA	KF013755	2011
3138-03	*Vitis vinifera*	Canada	JX559641	2011
-	*Vitis vinifera*	Palestin (Israel)	AY244516	2003
PA3	*Vitis vinifera*	Palestin (Israel)	AF007415	2003
LQ58	*Vitis*	China	DQ911145	2006
GTR1-1	*Vitis vinifera*	South Africa	DQ787959	2007
GTR1SD-1	grapevine cv. Shiraz	South Africa	DQ855081	2008
GTR1-2	*grapevine cv. Shiraz*	South Africa	DQ855086	2008
JP98	*Vitis vinifera*	South Africa	AF441235	2006
MSH18-1	grapevine cv. Shiraz	South Africa	DQ855085	2006
GTG11-1	grapevine cv. Shiraz	South Africa	DQ855084	2006
P163-1	*Vitis vinifera*	South Africa	AF441236	2001
92/778	*Vitis vinifera*	South Africa	AF441234	2001
-	*Grapevine*	Brazil	AY340581	2003
-	*Vitis vinifera*	Jordan	AY594176	2004
Is 151	*Grapevine*	Italy	X75433	2006
MT43/25	grapevine	Czech Republic	EU008560	2007
p25	*Grapevine*	Iran	MG977013	2016
p26	*Grapevine*	Iran	MG977014	2016

Table 2. Genetic characteristics of GVA coat protein from different populations around the world.

Phylogroup	N	H	Hd	S	η	K	π	SS	NS	dS	dN	ω
Group I	15	14	0.990	129	179	49.333	0.11635	102.42	317.58	0.40935	0.02327	0.0568
Group II	3	3	1.000	84	91	58.333	0.09771	141.94	452.06	0.38265	0.00885	0.0231
Group III	6	6	1.000	80	88	38.400	0.06432	140.69	453.31	0.24028	0.01015	0.0422
Total	24	23	0.996	165	259	70.413	0.16607	102.56	317.44	0.47712	0.06764	0.1417

N, number of isolates; H, number of haplotypes/isolates; Hd, haplotype diversity; S, number of polymorphic (Segregating) sites; η (Eta), total number of mutations; k, average number of nucleotide differences between sequences; π nucleotide diversity; SS, total number of synonymous sites analyzed; NS, total number of non-synonymous sites analyzed; dS, synonymous nucleotide diversity; dN, non-synonymous nucleotide diversity Maximum respective values between groups are shown in bold.

Table 3. Representation of parameter estimates and test statistics for demographic trends in GVA populations around the world include new Iranian isolates.

Phylogroup	π	Tajima's D	Fu and Li's D*	Fu and Li's F*
Group I	0.11635	-0.45884^{ns}	0.11652^{ns}	-0.05200^{ns}
Group II	0.09771	nd	nd	nd
Group III	0.06432	-0.02347^{ns}	0.27486^{ns}	0.23191^{ns}
Total	0.16607	0.06127^{ns}	0.73677^{ns}	0.61349^{ns}

P>0.10.
nd: Four or more sequences are needed to compute Tajima's and Fu and Li's statistics.
π: Nucleotide diversity per site.

differentiation including KS*, KST*, Z*, Snn and FST (Hudson *et al.*, 1992; Hudson, 2000) between phylogroups and geographical populations were carried out using DnaSP v.6.10.01 for the CP gene (Tables 2 and 3). The nearest neighbor of sequences were measured by the Snn test static, whose value ranges between 1 (when population is distinctly differentiated) to one-half in the case of panmixia (Hudson, 2000). Finally, the coefficient of FST (genetic differentiation) was used to estimate inter-population diversity (Hudson *et al.,* 1992; Tsompana *et al.,* 2005).

RESULTS

Symptoms and GVA detection by RT-PCR

Most of the visited vineyards from different regions in Zanjan showed a variety of symptoms including leaf deformation, vein banding, vein necrosis, mottling, proliferation, yellowing and green shoot deformation. The most common symptoms were yellowing, vein clearing, vein necrosis, leaf deformation, rugose wood and stem pitting (Figure 1).

