Prevalence and diversity of *Grapevine fanleaf virus* in southern Spain

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Short title: GFLV infecting vineyards in southern Spain

The occurrence of *Grapevine fanleaf virus* (GFLV) in 74 vineyard samples in grapevine-growing areas of Andalusia, southern Spain, was investigated as well as the genetic variability of the coat protein (CP) gene on RNA2. Overall, the prevalence of GFLV (the percentage of vineyards with GFLV) was 24.3%, and it was common in Jerez-Xérès-Sherry & Manzanilla-Sanlúcar de Barrameda denomination of origin (D.O.) area (29.4%) and Condado de Huelva D.O. (24.0%), followed by Montilla-Moriles D.O. (13.3%). GFLV-infected plants showed fanleaf and yellow mosaic symptoms together with general vine decline. In almost all samples, except one in Jerez-Xérès-Sherry & Manzanilla-Sanlúcar de Barrameda D.O. and another one in Condado de Huelva D.O., GFLV-infected plants showed soil infestation by the virus vector nematode species *Xiphinema index* and/or the potential vector *X. italiae*. Reverse transcription polymerase chain reaction (RT-PCR) successfully amplified the partial CP gene product of the expected size (555 bp) in leaf and nematode vectors. A total of 135 clones of the partial GFLV CP gene were analyzed for sequence variation using single-strand conformation polymorphism, yielding 12 different haplotype patterns. Haplotypes were separated among the three different D.O. The haplotypes patterns MOB, MOC, MMA, MMC and HB were detected in leaf and nematode vector for each locality, except for Jerez de la Frontera-429. Sequence analysis of the GFLV haplotypes revealed sequence variability within the haplotypes and greater abundance of some variants than others in the same sequenced haplotype pattern. Consequently, GFLV in southern Spain exists as a “quasispecies”, as it has been reported in other grapevine-growing areas of the world. This paper is the first report on molecular variability of GFLV in Spain.

Key words: coat protein gene sequence, dagger nematodes, genetic variability, GFLV,

grapevine, fanleaf degeneration, SSCP, *Xiphinema index*, phylogenetic analysis.

Introduction

Grapevine (*Vitis vinifera* L.) is the second most extensive fruit crop in Andalusia, southern Spain, after olive (MARM, 2008). It is used for table grape and wine grape production. Viticulture in southern Spain is located primarily in the provinces of Cádiz, Córdoba and Huelva. These provinces comprise three geographically-separated climatic zones that include the three major wine-producing areas corresponding to officially recognized “wine denomination of origin (D.O.) zones”: Condado de Huelva D.O. (= CH-D.O.) (Huelva province), Montilla-Moriles D.O. (=Mm-D.O.) (Córdoba province), and Jerez-Xérès-Sherry & Manzanilla-Sanlúcar de Barrameda D.O. (= JMSB-D.O.) (Cádiz province).

Grapevine is under constant threat of viral diseases that may cause severe economic losses (Martelli, 1993). Grapevine fanleaf disease caused by *Grapevine fanleaf virus* (GFLV, genus *Nepovirus*, family *Secoviridae*) is one of the most severe viral diseases of grapevines worldwide, causing extensive leaf yellowing, reduction of fruit quality, and shortening of the plant life-span that leads to up to 80% yield reductions and significant economic losses (Andret-Link *et al.*, 2004a; Esmenjaud & Bouquet, 2009). Three main leaf symptom types have been associated with the disease: fanleaf, yellow mosaic, and vein banding, but no association between these symptoms and virus strains has been observed (Liebenberg *et al.*, 2009). GFLV is specifically transmitted from plant to plant by the ectoparasitic nematodes *Xiphinema index* Thorne & Allen, 1950 under natural conditions (Andret-Link *et al.*, 2004b). There are indications that *Xiphinema italiae* Meyl, 1953 can transmit GFLV under controlled experimental conditions, but this has never been confirmed (Catalano *et al.*, 1992).

