Characterization of the full length coat protein gene of Iranian *Grapevine fanleaf* virus isolates, genetic variation and phylogenetic analysis

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Abstract
The full-length coat protein gene of *Grapevine fanleaf virus* (GFLV) isolates from Iran was characterized by reverse transcription polymerase chain reaction (RT-PCR) and sequencing. The expected 1515 bp coat protein (CP) gene amplicon was obtained for 16 isolates out of 89 that were identified by double antibody sandwich enzyme-linked immunesorbent assay (DAS-ELISA) in a population of 330 symptomatic grapevine leaf samples. CP products of eight isolates were cloned and nucleotide sequences were determined. Parsimonious trees indicated that GFLV isolates from Iran formed a distinct cluster, suggesting an independent evolution.
Keywords**:** Cloning; Coat protein; DAS-ELISA; GFLV; Parsimonious; RT-PCR; Sequencing

INTRODUCTION

Among 60 virus species that can infect grapevine (Martelli, 2009), *Grapevine fanleaf virus* (GFLV) is one of the oldest known viruses of grapevine and has been a major component of grapevine degeneration (Martelli and Savino 1990). GFLV belongs to the genus *Nepovirus* in the family *Secoviridae* (Sanfaçon *et al*., 2009) and possesses isometric particles of about 30 nm in diameter (ICTVdB- The Universal Virus Database, version 4, April 2006 [online], http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/). It causes grapevine fanleaf degeneration worldwide and severe losses up to 80%, poor fruit quality and reduced grapevine longevity (Andret-Link *et al*., 2004; Martelli and Savino, 1990; Raski *et al*., 1983). The disease occurs in almost all temperate regions where *Vitis vinifera* and hybrid rootstocks are cultivated. This disease was first reported in *V. vinifera* from Austria in 1883 and later it was speculated that the virus had originated from ancient Persia (Vuittenez, 1970).

In nature, GFLV is transmitted only by the ectoparasitic dagger nematode, *Xiphinema index* (Andret-Link *et al*., 2004; Martelli and Savino, 1990; Raski *et al*., 1983). It is believed that propagating material, particularly of tolerant *V. vinifera* cultivars has played a major role in long distance spread of GFLV.

Symptoms of the disease may vary according to different host species and GFLV isolates that may cause leaf symptoms ranging from fanleaf to yellow mosaic, vein banding, line pattern, chlorotic ringspots and mottling. These symptoms have been described in numerous grapevine varieties in several countries (Martelli and Savino, 1990) and in herbaceous hosts (Huss *et al*., 1989; Vuittenez *et al*., 1964).

The virus genome is composed of two single-stranded positive polarity linear RNA segments (RNA1 and RNA2), each containing one open reading frame (ORF) and coding for a polyprotein. The polyprotein encoded by RNA1 (P1) is processed into five proteins, from N- to C-terminus, including a putative proteinase cofactor, putative helicase, virus genome-linked protein (VPg), cysteine proteinase and a putative RNA-dependent RNA polymerase (RdRp). RNA2 codes for P2 polyprotein which is proteolitically processed into three proteins including a homing protein (HP), movement protein (MP) and coat protein (CP), from N- to C-terminus, respectively. Both RNA1

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and RNA2 of GFLV strain F13 have been sequenced which are 7342 and 3774 nucleotides (NTs) in length, respectively. GFLV isolates from Germany, France, USA, Tunisia, Slovenia, Jordan and Iran varying genetically in the CP region have been reported (Sokhandan Bashir *et al*., 2007 b; Vignet *et al*., 2005, 2004 a,b; Anfoka *et al*., 2004; Izadpanah *et al*., 2003; Naraghi-Arani *et al*., 2001).

We have previously molecularly characterized GFLV isolates from the northwest region of Iran (Sokhandan Bashir *et al*., 2007 b). However, cDNA fragments lacking 50 bps at the 5′ end of the CP gene were amplified with previously designed primers that were not intended for amplification of the full-length CP gene. In addition, while using such primers many samples did not give amplification of the expected fragments. The present study aimed at optimizing a protocol with new primers in order to amplify the complete CP gene (1515 bp including the stop codon) from local isolates. These sequences were used to assess the CP genetic diversity and to perform phylogenetic analyses. Besides, the amplification of complete CP provides foundation for preparation of recombinant antibody against the virus which is currently going on in our lab.