RT-PCR successfully detected GVA from samples in different regions of Zanjan province such as Zanjan, Abhar, Tarom, and Khoramdareh, two, three, one, and, three positive samples from each of the regions, respectively. The infection rate of GAV in vineyards was around 4%, 6%, 2%, and 6% in Zanjan, Abhar, Tarom, and Khoramdareh, respectively. Complete CP gene with a part of ORF5 fragment around 800 bp in length was amplified from nine out of 57 samples (Figure 2). The amplified DNA fragments showed the expected size based on the positions of the primers on the published nt sequence of GVA. No fragment was amplified from the healthy controls.

Genetic characterization and phylogenetic analysis of GVA isolates

The nucleotide sequences of the newly characterized isolates were blasted with that of previously reported

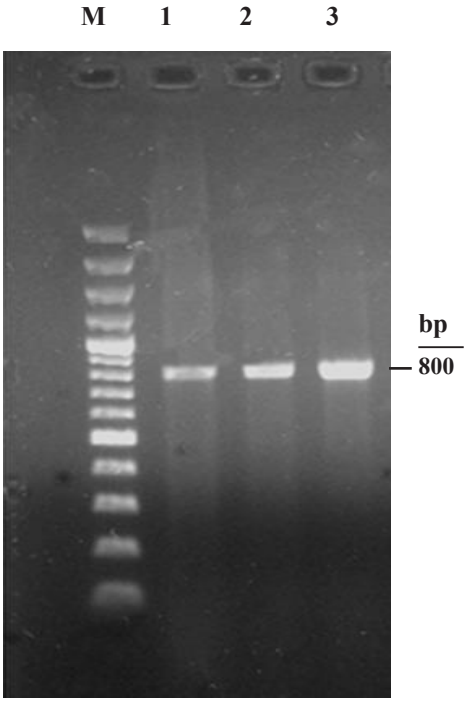

Figure 2. RT-PCR with specific primers (GVA-H557F andGVA-C7273R). Line M: 100 bp Ladder DNA Marker, Lanes 1-3: amplifications of the expected fragment from different isolates.

GVA isolates in NCBI, it appeared that the newly generated sequences corresponded to the CP gene of GVA. When the sequences of new isolates of GVA were subjected to BLAST analysis, 76 to 95% similarity was revealed between these new isolates and some other GVA isolates previously reported from Palestine (Israel), USA, Czech Republic, Jordan, Italy and South Africa. Furthermore, alignment of the deduced amino acid sequences showed that the identities between the isolates from Iran and the other isolates were 82- 98%.

The phylogenetic tree grouped the isolates in three main subgroups I, II and III. Group I include the new Iranian isolates and isolates from South Africa, Brazil,