The only practical and economical method of controlling viral diseases in viticulture is the use of planting material derived from certified, virus-tested stocks in vineyard replant sites that are free from nematode vectors. Consequently, sensitive, reliable and rapid identification methods of GFLV in infected planting material and nematode vectors have been developed as tools for effective disease control measures and epidemiological studies. Diagnostic methods available for testing of GFLV infections include immunological techniques such as double antibody sandwich enzyme-linked immuno-sorbent assay (DAS-ELISA) and ImmunoStrips, and molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) (Clark and Adams, 1977; Demangeat *et al.*, 2004; Liebenberg *et al.*, 2009). The genome of GFLV comprises two single-stranded RNAs, RNA1 and RNA2, both of which are required for infectivity. Each of the RNA molecules encodes a polyprotein that is cleaved by a viral protease into mature proteins. RNA1 encodes proteins involved in viral replication and polyprotein maturation, including a putative proteinase cofactor (1A). RNA2 encodes proteins involved in replication, a homing protein (2A^{HP}), the movement protein (2B^{MP}), and the coat protein (2C^{CP}) (Andret-Link *et al.*, 2004a; Mekuria *et al.*, 2009; Demangeat *et al.*, 2010). The coat protein (CP) is a multifunctional protein needed for the specific virus transmission by *X. index*, encapsidation of genomic RNAs, and systemic spread in plants (Andret-Link *et al.*, 2004b; Mekuria *et al.*, 2009). Recently, a putative ligand-binding pocket in the CP has been determined to be essential in virus transmissibility by *X. index* using a high structure resolution (Schellenberger *et al.*, 2011). GFLV, as with all RNA viruses infecting plants, has a great potential for genetic variation because host plants remain infected for long periods of time and the virus replication process is error-prone since no proof-reading correction mechanism is associated with the RNA1-encoded RNA-dependent RNA polymerase (Andret-Link *et al.*, 2004a). Nevertheless, some studies suggest

that molecular diversity of GFLV isolates are mainly dominated by one or few predominant haplotypes for the CP gene (Vigne *et al.*, 2004a,b).

Several studies have been conducted, to determine the molecular variability within the complete RNA2 or the CP gene, including isolates from Africa, Europe, the Middle East, and North America (Vigne *et al.*, 2004a; Liebenberg *et al.*, 2009; Mekuria *et al.*, 2009; Oliver *et al.*, 2010). PCR-coupled mutation detection techniques, such as denaturing gradient gel electrophoresis (DGGE) and single-strand conformation polymorphism (SSCP), provide useful and cost effective options for the direct analysis of genetic variation when large numbers of samples are required for analysis (García-Arenal *et al.*, 2001). SSCP allows determination of GFLV population diversity, including the number of haplotypes present in the population, as well as the frequency at which each haplotype is present in the population (García-Arenal *et al.*, 2001).

GFLV has been reported in most grapevine-growing areas in Spain. The virus has been detected infecting grapevines in Jerez (Cádiz province, southern Spain) in 1970 (Alfaro, 1971), and later in Castilla y León (García Benavides *et al.*, 1994) and Castilla-La Mancha in central Spain (Fresno *et al.*, 2001), Alicante province (Bertolini *et al.*, 2010) in the Valencia region, and Majorca in the Balearic islands (Cretazzo *et al.*, 2010). Additionally, it has been detected in some localities of Córdoba, and Huelva, provinces of Andalusia using DAS-ELISA (Troncoso *et al.*, 2004). Recent studies on plant-parasitic nematodes infesting vineyards in Andalusia revealed a prevalence of 12.5% to 30.3% for *X. index* and 10.9% to 13.2% for *X. italiae* among the sampled sites (Téliz *et al.*, 2007; Gutiérrez-Gutiérrez *et al.*, 2011). However, no information is available about the prevalence of GFLV infections in grapevine-growing areas of Andalusia. In addition, information on the molecular variability of the CP gene for GFLV isolates in Spain is lacking. For those reasons, we conducted an

extensive survey of grapevines for GFLV as well as for viruliferous nematode vectors in the three main D.O. wine zones of Andalusia. The specific objectives of the research were: *i*) to determine the prevalence of GFLV in vineyards in Andalusia; *ii*) to characterize the molecular diversity of GFLV isolates from plants, *X. index* and *X. italiae*; and *iii*) to study the genetic variability and phylogenetic relationships of the GFLV isolates identified in each D.O. with GFLV isolates infecting grapevines worldwide.