MATERIALS AND METHODS

Sampling, initial screening and inoculation tests: Grapevine leaf samples with GFLV symptoms were collected from vineyards in East- and West-Azarbaijan and Ardebil provinces during late spring, summer and early autumn 2007. For each plant the same sample of 0.5 g of leaf tissues was subjected to double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977) and to reverse transcription polymerase chain reaction (RT-PCR). DAS-ELISA was carried out using a 1:200 dilution of polyclonal rabbit anti-GFLV IgG and its alkaline phosphatase conjugate (Loewe, Germany). The absorbance values were measured at 405 nm wavelength in Anthus-2020 ELISA plate reader (Anthus, Austria). For the inoculations, leaf tissue (1g) from the DAS-ELISA-positive samples was ground in 5 ml of 0.1 M potassium phosphate buffer pH 7.2 containing 0.15% 2-mercaptoethanol and 2.5% nicotin in a sterile mortar and rubbed onto carborundum- dusted leaves of *Chenopodium quinoa* plants at 4-, 6- or 8-leaf stage. The plants were kept in glasshouse under 20ºC, 50-60% relative humidity and 16 h photoperiod, and were regularly checked for the appearance of symptoms.

Primer design: Nucleotides sequences of CP from previously reported GFLV variants including isolates from Iran (Sokhandan Bashir *et al*., 2007b) were aligned and a consensus sequence was determined. Then, two specific primers, GFLV2048 and GFLC3559, were designed according to the consensus sequence using Oligo 5.0 software (Wojciech, 1994). GFLV-2048 (5′-ACGGATCCGGATTAGCTGGTAGAG-GAG-3′) and GFLV-3559 (5′-GTCAAAGCTTCTA-GACTGGGAAACTGG-3′) corresponded to nucleotides 2048-2066 and 3541-3559 of GFLV strain F13 RNA2, respectively. The restriction sites *Bam* HI (GGATCC), *Hind* III (AAGCTT) and *Xba* I (TCTAGA) were engineered in the primers to facilitate cloning. The restriction sites were incorporated in a manner to bear the least heterogeneity with the virus genome (GFLV-F13). Accordingly, only 5 NT mismatches and at the 5′ ends were imposed to each primer.

RT-PCR: Total RNA from 0.1 g leaf tissue from DAS-ELISA-positive, symptomatic ELISA-negative and healthy grapevines was extracted according to Rowhani *et al*. (1993) and suspended in 25 µl sterile distilled water. Reverse transcription with oligo $d(T)_{18}$ and amplification using GFLV2048/ GFLC3559 were performed as described in Sokhandan Bashir *et al*. (2007 b). The initial denaturing was done at 94ºC for 90 s, followed by 35 cycles of 94ºC for 30 s, annealing at 48°C for 30 s and extension at 72ºC for 2 min and a final extension at 72°C for 7 min. Then, PCR products were run on 1.2% agarose in 0.5 × TBE buffer containing 0.5 µg/ml ethidium bromide (Sambrook and Russell, 2001).

Sequence determination of the CP gene: About 20 ng of each PCR product was ligated into 50 ng of the T/A cloning plasmid, pTZ57R/T (Fermentas, Lithuania) in a 10 µl reaction. The ligation mix was prepared according to the manufacturer's protocol and kept at 4°C overnight. Competent cells of *Escherichia coli* strain TG1 was prepared according to Chung *et al*. (1989) and transformed with 1 µl of the ligation mix. After selection of desired colonies on ampicillin/ IPTG/ X-Gal plate and subjecting to plasmid extraction and restriction analysis, they were subjected to single-cell isolation. Then, plasmids from three clones of each sample (isolate) were subjected to dideoxy terminator cycle sequencing in Macrogen Inc. (Seoul,

Table 1. *Grapevine fanleaf virus* strains/ isolates and *Arabis mosaic virus* strain S (ArMV-S) whose coat protein gene sequences were retrieved from GenBank for the parsimonious analysis.

