

SHORT NOTES

## Detection and phylogenetic analyses of fig-infecting viruses in Bosnia and Herzegovina and Montenegro

DUŠKA DELIĆ<sup>1\*</sup>, TATJANA PEROVIĆ<sup>2\*</sup>, SNJEŽANA HRNČIĆ<sup>3</sup>, BILJANA LOLIĆ<sup>1</sup>, GORDANA ĐURIĆ<sup>1</sup> and TOUFIC ELBEAINO<sup>4</sup>

<sup>1</sup> University of Banjaluka, Faculty of Agriculture, Bulevar vojvode Petra Bojovića 1A, 78000 Banjaluka, Bosnia and Herzegovina

<sup>2</sup> University of Montenegro, Biotechnical Faculty, Center for subtropical cultures, Bjelisi bb, 85000 Bar, Montenegro

<sup>3</sup> University of Montenegro, Biotechnical Faculty, Centre for Plant Protection, Mihaila Lalića 1, 81000 Podgorica, Montenegro

<sup>4</sup> Istituto Agronomico Mediterraneo di Bari (IAMB), Via Ceglie 9, 70010, Valenzano (BA), Italy

**Summary.** During spring 2016, a survey was carried out in Bosnian-Herzegovinian (BiH) and Montenegrin (MNE) fig orchards, germplasm collection plots and outdoor gardens, to investigate the presence of unreported fig viruses possibly present in both countries, *i.e.* Fig leaf mottle-associated virus 2 (FLMaV-2), *Fig latent virus 1* (FLV-1), *Fig cryptic virus 1* (FCV-1), Fig fleck-associated virus (FFkaV) and *Fig badnavirus 1* (FBV-1); as well as those previously reported, *i.e.* Fig leaf mottle-associated virus 1 (FLMaV-1), Fig mild mottle-associated virus (FMMAV) and *Fig mosaic emaravirus* (FMV). A total of 84 fig samples (49 from BiH and 35 from MNE) were collected and tested by PCR/RT-PCR using sets of virus-specific primers. Results showed that FBV-1 was the prevailing virus with all samples (100%) infected, followed by FLMaV-1 (54%), FMV (35%), FMMAV (7%), FFkaV (6%) and FLMaV-2 (1%); whereas FLV-1 and FCV-1 were not detected. Excluding the FBV-1 detection, 35% of tested trees were infected with at least one other virus. Sequence analyses of PCR/RT-PCR fragments obtained from different viruses showed that FBV-1 was the least variable (0.9% of nucleotides divergent) compared with FLMaV-1 (15.7% sequence variation), FLMaV-2 (17.4%), FMMAV (14.9%), FMV (16.9%) and FFkaV (14.3%). Phylogenetic trees constructed with obtained sequences, together with their homologues retrieved from the Genbank database, showed distinct separation of the BiH and MNE isolates from those of different origins, in particular for FFkaV and FMV; whereas for closteroviruses (FLMaV-1, FLMaV-2 and FMMAV), there was no distinction between the isolates. This is the first report on sequence analyses of fig viruses in this geographical region, and of the presence of FBV-1 in BiH and MNE, and of FLMaV-2 and FFkaV.

**Key words:** *Ficus carica*, PCR, sequence analyses.

### Introduction

Fig (*Ficus carica* L.) production in Bosnia and Herzegovina (BiH) is concentrated in Herzegovina, and Montenegro (MNE) along the Adriatic coastal regions, where mild Mediterranean climate prevails. In both countries the common fig is traditionally grown as individual or scattered trees in outdoor gardens. Although its cultivation is increasing in commercial orchards with intensive production, little is known

about this crop, particularly relating to virus infections. Only a few appreciated and varieties are selected and multiplied in collection plots in both countries. To date, nine viruses detectable by PCR/RT-PCR assays have been recorded in fig. These are: Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottle-associated virus (FMMAV), *Fig mosaic emaravirus* (FMV), *Fig latent virus 1* (FLV-1), *Fig cryptic virus 1* (FCV-1), Fig fleck-associated virus (FFkaV), *Fig badnavirus 1* (FBV-1) and *Strawberry latent ringspot virus* (SLRSV) (Elbeaino *et al.*, 2007a; 2009; 2010; 2011a; 2011b; 2012; 2015; Gattoni *et al.*, 2009; Laney *et al.*, 2012). However, other viruses belonging to different

Corresponding author: T. Elbeaino

E-mail: elbeaino@iamb.it

\* These authors contributed equally to this work

genera such as *Potyvirus*, *Carlavirus*, *Umbravirus*, *Luteovirus*, *Cripavirus*, and *Sobemovirus* have also been reported to infect this crop (Martelli, 2011).