Materials and Methods

Survey and sample collection

Surveys were conducted during the spring season in 2007 and 2008 in 74 commercial vineyards arbitrarily selected and located in the three main D.O. zones of Andalusia, namely = Mm-D.O. (Córdoba province), JMSB-D.O. (Cádiz province), and CH-D.O. (Huelva province) (Table 1). Samples of young leaves (approximately 1.5-2.0 g) were arbitrarily collected from four to five plants arbitrarily chosen in each vineyard and maintained at -80°C until further use for viral RNA extraction. Each of the sampled vineyards was georeferenced so that the same vineyard could be sampled again. The virus genetic diversity was determined using four to five leaves arbitrarily collected from a single plant or from ten individual nematodes from the same plant. Additionally, different plant organs and nematodes were sampled to characterize the temporal and spatial molecular diversity of GFLV. For this purpose, samples were taken during the spring and autumn of 2010 in the locality Montemayor-116 (Mm-D.O.). Briefly, roots (“1103P” rootstock) and soil were sampled from

the same plant, similarly leaves and stems were taken from the scion (“Shiraz”) from the same plant in spring 2010 and autumn 2010, respectively. Also, two samples (Moriles-393-6.4.2 and Moriles-393-10.13.1) (Mm-D.O.) were collected in spring 2009 in two places with high infestation levels of *X. index* within the same vineyard for comparison.

Prevalence of GFLV and infestation by vector nematodes was calculated as the percentage of vineyards in which GFLV or vector nematodes were detected, in relation to the total number of vineyards in a given D.O. area.

Nematode identification

Soil samples were collected with a shovel from the rootstock rhizosphere of the sampled grapevines in each vineyard at a 50 cm depth. Nematodes were extracted from 500 cm³ of soil by centrifugal flotation and a modification of Cobb’s decanting and sieving methods (Gutiérrez-Gutiérrez *et al.*, 2011). Nematode vectors were identified by a combination of molecular analysis, morphology and morphometric measurements (Gutiérrez-Gutiérrez *et al.*, 2011).

Preparation of total RNA extracts from plants and nematode vectors

Sampled leaves were homogenized by grinding in liquid nitrogen and total RNA was extracted with the RNeasy plant mini kit (Qiagen, Valencia, CA) following the manufacturer’s instructions, except that the RLT lysis buffer was supplemented with 1% PEG 6000 to eliminate polyphenols and polysaccharides (Tattersall *et al.*, 2005). Total RNA was recovered in 30 µl of RNase-free water and either used immediately in RT-PCR experiments or stored at –80°C.

In selected samples of each D.O. area, total RNA was isolated from ten homogenized

nematodes using TRizol® LS Reagent (Invitrogen Corp., Carlsbad, CA) as described by Finetti Sialer & Ciancio (2005). Total RNA isolated with Trizol was recovered in 30 µl of RNase-free water and either used immediately in RT-PCR experiments or stored at -80 °C.

RT-PCR analyses

cDNA was obtained using Script cDNA Synthesis Kit (BIO-RAD, Hercules, California, USA) according to the manufacturer's instructions. 2.5 µl of cDNA suspension were used in PCR with the primers EV00N1 (5' GACTATCTAGACACATATATACACTTGGGTCTTTTAA '3) and CPS (5' TTGTGCGCCCAGATCTCTCTTTACCA '3) according to conditions described by Demangeat *et al.* (2004). The presence of GFLV was detected by PCR amplification of a 555 bp fragment from the GFLV CP gene. Positive and negative controls for RT-PCR assays consisted of leaves from GFLV-infected and non-symptomatic grapevines maintained under growth chamber conditions, respectively. The RT-PCR products were analyzed by electrophoresis in 1.2 % agarose gels after ethidium bromide staining, and observed under UV light.

PCR purification and cloning

GFLV RT-PCR products were purified with the GeneClean turbo gel extraction kit (Q-BIOgene SA, Illkirch Cedex, France), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and transformed into *Escherichia coli* JM109 High Efficiency Cells (Promega Corporation, Madison, WI, USA) according to the manufacturers' instructions. Several *E. coli* colonies were studied using PCR analysis with

EV00N1 and CPS primers in order to check the presence of the correct GFLV-fragment. Additionally, the purified plasmid DNA containing the insert from the positive *E. coli* colonies were digested using *EcoRI* restriction digestion at 37 °C for 1h followed by electrophoresis in order to know the correct size of the PCR product inserted. Approximately 15 GFLV clones were selected for SSCP analysis.