Accession(s)[a]	Isolate/strain	Host	Country
DQ362932	FA31	*Vitis rupestris* St George/Favorita	Italy
DQ362934	LGR12	*V. vinifera*/Lambrusco	Italy
DQ362933	LS2	Grasparossa	Italy
DQ362923-	SG10, SG11, SG12,	*V. vinifera*/Lambrusco Salamino	Italy
DQ362926	SG16	*V. vinifera*/Sangiovese	
U11768	Aust	Grapevine (no cv was specified)	Austria
DQ362921 -	SG1, SG4	*Vitis vinifera*/Sangiovese	Italy
DQ362922			
DQ362928 -	NE166, NE83,	*V. rupestris* St. George/Nebbiolo	Italy
DQ362930	NE185		
AY370941	A2b	*V. vinifera* cv Chardonnay	France
AY370944	A6e	*V. vinifera* cv Chardonnay	France
AY370946	A7a	*V. vinifera* cv Chardonnay	France
AY370949	A10a	*V. vinifera* cv Chardonnay	France
AY370951	A12a	*V. vinifera* cv Chardonnay	France
AY370953	A14a	*V. vinifera* cv Chardonnay	France
AY370961 -	A19a, A19c	*V. vinifera* cv Chardonnay	France
AY370962			
AY370965	A21a	*V. vinifera* cv Chardonnay	France
AY370969	A26b	*V. vinifera* cv Chardonnay	France
AY370973	A30b	*V. vinifera* cv Chardonnay	France
AY370975	A30f	*V. vinifera* cv Chardonnay	France
AY370982	A32e	*V. vinifera* cv Chardonnay	France
AY370984	A33a	*V. vinifera* cv Chardonnay	France
AY370986 -	A36a, A38b	*V. vinifera* cv Chardonnay	France
AY370987			
AY370990	A39a	*V. vinifera* cv Chardonnay	France
AY370992	A40a	*V. vinifera* cv Chardonnay	France
AY371000	B11c	*V. vinifera* cv Chardonnay	France
AY371004 -	B13c, B16a	*V. vinifera* cv. Chardonnay	France
AY371005			
AY371008	B19b	*V. vinifera* cv Chardonnay	France
AY371011 -	B2a, B3a	*V. vinifera* cv Chardonnay	France
AY371012			
AY371015 -	B4c, B4e	*V. vinifera* cv Chardonnay	France
AY371016			
AY371018 -	B6a, B6b	*V. viniferaa* cv. Chardonnay	France
AY371019			
AY371023	B8a	V. vinifera cv Chardonnay	France
AY371025	GFLV	*V. vinifera* cv Cabernet franc	France
AY371026	Hungary	cv Gloria Hungariae	Hungary
AY780899	A17a	*V. vinifera* cv Chardonnay	France
DQ362927	MS43	*V. rupestris* St. George/Moscato	Italy
DQ362931	Do64	*V. rupestris* St. George/Dolcetto	Italy
DQ362935	FR113	*V. rupestris* St. George/Freisa	Italy
DQ526452	Ch-80	*V. vinifera* cv Chardonnay	France
AY997693	S1	*V. vinifera*	Iran
AY997694	S1	*V. vinifera*	Iran
AY997695	B5	*V. vinifera*	Iran
AY997696	B5	*V. vinifera*	Iran
AY997697	SH3	*V. vinifera*	Iran
AY997698	SH3	*V. vinifera*	Iran
AY997699	SH3	*V. vinifera*	Iran

Continue in next page.

Accession(s)[a]	Isolate/strain	Host	Country
GU972580	CACSB5	*V. vinifera* cv Chardonnay	USA
GU972578 -	CACSB3,	*V. vinifera* cv Cabernet Sauvignon	USA
GU972579	CACSB4	*V. vinifera* cv Cabernet Sauvignon	USA
GU972583	CACSC3		
GU972571	CAZINA1	*V. vinifera* cv Zinfandel	USA
GU972573	CAZINA3	*V. vinifera* cv Zinfandel	USA
GQ332370	WAME1492	*V. vinifera* cv Merlot	USA
GQ332364	WACH911	*V. vinifera* cv Chardonnay	USA
GQ332368 -	WAPN173,	*V. vinifera* cv Pinot Noir	USA
GQ332369	WAPN8133		
X81815	ArMV-S	Not cited	France

[a]Successive accessions sharing similar characteristics are merged.