Some of these viruses, *i.e.* FLMaV-1, FMaV and FMV, were only recently reported from BiH and MNE, in two surveys conducted on a limited number of samples (26 fig trees) collected from a germplasm collection plot and outdoor gardens (Delić *et al.*, 2016; Perović *et al.*, 2016); with most trees exhibiting mosaic symptoms, typical of FM disease (Elbeaino *et al.*, 2009). The aims of the present research was to further evaluate the virus status of fig in both countries by assaying a greater number of samples and viruses (FLMaV-1, FLMaV-2, FMaV, FMV, FCV-1, FLV-1, FFkaV and FBV-1), and to study their sequences in comparison with homologues reported in the Genbank database.

## Materials and methods

During spring 2016, a survey was carried out in different fig-growing areas located at Mostar, Trebinje, Ljubuski, Grude (BiH) and Podgorica, Bar (MNE), to collect samples from fig germplasm collection plots, orchards and outdoor gardens. In total, 84 leaf samples (49 from BiH and 35 from MNE) were taken from symptomatic trees showing different symptoms including, leaf discolouration, chlorotic ring spotting and leaf malformation. Only the most important local fig varieties in both countries were surveyed, *i.e.* Petrovača bijela, Petrovača crna, Tenica, Sušilica and Zimica.

Total RNA and DNA were extracted from leaf midribs using, respectively, RNeasy and DNeasy plant mini kits (Qiagen), following the manufacturers' instructions. For RT-PCR assays, RNA templates (10 µL) were reverse-transcribed into cDNA using random hexamers (0.5 µg) (Invitrogen), after denaturation at 94°C for 5 min and quickly chilling in ice, in the presence of M-MLV reverse transcriptase enzyme (200 units µL<sup>-1</sup>) (BioLabs), in a final volume of 20 µL, for 1 h. at 39°C. A final step for enzyme denaturation was conducted at 70°C for 10 min. RT-PCR and PCR assays for FBV-1 were performed using 2.5 µL of cDNA in a total volume of 25 µL, using virus-specific primers (Supplementary Table 1) and conditions described in Elbeaino *et al.* (2006; 2007a; 2009; 2010; 2011a; 2011b) and Laney *et al.* (2012). PCR products were each analyzed in a gel of 1.2% agarose in TAE buffer and stained with

GelRed (Biotium) and visualised under a UV transilluminator.

PCR amplicons were sequenced bi-directionally with forward and reverse virus-specific primers using the Sanger method on a Macrogen 3730XL05-16108-002 instrument. Nucleotide sequences were analyzed with the assistance of the DNA Strider 1.1 program (Marck, 1988). Multiple alignments of nucleotide sequences were obtained using the default options of CLUSTALX 1.8 (Thompson *et al.*, 1997). Search for homologies with proteins from the Protein Information Resources database (PIR, release 47.0) was carried out with the FASTA (Pearson and Lipman, 1988) and BlastX programs (Altschul *et al.*, 1990). Tentative phylogenetic trees were constructed using the NJPLOT package, with 1000 bootstrap replicates (Perrière and Gouy, 1996).

## Results and discussion

Based on PCR/RT-PCR results, six viruses (of eight here investigated) were present in the collected fig samples from both countries, with different levels of infection. Among the investigated viruses, FBV-1 was the most prevailing with an infection rate of 100%. Infection rates for the other detected viruses were less: for FLMaV-1, 54% of samples; FMV, 35%; FMaV, 7%; FFkaV, 6%; and for FLMaV-2, 1% found only in BiH (Table 1). FLV-1 and FCV-1 were not detected in any of the samples. The six detected viruses were found in all fig varieties, often as multiple infections.