Single-strand conformation polymorphism (SSCP) analysis

During the spring of 2009, one GFLV-infected individual plant from each of the D.Os. was selected and assayed by RT-PCR, as well as a pool of 10 nematode vectors infesting the rhizosphere of the same plants in order to study GFLV molecular diversity. Additionally, in the spring and autumn of 2010 a GFLV-infected grapevine was arbitrarily selected in Mm-D.O. to study the molecular diversity of the virus in leaves and stems of the scion, in roots, as well as in vector nematodes infesting the rhizosphere of the sampled plant. Approximately 15 PCR products from 15 GFLV clones (one clone per PCR product) obtained from a single RT-PCR assay per plant or groups of 10 viruliferous nematodes were processed for SSCP analysis. For SSCP analysis, 5 µl of PCR product were mixed with 5 µl of loading dye (99 % formamide, 1 % of NaOH 1M, and bromophenol blue), denatured for 10 minutes at 94°C, and placed immediately in a cooler block (0-4 °C) until loading on a non-denaturing MDE[®] Gel Solution (Cambrex, Rockland, USA) as described by the manufacturer (0.5 mm and 5 % glycerol). The conditions for electrophoresis were 80 V at 4°C for 12 h. After electrophoresis, haplotype patterns were visualized by DNA silver staining following the protocol described by Caetano-Anollés & Gresshoff (1994). GFLV isolates and nematodes from each population were tested in different gels. The commercial molecular DNA marker O'GeneRuler[™] (Fermentas, Burlington, Canada) and a GFLV haplotype sample (MMA= Montemayor

haplotype A, reference haplotype) from isolate Montemayor-116 were included in each gel for comparing band positions among different gels.

Sequencing and phylogenetic analyses

Samples from different GFLV clones showing different or indistinguishable SSCP patterns were sequenced in both directions with universal cloning vector primers using a terminator cycle sequencing ready reaction kit (BigDye, Perkin-Elmer Applied Biosystems) according to the manufacturer's instructions. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyzer, Applied Biosystems, Foster City, CA, USA) at the STABVIDA sequencing facilities (Monte da Caparica, Portugal). Primers were removed from the sequences and then deposited in the National Center of Biotechnology Information (GenBank) database under accession number JN585765-JN585701.

The nucleotide and deduced amino acid sequences of the partial GFLV CP gene were aligned with related sequences obtained from GenBank with default parameters and manually edited using BioEdit (Hall, 1999). Only the coding regions were included in the phylogenetic analysis. Outgroup sequences were chosen according to previous published data (Liebenberg *et al.*, 2008). Phylogenetic analyses of the datasets were performed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis under a partitioned (site specific) rate model for nucleotide data and estimation of fixed rate model option implemented in MrBayes 3.1.2 for amino acid data was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further

analysis. The topologies were used to generate a 50% majority rule consensus tree. Maximum likelihood (ML) analysis for phylogeny with all GFLV CP gene sequences available in GenBank, was performed using a distant server (<http://phylobench.vital-it.ch/raxml-bb/index.php>) running the program RAxML-VI-HPC v. 4.0.0 (Randomized Accelerated Maximum Likelihood for High Performance Computing (Stamatakis *et al.*, 2008) using 100 bootstraps. The best fit model of DNA evolution was obtained using the program jModelTest ver. 0.1.1 (Posada, 2008) with the Akaike Information Criterion (AIC). The best fit model of amino acid evolution was obtained using the program MEGA ver. 5.05 (Tamura *et al.*, 2011) with the Akaike Information Criterion (AIC). Trees were visualized using MEGA ver. 5.05 (Tamura *et al.*, 2011).

Results

Symptoms and prevalence of nematode vectors and GFLV in vineyards in southern Spain

The overall prevalence of infestations (percentage of fields with the specific studied nematode) by nematode vectors in the surveyed commercial vineyards in southern Spain was 27.0% for *X. index* and 12.2% for *X. italiae*. Overall *X. index* population density ranged from 0 to 235 nematodes per 500 cm³ of soil and that of *X. italiae* ranged from 0 to 12 for nematodes per 500 cm³ of soil. *Xiphinema index* was rather extensively distributed among the three studied D.O. zones, with a prevalence of 29.4%, 28.0%, and 20.0%, in JMSB-D.O., CH-D.O., and Mm-D.O., respectively. However, *X. italiae* was more prevalent in CH-D.O. (28.8%) than in Mm-D.O. (13.3%) and it was not detected in JMSB-D.O. Four soil samples

(5.4%) were co-infested by both nematode vectors, including one in Mm-D.O., and three in CH-D.O. (Table 1).