South Korea) with the universal primers M13 F and M13 R.

Sequencing and phylogenetic analyses: The CP clones from the Iran isolates of GFLV were aligned with CP sequences of previously reported GFLV isolates and *Arabis mosaic virus strain* S (ArMV-S) using GeneDoc (Nicholas and Nicholas, 1997). Phylogenetic trees were inferred based on distance and parsimony approaches by the use of Phylip package 3.65 (Felsenstein, 2004). Both NT and deduced amino acid (AA) sequences were subjected to 100-replicates of bootstrap by the use of SEQBOOT before further analyses.

Initially, a distance tree was generated on the basis of nucleotide sequences of 202 GFLV strains/ isolates including the isolates reported in this study, our previous study (Sokhandan Bashir *et al*., 2007 b), and the isolates reported from other parts of the world together with ArMV-S as the outgroup. The bootstrapped alignment was subjected to DNADIST program wherein Kimura distance correction method was applied (Kimura, 1980) because of frequency of transitional substitutions. Then, neighbor joining trees were constructed by the use of Neighbor program and followed by choosing a maximum likelihood (ML) tree by CONSENSUS program wherein branches with <70% bootstrap values were unresolved. Since there were many branches on the tree making interpretation uneasy, one or two representative isolates were chosen from each cluster bearing isolates with <2% distance apart. Thus, an alignment of 77 GFLV strains/ isolates and ArMV-S (Table 1) were subjected to parsimonious analysis as follows. The NT or AA sequence alignment was subjected to DNAPARS or PROTPARS, respectively, with multiple data set analysis option and followed by choosing a consensus tree from among the parsimonious trees by the use of CONSENSUS program. The inferred trees were opened and viewed in Treeview program (Page, 1996).

RESULTS

Screening by DAS-ELISA and inoculations: GFLV was detected by DAS-ELISA in 89 of the 330 grapevines from all the three provinces. The infectivity assays provided additional evidence as to the identity of the infecting virus being GFLV. The inoculated plants showed leaf distortion, vein banding and chlorotic spots 15 dpi (days post inoculation) corresponding to GFLV infection in *C. quinoa* (Izadpanah *et al*., 2003). We detected GFLV by DAS-ELISA in the inoculated plants 20 dpi (data not shown).

RT-PCR amplification: This reaction was optimized by performing PCR in a range of annealing temperatures from 48 to 60°C with an increment of 2°C. Annealing at 48°C for 30 s resulted in amplification of an intense expected ~1520 bp DNA band from 16 grapevines including samples from East- and West- Azarbaijan provinces (data not shown), whereas no amplification was obtained from the healthy grapevine (negative control). Seventy three of the 89 samples that were positive in DAS-ELISA gave no amplification in RT-PCR. Inversely, there were two samples, F4 and F12, that produced the expected ~ 1520 bp DNA fragment in RT-PCR although they had not given positive reactions in DAS-ELISA (Table 2).

Cloning and sequencing and phylogenetic analysis: Fragments amplified from samples S4, KH12, KH11, KH4, MG28, KJ16, KJ18, and MK14, representing 8

Table 2. Characteristics of infected grapevine samples from the northwest region of Iran giving amplification of *Grapevine fanleaf virus* coat protein gene in RT-PCR.

Sample(s)	Location[a]	Symptoms	DAS-ELISA	RT-PCR
S4[b]	Sardroud	Fanleaf	+	+
(KH4, KH11, KH12)[b], KH23	Khal'at pushan	Vein banding	+	+
MG28[d]	Marageh	Vein banding	+	+
(KJ16 and KJ18)[b]	Kheldjan	Vein banding	+	+
T1	Tabriz	No symptoms	+	+
MK3 and MK14[b]	Malekan	Vein banding	+	+
F4 and F12	Fakhrabad	Vein banding	-	+
L7, L13 and L14	Lahroud	Vein banding, fanleaf		+