The high incidence of virus infections (100%) observed for FBV-1 was expected, given the widespread incidence of this virus in many countries (Iran, Italy, Turkey, Tunisia, Palestine) (Elci *et al.*, 2012; Alimoradian *et al.*, 2014; Alkowni *et al.*, 2015; Elair *et al.*, 2015). However, less expected was the high incidence of FLMaV-1 (54%) which was the second most prevalent virus in the samples, and was more commonly detected than FMV. FMV is known to be the most widespread virus in fig orchards, together with FBV-1, with cosmopolitan distribution and fast transmission through virus vectoring eriophyid mites (*Aceria ficus*). The high incidence of FLMaV-1 could be due to the high populations of an insect likely to be a typical vector, such as pseudococcids (including *Planococcus ficus*, *Pl. longispinus*) (T. Elbeaino, personal communication). These insects are also known for their transmission of closterovir-

**Table 1.** Levels of infection for Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottle-associated virus (FMMaV), Fig mosaic emaravirus (FMV), Fig fleck-associated virus (FFkaV), Fig latent virus 1 (FLV-1), Fig cryptic virus 1 (FCV-1), Fig badnavirus 1 (FBV-1) from a virus survey of fig trees in Bosnia and Herzegovina and Montenegro during spring, 2016.

Bosnia and Herzegovina						Montenegro				
FBV-1	FLMaV-1	FMV	FMMaV	FFkaV	FLMaV-2	FBV-1	FLMaV-1	FMV	FMMaV	FFkaV
49/49 (100%)	25/49 (51%)	20/49 (41%)	4/49 (8.1%)	4/49 (8.2%)	1/49 (2%)	35/35 (100%)	20/35 (57%)	9/35 (26%)	2/35 (5.7%)	1/35 (2.8%)

ids in general (Tsai *et al.*, 2010). Pseudococcids were found to be abundant on fig trees during the surveys we carried out. In general, the levels of virus infections here obtained were similar to those previously reported from the other Mediterranean countries (Aldhebani *et al.*, 2015; Alkowni *et al.*, 2015; Elair *et al.*, 2015; Alimoradian *et al.*, 2014; Danesh-Amuz *et al.*, 2014; Alhudaib *et al.*, 2012; Elci *et al.*, 2012; Elbeshehy and Elbeaino, 2011; Elbeaino *et al.*, 2009a, 200b, 2007; 2006). However, it was difficult to determine the nature of some virus-like symptoms observed in some fig plants, for which no viruses were detected. Whether these symptoms are induced by unknown biotic or abiotic factors are yet to be investigated.