RT-PCR assays using the primer pair EV00N1 and CPS successfully amplified the expected 555-bp target product from viruliferous nematodes and GFLV-infected grapevine leaf tissue sampled in Andalusia, southern Spain. Overall GFLV prevalence among the 74 vineyards was 24.3%; prevalence was 29.4% in JMSB-D.O., 24.0% in CH-D.O. and 13.3% in Mm-D.O. With the exception of one sample from JMSB-D.O. and another one from CH-D.O., all the GFLV-infected plants have the rhizosphere soil infested with at least one of the two nematode vector species (Table 1). Also, GFLV-infected plants were always co-infested with *X. index*, except for one vineyard sampled in JMSB-D.O. (sample 430) and two vineyards in CH-D.O. (473 and 479) where no vector nematode species were detected, and one vineyard in CH-D.O. (471) that was infested only by *X. italiae* (Table 1). Conversely, for 82.1% of vineyards that were GFLV-free none of the two vector nematode species were present, but 10 sampled vineyards (17.9%) were infested with at least one of the *Xiphinema* species. Specifically from these GFLV-free vineyards, two vineyards had both nematode species, while four vineyards were infested with *X. index* and another four with *X. italiae* (Table 1). GFLV-infected plants showed fanleaf and yellow mosaic symptoms together with a general growth decline (Table 1, Fig. 1). Both fanleaf and yellow mosaic were observed in all three D.O. studied areas, with yellow mosaic found to be predominant (83.3%) (Table 1). Additionally, GFLV symptomatology associated with grapevine decline was found in sample 470 from Rociana (CH-D.O.) that had been grafted onto rootstock *V. rupestris* du Lot. Nematode-infected roots showed enlarged swellings of root tips irrespective of rootstock or D.O. area (Fig. 1).

Analyses of GFLV molecular variability and phylogeny using a partial CP gene sequence

A total of 135 GFLV clones were analyzed for sequence variation using SSCP of the 555-bp fragment from the CP gene, yielding 12 different haplotypes (Table 2). Haplotypes were unequally distributed among the three different D.O. Haplotypes MOB (= Moriles haplotype B), MOC (= Moriles haplotype C), MMA, MMC (= Montemayor haplotype C) and HB (= Huelva haplotype B) were detected both in plants and nematodes in each vineyard where these haplotypes were found, except for one sample from Jerez de la Frontera-429. However, haplotypes MOA (= Moriles haplotype A), MMB (= Montemayor haplotype B) and JC (= Jerez haplotype C) were only detected in plants whereas haplotypes MMD (= Montemayor haplotype D), JA (= Jerez haplotype A), JB (= Jerez haplotype B) and HA (= Huelva haplotype A) were present only in nematode vectors. Montemayor-116 had the highest number of haplotypes, probably because it included more samples. The number of haplotypes per vineyard ranged from 1 (Moriles-393_10.3.1) to four (Montemayor-116). However, the highest number of haplotypes per sample, plant or nematode, was one or two.

Temporal sampling in Montemayor-116 showed that occurrence of haplotypes varied from spring 2010 (MMA) to autumn 2010, respectively (MMB, MMC and MMD). Haplotype patterns differed between grapevine organs (leaf-stem and root-stock) and *X. index* were different depending on the period of sampling. In this case, spring sampling showed only one haplotype (MMA) in both plant organs and nematode vectors. This result was different from the same grapevine plant in autumn, in which three haplotypes (MMB, MMC and MMD) were detected; with haplotype MMB being detected both in the rootstock and stem of the scion variety, haplotype MMC being found in the stem of the scion variety and

the nematode vector, whereas haplotype MMD was present in the nematode vector only.

Indistinguishable SSCP patterns (considered as one haplotype) showed sequence variation between different clones sequenced, with the exception of the single occurrence of haplotypes (JA) or haplotypes in which only one clone was sequenced (HA, MMC and MMD). Differences among sequence variants within a SSCP pattern ranged from 1 to 12 nucleotides, however, the haplotype pattern was reproducible between the samples. Differences in nucleotide sequences among and within SSCP patterns were not all silent and changes in amino acid sequences were observed. The lowest nucleotide sequence identities were detected between MOA and JB SSCP patterns (81.3-82.6%) and these differences corresponded also with the lowest identities at amino acid level (88.7-90.7%). Among isolates from the same sampling site, the nucleotide identities of the partial CP gene ranged from 99.1-99.7% (1-4 nucleotides) for sample Moriles-393-10.13.1 to 86.7-99.7% (1-62 nucleotides) for sample Moriles-393-6.4.2, while identities at amino acid level ranged from 98.7-99.3% (1-2 amino acids) for sample Moriles-393-10.13.1 to 93.2-100% (0-11 amino acids) for sample Moriles-393-6.4.2 (Table 3). Identities between sequences found in this study and sequences deposited in GenBank ranged from 82.0 to 97.6% at the nucleotide level and from 96.5 to 100% at the amino acid level.