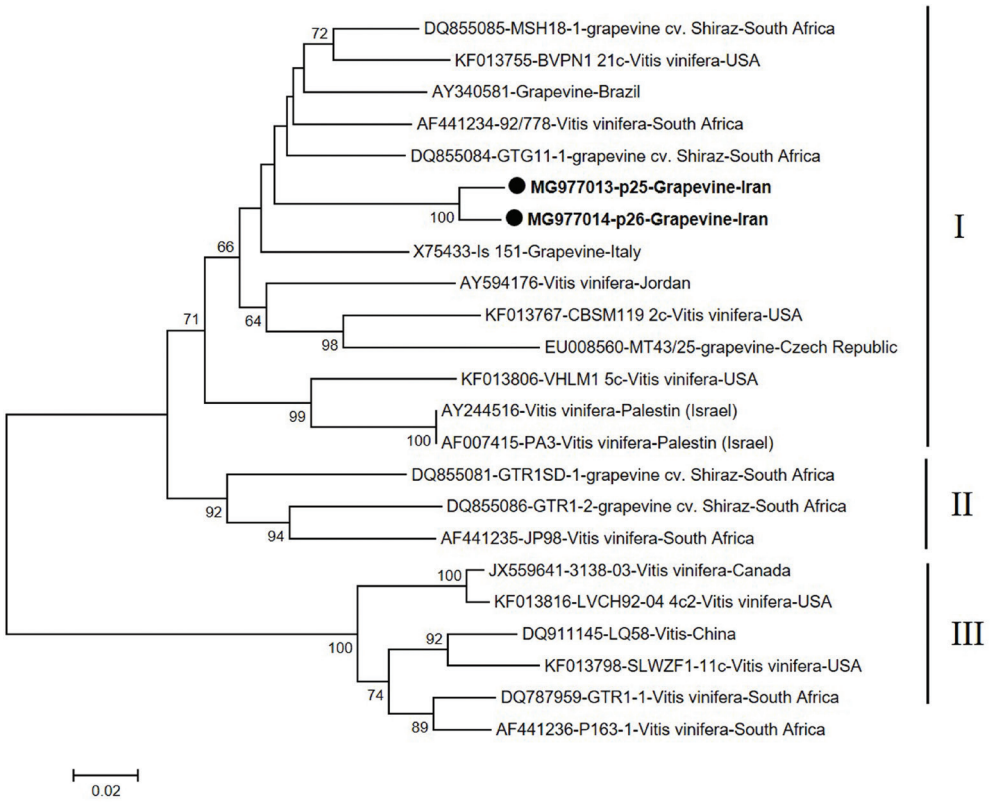

Figure 3. Neighbour joining phylogenetic trees based on nucleic acid sequences of CP gene of GVA isolates generated by Mega 7 program. Branches with bootstrap value of <50% are unrevealed. Iranian Isolate are shown by circle in bold.

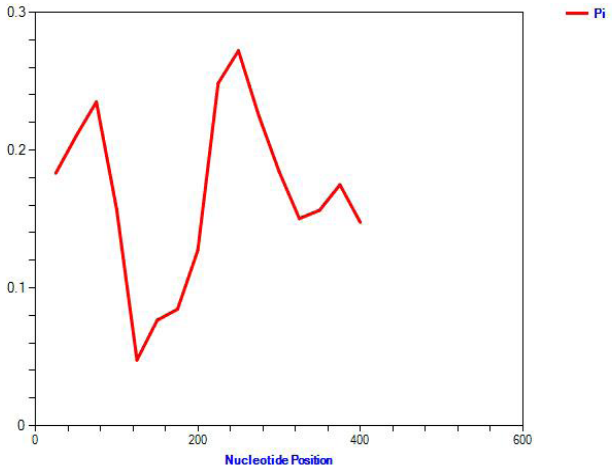

Figure 4. Trend of polymorphism along the coat protein gene in the GVA population, using data from 24 strains/isolates. Pi stands for nucleotide diversity. The curves were generated by sliding windows with 50 and 25 as the window and step sizes, respectively.

Palestine (Israel), Czech Republic, Jordan, Italy; group II was an independent isolate from South Africa, and the isolates from South Africa, USA and China were clustered in group III (Figure 3).

For genetic characterization of the GVA population and phylogroups based on the CP sequences, several genetic diversity parameters were calculated (Table 2). The largest nucleotide diversity (π=0.1163), non-synonymous to synonymous nucleotide diversity ratio (ω=0.0568), and mutations within the segregating sites (η=179 nt) were obtained for the phylogroup I. However, overall average number of differences, (k=58 nt) were calculated for the phylogroup II. Nevertheless, the