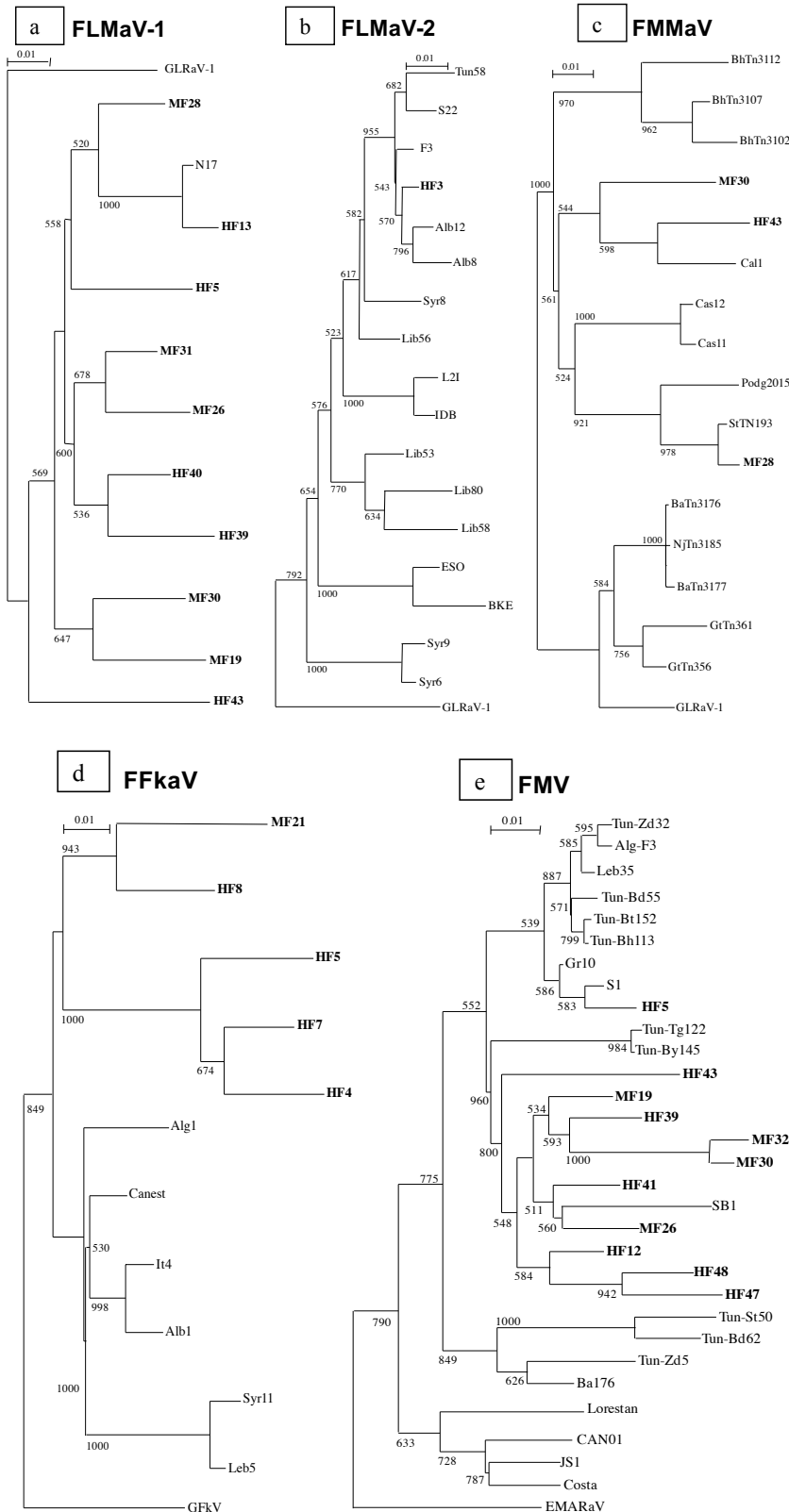
At the sequence level, 20 viral DNA were obtained from sequencing RT-PCR amplicons generated from different fig trees infected with FLMaV-2, FMMaV, FFkaV and FBV-1. These were used to determine the genetic variability of the newly recorded viruses in BiH and MNE, in comparison with previous reports. Accession numbers of sequences obtained, together with those retrieved from the Genbank database, are reported in Supplementary Table 2.

The nucleotide analyses of the three newly recorded viruses in BiH and MNE (FBV-1, FFkaV and FLMaV-2) showed that sequences of FBV-1 amplicons (five from BiH and eight from MNE, all generated from different infected plants) had 99.1–100% identity between them, and with isolates reported in the Genbank. In contrast, sequences of FFkaV, obtained from five different samples (four from BiH and one from MNE), of FLMaV-2 (one from BiH), and of FMMaV (one from MNE) showed identities that ranged from 86 to 90% for FFkaV, 82.6 to 91% for FLMaV-2, and 85.1 to 86.3% for FMMaV, with their homologues in the database. Additional sequence analyses were conducted on previously reported

FMV viruses from BiH and MNE, which showed 83.1 to 96.6% of identity (Delić *et al.*, 2016; Perović *et al.*, 2016). The phylogenetic trees constructed, based on the sequences here obtained and those retrieved from the Genbank for each virus, showed no distinct separation between all isolates of FBV-1 from different origins (tree not shown). However, for FFkaV, all BiH and MNE isolates were grouped according to their geographical origins in one cluster, separated from the Mediterranean isolates making two defined clades [Italy (Canest, It4); Albania (Alb1); Syria (Syr11), Lebanon (Leb5) and Algeria (Alg1)] (Figure 1d). In the case of FMV, BiH and MNE isolates were closer to Mediterranean isolates [Algeria (Alg-F3); Greece (Gr10); Italy (S1); Lebanon (Leb35): Tunisia (TUn-Zd32, Tun-Bd55, Tun-Bt152, Tun-Bh113, Tun-Tg122, Tun-By145, Tun-St50, etc.)] compared to those from other regions such as sequences from Canada (CAN01), Iran (Lorestan), Japan (JS1), or Costa Rica (Costa) (Figure 1e).