When the CP sequence was studied phylogenetically, the majority of variants that showed the same SSCP haplotype clustered together. However, in the majority of cases, the CP sequences did not show a clear relationship with the origin of the isolate at either the amino acid or nucleotide levels. The relationship between isolate origin and sequence was stronger with nucleotide sequences than when analysing amino acids. For example, nucleotide sequences from Moriles-393 formed three distinct clades according to the haplotypes MOA, MOB and MOC, sequences from Montemayor-116 formed two clades

according to haplotypes MMA and MMB-MMC, and sequences from Rociana-474 formed one clade according to haplotype HB (Fig. 2). Haplotypes MMD and HA were represented by single clones and grouped separately from the other haplotypes of the same location. Some variants were more abundant than others in the clones sequenced from the same haplotype, as indicated by the clone numbers in the labels in Fig. 2.

A more extensive phylogenetic analysis using 270 sequences and a matrix of 489 nucleotides under TIM1 + G model and 163 amino acids under JTT + G was done using RAxML (Stamatakis *et al.*, 2008). The phylogenetic analyses based on GFLV nucleotide or amino acid sequences did not show any clear pattern of clustering according to geographical origin (Fig. 3). For both nucleotide and amino acid sequences, only some minor clades were well supported by clade posterior probability and the majority of the haplotypes in our study were distributed throughout the tree.

Discussion

The primary objective of this study was to determine the prevalence of GFLV in the main grapevine-growing areas belonging to the main “wine denomination of origin (D.O.) zones” of Andalusia. Our results indicate that GFLV is widespread in vineyards at that region with an overall prevalence of 24.3%. Troncoso *et al.*, (2004) studied the incidence of GFLV in grapevine plants in the provinces of Córdoba and Huelva using DAS-ELISA, but no data concerning the prevalence of affected vineyards was provided. In our study, some prevalence differences were detected between JMSB-D.O. (29.4%) and CH-D.O. (24.0%), and lower in Mm-D.O. (13.3%). Although these differences are difficult to explain, the two vineyards

from Mm-D.O. infested with GFLV have new varieties introduced in this wine D.O., ‘Shiraz’ and ‘Macabeo’, and not the traditional ‘Pedro Ximenez’ variety, for this reason, we hypothesize that grafting with infected material or the plantation in an infested soil with viruliferous nematodes may have occurred. Previous studies in other grapevine-growing regions of Spain reported GFLV being widespread in the country. A mean prevalence of GFLV infections of 10% in several Spanish D.Os. was first reported from a general survey following a northwest to southeast direction in Spain (Arias *et al.*, 1994). Subsequently, higher prevalence of GFLV, 50% and 95.8%, were reported in vineyards of local grapevine varieties from Valencia and Mallorca, respectively (Cretazzo *et al.*, 2010; Bertolini *et al.*, 2010). In our study, nematode vectors were clearly related to the prevalence of GFLV since all samples except one in D.O. JMSB-D.O. Condado de Huelva had rhizosphere soil infested with at least one vector nematode species (Table 1). Although *X. italiae* has never been confirmed as a vector of GFLV (Catalano *et al.*, 1992), in our case, sample Rociana-471 was positive for GFLV with *X. italiae* being the only *Xiphinema* species detected. Additionally, GFLV haplotypes found in this nematode were similar to those found in *X. index* (Table 2), however, it would be interesting to perform additional experiments under controlled conditions to test if *X. italiae* is a vector of GFLV.

The overall prevalence of *X. index* in each D.O. area was similar to that described by Gutiérrez-Gutiérrez *et al.* (2010) (27% vs. 30%) and similar results were also obtained for *X. italiae* (12.2 vs. 13.2%). The passive dispersal of *X. index* carrying GFLV through wind or field machinery among vineyards is very restricted (Villate *et al.*, 2010), because these nematodes do not have proper defence mechanisms against quick dehydration and their movement in the soil is very slow. Consequently, the most plausible way of introducing the virus in vineyards should be in *X. index*-infected planting material. GFLV symptoms were