[a]Sardroud, Khal'at pushan, Kheldjan, Marageh, Malekan, and Tabriz in East Azarbaijan; Fakhrabad and Lahroud in Ardebil province. [b]Cloned, sequenced and the data were submitted to GenBank and assigned the accession numbers from FJ513376 to FJ513386.

isolates, were selected for further analyses because of feasibility of cloning and sequencing due to their intensities. Alignment of sequencing data from 8 new GFLV isolates with those of previously reported GFLV strains/ isolates and ArMV-S showed that a 1515 bp fragment was amplified from new isolates corresponding to the complete CP region of GFLV. This provided further evidence as to the GFLV identity of the infecting virus. At the NT level, there were over 99% similarities within clones from each isolate. Between clones from different isolates, the lowest NT similarity (92%) was found between KH4-5-3 and S-4-2-1 or KJ-16-2-3; the highest (98%) between KH4-5-3 and MG-28-1-3. At deduced AA level, the lowest similarity (95%) was found between KH4-5-3 and S-4-2-1. When the previously reported isolates from Iran (partial sequences, 1461 NTs) (Sokhandan Bashir *et al*., 2007 b) were included in the comparison, the lowest similarity was observed between clones of B5 and several other isolates. The majority of substitutions were transitions that were also observed with the previously reported isolates from Iran.

On the ML parsimonious trees regardless if NT or AA data were used, the Iran GFLV isolates formed a distinct cluster except for a previously reported isolate from Iran, B5, that clustered with other isolates when the analysis was based on NT sequences (Sokhandan Bashir *et al*., 2007 b) (Fig. 1). Interestingly, GFLV isolates from Iran were the only geographical isolates forming a distinct cluster although a fraction of GFLV isolates from some other countries, particularly France, also stood distinct. There, seemed to be a correlation between geographical origin of the isolates and their phylogenetic positions at large scale (world) as the isolates from Iran stood distinct. However, among these new isolates (at regional scale) no correlation could be drawn with respect to their origin and CP genotype. For instance, KH4 was more homologous to MG28 than to KH11 and KH12 although KH4, KH11 and KH12 shared the same origin (Khal'at pushan, Tabriz) whereas MG28 came from another region (Maraghe).

DISCUSSION

In this study, complete CP gene was cloned and sequenced from GFLV isolates from vineyards in the northwest region of Iran. Along with the results from our previous studies (Sokhandan Bashir *et al*., 2007 b), the current research further demonstrated a frequent occurrence of the virus in these vineyards. However, as observed in the previous studies, the majority of the diseased grapevines did not appear to contain GFLV as revealed by DAS-ELISA and RT-PCR. Such results are not uncommon in the surveys for plant viruses because similar symptoms can be caused by different virus species or even strains (Hull, 2002). As an example, GFLV was not detected in majority of symptomatic grapevine samples from Jordanian vineyards (Anfoka *et al*., 2004) and, as a result in that study, GFLV-like symptoms on 241 ELISA-negative grapevines were attributed to herbicides, other grapevine-infecting viruses or *Grapevine yellow speckle viroid*. In the present study, majority of DAS-ELISA- positive samples (73 of 89) gave no amplification of any DNA fragment in RT-PCR although they showed symptoms similar to those caused by GFLV. These diseased grapevines might be infected with distant variants of GFLV and/ or perhaps other grapevine infecting viruses such as ArMV that serologically cross reacts with anti-GFLV polyclonal IgG (Wetzel *et*