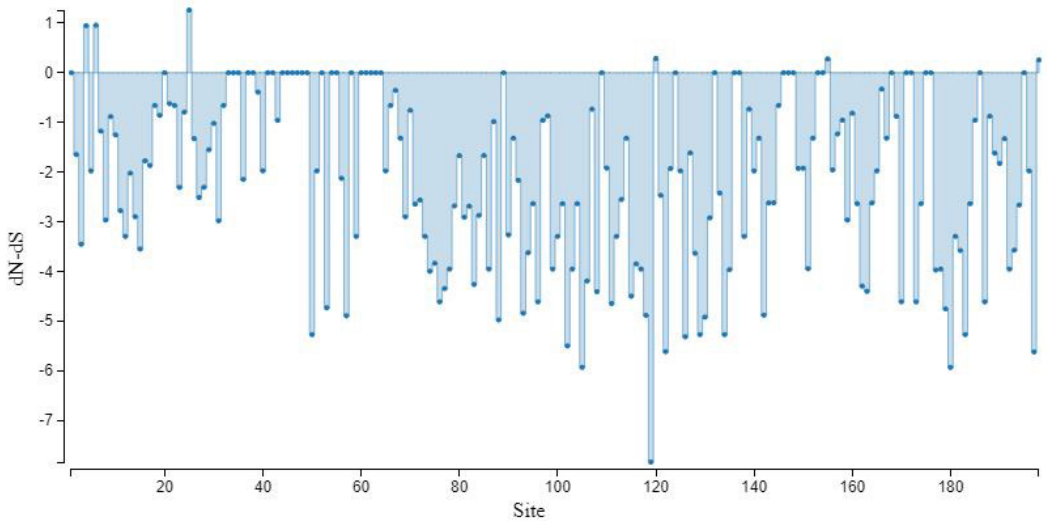

Figure 5. SLAC site graph to identify positive and negative codons/sites.

lowest π=0.0.064, η=88 nt, k=38 nt, and ω=0.042 were estimated for the phylogroup III.

Analysis of nucleotide diversity by "sliding window" options to (100) and step size to (25) revealed a low polymorphism at near N-terminal of CP between nucleotides 101 -200 (π=0.065) of CP-ORF (Figure 4) suggesting that this part of genome is suitable to design primers.

Positive selection

The ratio of non-synonymous to synonymous polymorphic sites (dN/dS) in new Iranian sequences was 0.141, which provided evidence of purifying selection in the CP gene acting to remove deleterious nonsynonymous variants. In details, pervasive positive selection was detected only in one site (codon 25) by the two methods, SLAC and FEL (Figure 5).

Differentiation of phylogroups

Analysis of differentiation of GVA pylogroups showed that three phylogroups with significant Ks*, Kst*, Z* and Snn (1.000) are completely distinct. It is also confirmed by high F_{ST} (>0.26).

DISCUSSION

Viral diseases are serious problems in the vineyards in Iran. Detection of plant virus isolates and assessment of their characteristics are effective steps for controlling grapevine viruses. Also, a rapid and precise identification of viruses is essential for proper application of the control measures. We observed a wide range of viral symptoms including yellowing, vein clearing, leaf deformation, rugose wood, rupestris stem pitting, corky bark, and kober stem grooving in vineyards in the northwest of Iran incurring significant losses (Figure 1). A large number of GVA isolates have been characterized from different vineyards around the world (Haidar *et al.,* 1996; Alkowni *et al.,* 1998; Alkowni *et al.,* 2004; Mslmanieh *et al.,* 2006; Fattouh *et al.,* 2014). As noted before, virus symptoms were observed in most vineyards visited; however, vein necrotic, leaf deformation, yellowing and decline were most common symptoms seen in spring up to fall. It needs to be mentioned that most symptoms were observed at early summer and fall. Therefore, the symptoms decreased in the middle of the summer when the weather turned hot. GVA was detected in most of the regions surveyed in Zanjan province. Previous studies have reported GVA from different areas around the world and Iran (Boscia *et al.,* 1995; Anfoka *et al.*, 2004; Rakhshandehroo *et al.,* 2005; Basso *et al.,* 2017).

When a pair of primers encompassing complete CP gene of GVA was used to detect the virus by RT-PCR (Figure 2), the CP fragment was amplified from nine samples. However, GVA was not detected in some samples showing the typical symptoms of virus infection. This might be due to the suboptimal PCR conditions and particularly the heterogeneity of the primers. The low quality of the RNA preparations from grape tissues which contain lots of inhibitors could be assumed as another reason for not detecting the GVA isolates in some cases. It is better that RNA extraction be performed immediately after sampling. GVA was detected in 9 samples in different regions including Abhar, Zanjan, Tarom, Khoramdareh as the main regions that have vineyards in Zanjan province. Also, the symptoms of GVA are similar to other viruses especially; viruses belong to *Foveavirus* and *Vitivirus*

genera. In many cases symptoms shown by the infected plants are due to synergistic or combined action of two or more viruses (Meng *et al.,* 2017).