clearly associated with two of the three main syndromes described by Raski *et al.* (1983), fanleaf and yellowish mosaic, with yellowish mosaic being more prevalent than fanleaf symptoms in all studied areas. Vein banding or other symptoms were not found in our sampling. Additionally, a symptomatology associated with decline symptoms was found in a GFLV positive plant in a sample from Rociana locality using as rootstock Rupestris Du Lot, suggesting the influence of rootstock in the scion symptoms expressed and the importance of molecular detection in GFLV-infected plants. The detection of these infected plants is a factor of great importance in the management and control of the grapevine fanleaf disease (Arias *et al.*, 1994). The factors affecting expression of grapevine fanleaf symptoms are unknown but they might include virus strain, rootstock, scion variety, environmental conditions and perhaps the effects of other viruses co-infecting the plant (Martelli, 1993). In our sampling, there was no association between GFLV symptom in the plant and locality, vineyard age, scion variety or between symptom expression of GFLV and rootstocks, with the exception of this decline symptom associated with only one isolate and Rupestris du Lot as rootstock. This result is interesting because the same scion variety could have different symptoms in the same D.O, in which some of them are clearly dominated by unique scion varieties ('Palomino' or 'Zalema'). Only one sample negative for GFLV using RT-PCR showed a clear yellow mosaic and decline. This sample could be infected by other pathogens with similar symptomatology but not tested in this study (*Arabis mosaic virus* or *Grapevine yellow speckle viroid*) or other factors including mutations in primer sites; low level of virions in the period of sampling or exceptionally elevated levels of plant substances interfering with the reaction could be also possible for failure to detect the virus by RT-PCR.

Our analyses showed sequence variability among clones with the same SSCP pattern. This variability could be associated to several aspects in the SSCP technique as well as the

1 fragment size and the intrinsic secondary structure formed (Chen *et al.*, 1995). In our case,
2 the large size (555 bp) of our product could have influenced this variability inside each
3 pattern studied. In the phylogenetic analysis, the majority of the variants from the same
4 haplotype pattern grouped together and to some extent also according to geographic origin.
5 For these reasons, GFLV in Andalusia exists as a “quasispecies”, as it has been reported in
6 other grapevine-growing areas of the world (Oliver *et al.*, 2010). GFLV variability among
7 different locations was up to 18.7% and 11.3% between sample Moriles-393-6.4.2 and
8 sample Jerez de la Frontera-429 at the nucleotide and amino acid level, respectively. This
9 level of variability between variants of the virus is slightly higher than found in other
10 countries, where a variability of up to 17% and 9% has been reported for CP sequences at the
11 nucleotide and amino acid level, respectively (Oliver *et al.*, 2010). However, the genome
12 fragment studied in this work is only 555 bp and this is shorter than sequences analyzed in
13 other studies. The lack of association between phylogenetic relationship of GFLV and
14 geographic origin has been observed in several regions in the world, except for a few lineages
15 (Oliver *et al.*, 2010; Liebenberg *et al.*, 2009). Nevertheless, some relationships between
16 locality and nucleotide differences have been detected in other regions (Bashir & Hajizadeh,
17 2007). The main reasons for this lack of association between phylogenetic relationship of
18 GFLV and geographic origin may be the absence of GFLV resistance in grapevine, the
19 perennial nature of the host, vegetative propagation of infected material, trafficking of
20 vegetative material and recombination between virus variants. Additionally, considering the
21 key role of the CP gene in maintaining the virion structure and stability, movement in the
22 plant, and the interactions with host and vector ascribed, limited levels of genetic variation
23 can be tolerated in order to maintain viability (Andret-Link *et al.*, 2004a). In some cases the
24 intra-population variability was higher than between populations (Oliver *et al.*, 2010).

Interestingly, the same haplotype was found during maximum vegetative plant growth (spring), while different haplotypes were detected during declining plant growth (autumn) amongst the leaves of scion variety, roots of rootstock, and *X. index* specimens in one sampled plant in both periods. However, these results were only performed in one plant and further studies are needed to clarify this situation in comparison to other woody crops and in virus transmitted by nematodes. It is important, however, to indicate that lower levels of virions were found in this period of the year (autumn) for GFLV infecting grapevine (Čepin *et al.*, 2010) which could have implications for PCR performance and some plant-virus variants are sensitive or have better performance depending of temperature (Mansournia, 2008).

In conclusion, this work showed a high prevalence of GFLV in Andalusia and the existence of a certain degrees of molecular diversity in some of the most representative localities sampled. The presence of *X. italiae* in some localities with plants infected by GFLV could be important in order to understand the possibility of this nematode species vectoring GFLV in vineyards, however, additional work is required to verify this hypothesis. Genetic variability of GFLV in the analyzed genome region in isolates from Andalusia was higher than that shown for other grapevine-growing areas of the world, but other characteristics of the virus are similar. Finally, to the best of our knowledge, this is the first report on molecular variability of GFLV in Spain.

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11

FIGURE LEGGENDS

Fig. 1. Leaf and root symptoms observed in commercial vineyards in southern Spain. (a) Yellow mosaic. (b) Fanleaf. (c) Apical roots of 161-49C (*V. riparia* Michx x *V. berlandieri* Planchon), and (d) Richter110, (*V. berlandieri* x *V. rupestris* Scheele) rootstocks infected with the dagger nematode *Xiphinema index*.