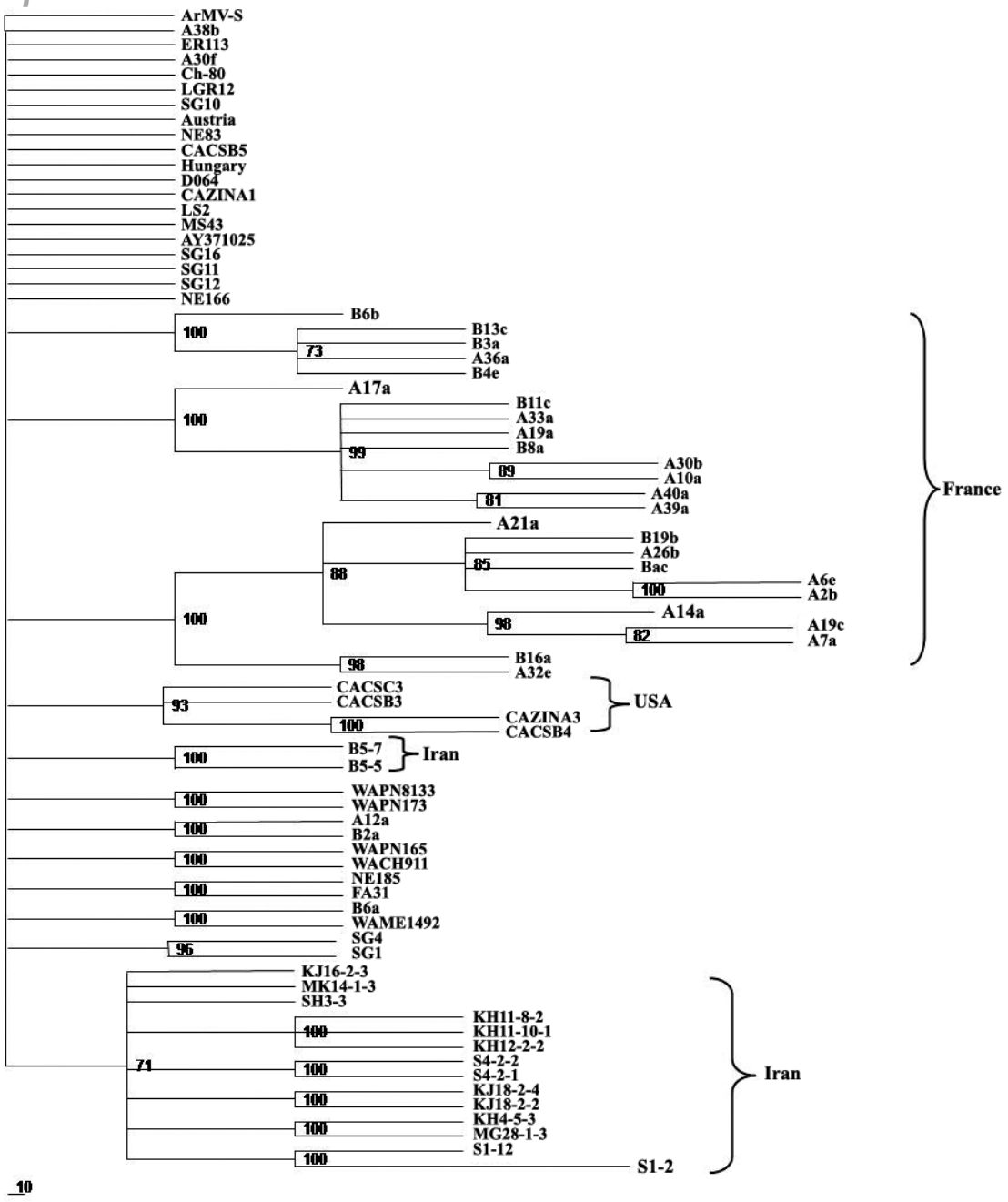

Figure 1. A maximum likelihood (>0.7 boostrap supports) parsimonious tree inferred from the nucleotide sequences of coat protein of *Grapevine fanleaf virus* isolates from Iran (right brace) and previously reported strains/ isolates of the virus plus *Arabis mosaic virus* strain S (ArMV-S) as the designated root. Numbers on the branches are bootstrap values and branch lengths are proportional to these values. The scale bar on the bottom left hand represents 10% bootstrap value.

al., 2002). Because of high conservation at the primer sites it was not thought that degenerate primers could be more useful. However, what can be drawn from this result is that there might be endogenous GFLV variants in these samples very different from so far characterized isolates. Inversely, there were two DAS-ELISA-negative samples including F4 and F12 (Table 2) that produced the anticipated ~1520 bp fragment in RT-PCR suggesting that the titer of GFLV in these samples might be too low to be detected by DAS-ELISA though enough for detection by the RT-PCR. No amplification could be achieved from samples collected from West Azarbaijan province. However, by DAS-ELISA, the virus was also detectable in such samples.