In the case of closterovirids (FLMaV-1, FLMaV-2 and FMMaV), all BiH and MNE isolates were closely related in the phylogenetic trees (Figure 1a,b,c). This occasional distribution of isolates in different clusters was probably conditioned by the limited numbers of sequences of isolates present in the database and by the unilateral origin of isolates; all from the Mediterranean basin.

This second study of the virus status of fig in BiH and MNE extends knowledge of the prevalence and spread of fig viruses in both countries, for which limited information was previously available. We report for the first time the presence of FBV-1 in BiH and MNE, and of FLMaV-2 in BiH and MNE. In addition, this study has confirmed the high the high presence of viruses in *F. carica* in these countries. On this basis there is a strong case to initiate a programme of certi-



**Figure 1.** Phylogenetic trees generated from the alignment of nucleotide sequences for partial genes of: a) Fig leaf mottle-associated virus 1 (FLMaV-1), b) Fig leaf mottle-associated virus 2 (FLMaV-2), c) Fig mild mottle-associated virus (FMMAV), d) Fig fleck-associated virus (FFkaV), e) *Fig mosaic emaravirus* (FMV) and BiH, MNE isolates, together with homologue sequences reported in the Genbank (Supplementary Table 2), using the neighbour-joining algorithm, p-distance method and bootstrap consisting of 1,000 pseudoreplicates. Nucleotide sequences of *Grapevine leafroll-associated virus 1* (GLRaV-1, acc.n: KU674797), *Grapevine fleck virus* (GFkV, acc.n:NC\_003347) and *European mountain ash ringspot-associated emaravirus* (EMARaV, acc.n: AY563040) were used as outgroup species to root the trees. Branch lengths represent bootstrap values. Bar represents 0.01 changes per site. Bootstrap values less than 50% are not shown as they are considered unreliable. Bosnia and Herzegovinian (HF) and Montenegrin (MF) isolates were from a virus survey in undertaken fig orchards during spring, 2016 are highlighted in bold.

fied clonal and sanitary selection for the production of virus-free plant material for fig growers in BiH and MNE.

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**Supplementary Table 1.** List of primers used in RT-PCR assays for Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottle-associated virus (FMaV), *Fig mosaic emaravirus* (FMV), Fig fleck-associated virus (FFkaV), *Fig latent virus 1* (FLV-1), *Fig cryptic virus 1* (FCV-1), *Fig badnavirus 1* (FBV-1) detection. HSP70, Heat shock protein 70 kDa; RdRp, RNA-dependent RNA polymerase; CP, Capsid protein; MP, Movement protein.

Virus	Primers (5'-3')	Amplicons	Genes	Reference
FLMaV-1	N17-s: CGTGGCTGATGCAAAGTTTA N17-a: GTTAACGCATGCTTCCATGA	350	HSP70	Elbeaino et al., 2006
FLMaV-2	F3-s: GAACAGTGCCTATCAGTTTGATTTG F3-a: TCCCACCTCCTGCGAAGCTAGAGAA	360	HSP70	Elbeaino et al., 2007a
FMaV	LM3-s: AAGGGGAATCTACAAGGGTTCG LM3-a: TATTACGCGCTTGAGGATTGC	311	HSP70	Elbeaino et al., 2010
FMV	E5-s: CGGTAGCAAATGGAATGAAA E5-a: AACACTGTTTTGCGATTGG	302	RdRp	Elbeaino et al., 2009
FFkaV	CPFk-s: ATGACGACTGTCAACTCCCT CPFk-a: TTAAGCCAGGGTGGGAGTGTG	668	CP	Elbeaino et al., 2011a
FCV-1	R1-s: TCGATTGTCTTTGGAGAGG R1-a: CGCATCCACAGTATCCCATT	353	RdRp	Elbeaino et al., 2011b
FLV-1	Ffup: CGCTTTGCCCAATGTGCAGAT Ffdow: TCGAAGGCCAGAGTTGATGCA	125	CP	Gattoni et al., 2009
FBV-1	1094F: ACCAGACGGAGGGAAGAAAT 1567R: TCCTTGCCATCGGTTATCTC	474	MP	Laney et al., 2012

**Supplementary Table 2.** List of isolates of Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottle-associated virus (FMaV), *Fig mosaic emaravirus* (FMV), Fig fleck-associated virus (FFkaV), and *Fig badnavirus 1* (FBV-1), used in sequence and phylogenetic analyses, together with their GenBank accession numbers and geographical origins; Albania (ALB), Algeria (ALG), Bosnia-Herzegovina (BiH), Canada (CAN), Costa Rica (CRI), Greece (GRE), Iran (IRA), Italy (ITA), Japan (JAP), Lebanon (LEB), Montenegro (MNE), Serbia (SER), Spain (SPA), Syria (SYR), Tunisia (TUN), Turkey (TUR), United Kingdom of Saudi Arabia (KSA).