The variability in the CP gene of GVA has been reported in several studies (Goszczynski and Jooste, 2003a; Anfoka *et al.,* 2004; Goszczynski *et al.,* 2008; Goszczynski and Habili, 2012). According to the CP gene sequences, the GVA isolates have been assigned to three groups including I, II and III (Anfoka *et al.,* 2004; Goszczynski and Habili, 2012). New Iranian GVA isolates were grouped into group I (Figure 3, 4), which consists of many isolates from different countries such as Palestine (Israel), USA, Czech Republic, Jordan, Italy, Iran and South Africa. Overall, results of this research and the available data in the literature (Meng *et al.,* 2017) indicate that placement of GVA isolates in one of the several main phylogroups is not correlated with the geographical or host origin of a given isolate.

Because the GVA populations from different countries formed several lineages and the presence of low genetic diversities within the phylogroups, it is possible that infections resulted from the founder effect (Delatte *et al.*, 2007) i.e., different GVA variants have been introduced into the vineyards in different regions. This conclusion is further supported by genetic differentiation estimates between phylogroups (Ks*, Kst* and Z* were significant, Snn values were unit (Snn=1.000) and F_{ST}>0.25) (Table 4).

The sequencing results of the GVA CP showed that new Iranian GVA isolates shared 97.3% nucleotide sequence similarity. In addition, the sequences of the GVA isolates reported in this study showed highest (90%) and lowest (76%) similarities with the isolates from South Africa (DQ855086) and China (DQ911145), respectively. It is suggested that genes other than the CP such as the movement protein could be investigated to differentiate between the GVA isolates.

The results revealed evidence on negative selection in the CP gene acting to remove deleterious nonsynonymous variants because the ratio of non-synonymous to synonymous polymorphic sites (dN/dS) was estimated to be 0.141 (Table 2). It could be concluded that mutations did not happen in important motifs. Using HyPhy software, positive selection value was observed only on one position (codon 25) with IEFL and SLAC methods, indicating the role of this position probably in virus survival and flexibility against evolutionary forces which requires further investigation. To the best of our knowledge, this is

Table 4. Genetic differentiation estimates for geographical populations of GVA isolates around the world with new Iranian isolates.

Comparisons	$^{a}K_S^*$	$^{a}K_{ST}^*$	*Ks*, Kst*p* value	$^{a}Z^*$	*P* value	Snn	*P* value	$^{b}F_{ST}$
GroupI (n=15)/GroupII(n=3)	3.85680	0.02225	0.0090**	3.74964	0.0010**	1.000	0.0020**	0.260
GroupI (n=15)/GroupIII(n=6)	3.73551	0.09984	0.0000***	3.65880	0.0000***	1.000	0.0000***	0.611
GroupII (n=3)/GroupIII(n=6)	3.69496	0.14164	0.0060**	2.05385	0.0060**	1.000	0.0130*	0.648

ns: not significant.
* 0.01<P<0.05; ** 0.001<P<0.01; *** P<0.001.
a K*, Kst*, Z* and Snn are test statistics of genetic differentiation.
b FST, coefficient of gene differentiation, which measures inter-population diversity.

the first report on the detection of positive selection pressure at a codon in the GVA CP gene.

Tajima's D and Fu and Li's D* and F* statistical tests were non-significant (P.0.10) for the GVA population and phylogroups isolates (Table 3) indicating that GVA populations may be at equilibrium. This result of neutrality statistical test is in agreement with those of the natural selection pressure. It is plausible to hypothesize that strong negative selection may not allow an excess of low or high frequency polymorphisms.

Using virus-tested certified plants in new vineyards is the most important method to control diseases caused by GVA. The infected-nurseries need to be separated from healthy commercial orchards to prevent or limit the disease. Removing infected trees to prevent new infections is one of the major strategies to control the GVA-infection in vineyards. Using the serological and molecular methods to detect symptomless, infected trees and virus free nursery stocks and scion woods to establish new orchards are useful to prevent the spread of GVA in a wide area.

In conclusion, attempts should now be made to investigate the population diversity among GVA isolates in certain locations during several years, taking into consideration the size of the sample and the regions on the virus genome that should be subjected to sequence analysis. Also, our analyses provide further evidence for several evolutionary mechanisms driving GVA evolution such as negative selection, recombination and founder effect by the exchange of infected plant materials among different geographic locations. These findings help to develop more effective strategies for detection and management of GVA.

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