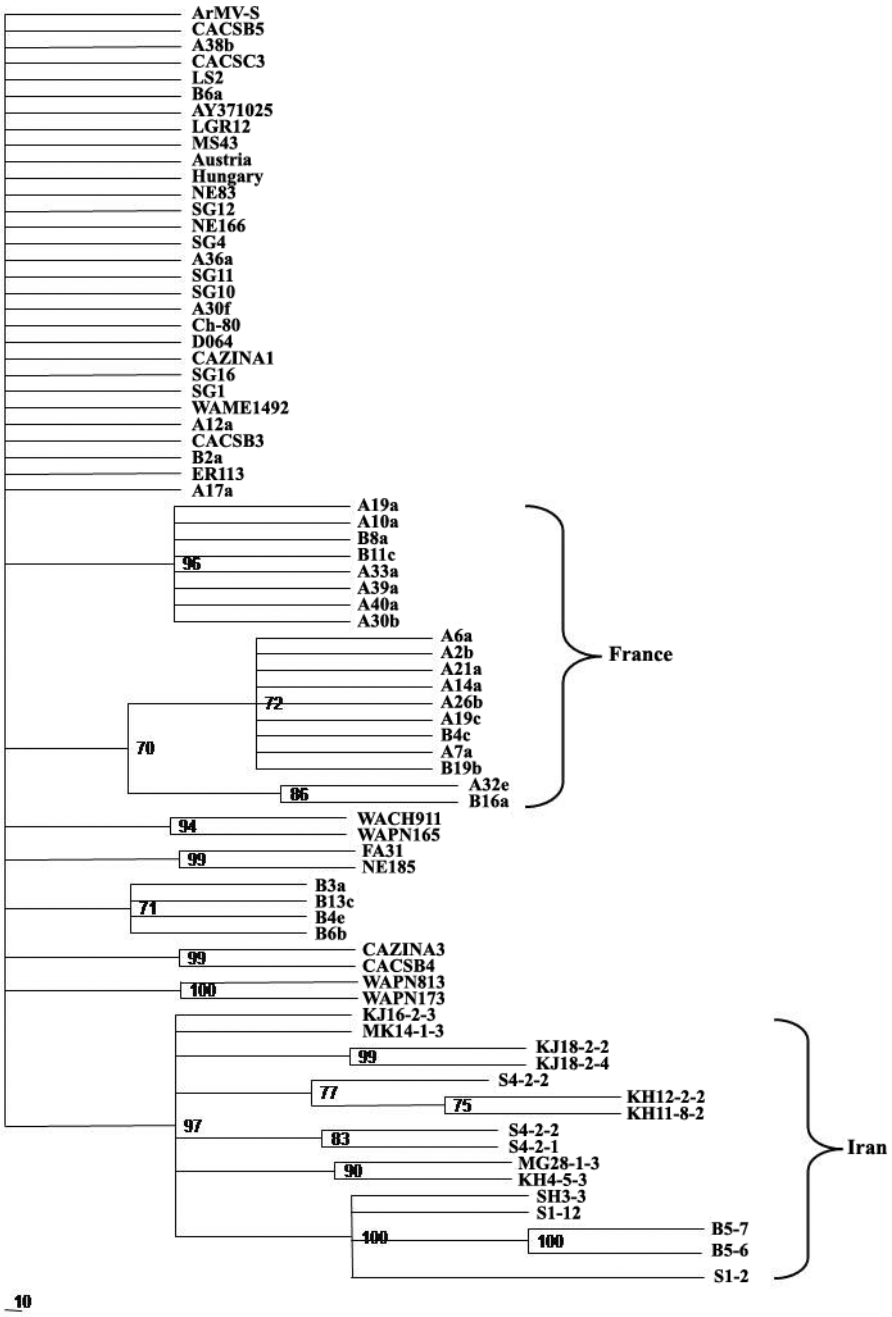

Figure 2. A maximum likelihood (>0.7 boostrap supports) parsimonious tree based on coat protein amino acid sequences of *Grapevine fanleaf virus* strains/isolates. Further characteristics of the tree are as described in Figure 1.