Virus	Isolate	Accession number	Origin
FLMaV-1	HF5	KU198378	BiH
	HF13	KU198379	
	HF39	KU198380	
	HF40	KU198381	
	HF43	KU198382	
	MF19	KU198384	
	MF26	KU198385	
	MF28	KU198386	
	MF30	KU198387	ITA
	MF31	KU198383	
	N17	AM113547	
	S12	LN873219	KSA

(Continued)

Supplementary Table 2. (Continued).

Virus	Isolate	Accession number	Origin
FMV	HF5	KU198367	BiH
	HF12	KU198368	
	HF39	KU198373	
	HF41	KU198369	
	HF43	KU198370	
	HF47	KU198371	
	HF48	KU198372	
	MF19	KU198374	MNE
	MF26	KU198375	
	MF30	KU198376	
	MF32	KU198377	
	TUN-Zd32	LN908801	TUN
	TUN-Bd55	LN908803	
	TUN-Bt152	LN908807	
	TUN-Bh113	LN908809	
	TUN-By145	LN908806	
	TUN-St50	LN908802	
	TUN-Bd62	LN908804	
	TUN-Zd5	LN908800	
	TUN-Ba176	LN908808	
	TUN-Tg122	LN908805	
	Alg-F3	LN908811	
	Leb35	LN908810	LEB
	Gr10	AM941711	GRE
	S1	LN873221	KSA
	SB1	AB697827	SER
	Lorestan	KT732024	IRA
CAN01	HQ703343	CAN	
JS1	AB697826	JAP	
Costa	KP796425	CRI	
FMMAV	HF43	KU198388	BiH
	MF30	KU198389	MNE
	MF28	KY473899	
	Podg2015	FJ611959	TUN
	BhTn3112	LN850117	
	BhTn3107	LN850115	
	BhTn3102	LN850114	
	TgTn108	LN8750116	
	BaTn3176	LN850119	
	NjTn3185	LN850118	
BaTn3177	LN850121		

(Continued)

Supplementary Table 2. (Continued).

Virus	Isolate	Accession number	Origin
FBV-1	GtTn361	LN850113	
	GtTn356	LN850112	
	StTn193	LN850120	
	Cas12	KC914283	SPA
	Cas11	KC914282	
	Ca1	FJ611959	ITA
	HF7	KY473906	BiH
	HF11	KY473907	
	HF36	KY473908	
	HF43	KY473909	
	HF46	KY473910	
	MF17	KY473911	MNE
	MF18	KY473912	
	MF19	KY473913	
	MF20	KY473914	
	MF22	KY473915	
	MF28	KY473916	
MF30	KY473917		
MF35	KY473918		
FFkaV	HF4	KY473901	BiH
	HF5	KY473903	
	HF7	KY473902	
	HF8	KY473904	
	MF21	KY473905	MNE
	Alg1	FR821256	ALG
	IT4	FR821254	ITA
	Canest	FM200426	
FLMaV-2	Alb1	FR281255	ALB
	Syr11	FR821257	SYR
	Leb5	FR821256	LEB
	HF3	KY473900	BiH
	Tun58	FN687747	TUN
	S22	LN873220	KSA
	IDB	FN687733	TUR
	ESO	FN668734	

(Continued)



Supplementary Table 2. (Continued).

Virus	Isolate	Accession number	Origin
	BKE	FN666270	
	L21	FN666271	
	F3	FJ473383	ALG
	Alb12	FN687736	ALB
	Alb8	FN687735	
	Syr8	FN687745	SYR
	Syr9	FN687746	
	Syr6	FN687744	
	Lib56	FN687739	LEB
	Lib53	FN687738	
	Lib80	FN687743	
	Lib58	FN687741	