This again might be suggestive of highly different genotypes of GFLV residing in such samples. Such a speculation could also be supported by the hypothesis that GFLV may have arisen from this part of the world (Vuittenez, 1970) so that because of going through a long evolutionary process, very divergent variants may have resulted. One efficient approach to dig out such genotypes from the diseased samples would be application of the virus detection methods based on probes that are made according to highly conserved regions of the virus genome. Alternatively, the novel metagenomic approach may be applied which begins with isolation of virus-like particles from plants (Melcher *et al*., 2008).

When a range of annealing temperatures from 48 to 60°C was used in the PCR it became evident that the

least applied temperature, 48°C, was optimal for the amplification. This might be suggestive of heterogeneities in the virus genotypes. Primers are crucial components of a PCR reaction and, as a result, an accurate primer design plays a basic role in the success of a PCR reaction. Here, we had previously used primers which were published by others (Wetzel *et al*., 2001) for amplification, cloning and sequencing cDNA segments of the virus RNA2 from the local isolates (Sokhandan Bashir *et al*., 2007 a,b). Alignment of these overlapping sequence data helped us to generate a consensus CP sequence that was used as the template to design new primers. Subsequently, amplifications from a larger number of samples were achieved compared to our previous report (Sokhandan Bashir *et al*., 2007 b).

Amplification of full length CP gene (1515 bp) from the GFLV isolates has the following important implications. First, assessment of virus variation at the full length CP region is an established procedure for studying genetic variability (e.g., Vigne *et al*., 2004 a and b; Naraghi-Arani *et al*., 2001). Second, the CP is important to the biology of the virus, i.e. it provides stability of the virus, dictates transmission by vector and directs synthesis of anti-virus antibody (e.g., Hull, 2002). Thus, preparation of recombinant viral antibodies will not be possible unless the CP gene is cloned and sequenced. This technique is gaining popularity and expected to become an applicable technique even in developing countries wherein virus purification equipment such as high speed- and ultra- centrifuges are generally unavailable (Bashir, 2007). Third, amplification of CP sets ground for routine RT-PCR amplification of the virus CP cDNA for downstream works. Here, the new primers were according to the local isolates and proved to be efficient in picking the virus genotypes for further studies.

Analysis of the CP sequence data revealed a genetic diversity of up to 8% between the new isolates; however, when previously sequenced clones were also taken into account diversity of up to 17% was revealed between the clones of isolate B5 and several other isolates. It was interesting to note that even in the previous study, B5 stood alone from other two isolates suggesting that this isolate might be imported into the region from other countries probably via rootstocks (Sokhandan Bashir *et al*., 2007 b). Another possibility would be more frequent occurrence of recent mutations in this isolate because based on AA sequences it is grouped with the remaining Iran isolates (Fig. 2). Accordingly, on the NT-based ML parsimony tree the isolate B5 remained distinct from other GFLV isolates from Iran (Fig. 1). Since all the Iran GFLV isolates, except B5, formed a distinct cluster, this maybe suggestive of an independent evolution pathway for these isolates. Taking into consideration that the origin of GFLV might be in Iran (ancient Persia) (Vuittenez, 1970) the current finding may provide further support for this hypothesis. Although the distinct position relates the geographical origin (Iran) of the isolates with their genotypes, at the regional scale (localities of vineyards) there appeared to be no such correlation perhaps implying that these isolates are the descendents of one ancestor.

For future direction, we plan to design newer primers by involvement of newly reported sequences. Such primers may pick more isolates in the local vineyards particularly in West Azarbaijan province where no amplification was made in this study. Also, preparation of recombinant anti-GFLV antibody is going on in our lab. By the availability of such antibody, initial screening may be done more efficiently in a wider range of vineyards. Then, more detailed information would be achievable on the virus diversity and evolution.

CONCLUSIONS

In this study, full GFLV CP cDNA for the first time was precisely a mplified by the use of newly-design primers from GFLV- infected samples from Iran. This opened new avenues as to the establishment of molecular detection protocol for the virus isolates, analysis of the virus diversity at the CP level, and preparation of the virus recombinant antibody.

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