Fig. 2. The 50% majority rule consensus trees from Bayesian analysis generated from the GFLV partial coat protein fragment from the different Andalusian Wine Denomination of Origin using the partitioned (site specific) rate model for nucleotide data and estimation of fixed rate model for amino acid data. Lengths of the alignments were 489 bp and 163 aa for nucleotide and amino acid sequences. Posterior probabilities more than 65% are given for appropriate clades.

Fig. 3. Best scoring ML trees with support values using the GFLV partial coat protein fragment using TIM1 + G and JTT + G for nucleotides and amino acids, respectively. Lengths of the alignments were 489 bp and 163 aa for nucleotide and amino acid sequences. Posterior probabilities more than 65% are given for appropriate clades. The compressed clades include isolates of different geographic origin, the number of sequences for each country are in brackets.

Table 1 Detection of dagger nematode vectors in soil and *Grapevine fanleaf virus* (GFLV) in leaf tissues by RT-PCR using the primer pair EV00N1 and CPS during the spring of 2007 and 2008 in commercial vineyards in southern Spain.

						Nematode virus		
Denomination of origin						vector		
(D.O.) zone/	Reference			Age	RT-PCR			
Localities	vineyard	Scion variety	Rootstocks	grapevine	GFLV	<i>X. index</i>	<i>X. italiae</i>	Symptoms
Montilla-Moriles D.O. (15)^a								
Montemayor	116	Shiraz	1103P	15	+	+	+	Fanleaf, decline
Moriles	393	Macabeo	161-49C	35	+	+	-	Yellow mosaic, decline
Moriles	518	Tempranillo	1103P	20	-	+	-	Yellow mosaic, decline
Montemayor	528	Pedro Ximenez	161-49C	35	-	-	+	No symptoms
Jerez-Xérès-Sherry & Manzanilla-Sanlúcar de Barrameda D.O. (34)^a								
Jerez de la Frontera	401	Palomino	1103P	40	+	+	-	Yellow mosaic, decline
Jerez de la Frontera	404	Palomino	161-49C	35	+	+	-	Fanleaf, decline
Jerez de la Frontera	405	Palomino	161-49C	20	+	+	-	Yellow mosaic, decline
Jerez de la Frontera	427	Palomino	161-49C	25	+	+	-	Yellow mosaic, decline
Jerez de la Frontera	429	Palomino	161-49C	40	+	+	-	Yellow mosaic, decline
Jerez de la Frontera	430	Palomino	161-49C	35	+	-	-	Yellow mosaic, decline
Jerez de la Frontera	431	Palomino	161-49C	40	+	+	-	Yellow mosaic, decline
Jerez de la Frontera	432	Palomino	161-49C	35	+	+	-	Yellow mosaic, decline
Jerez de la Frontera	MN	Palomino	161-49C	40	+	+	-	Yellow mosaic, decline
Jerez de la Frontera	498	Palomino	161-49C	20	-	+	-	No symptoms
Jerez de la Frontera	499	Palomino	161-49C	35	+	+	-	Yellow mosaic, decline
Condado de Huelva D.O. (25)^a								
Bollullos par del Condado	422	Zalema	110R	35	-	-	+	No symptoms
Bollullos par del Condado	423	Zalema	161-49C	25	-	+	+	Decline
Bollullos par del Condado	424	Zalema	161-49C	20	-	+	-	Decline
Bollullos par del Condado	425	Zalema	161-49C	20	-	+	+	No symptoms
Bollullos par del Condado	426	Zalema	110R	20	-	-	+	No symptoms
Almonte	467	Zalema	110R	15	-	-	+	Decline
Almonte	469	Zalema	110R	20	+	+	-	Yellow mosaic, decline
Rociana	470	Zalema	Rupestris du Lot	30	+	+	-	Decline
Rociana	471	Zalema	110R	20	+	-	+	Yellow mosaic, decline
Rociana	473	Zalema	110R	20	+	-	-	Yellow mosaic, decline
Rociana	474	Zalema	110R	20	+	+	+	Yellow mosaic, decline
Bonares	479	Zalema	110R	25	+	-	-	Fanleaf, decline
Bollullos par del Condado	484	Zalema	110R	10	-	+	-	No symptoms

^a Total number of vineyards sampled in each Denomination of origin zone

TABLE 2 Distribution of *Grapevine fanleaf virus* (GFLV) coat protein gene haplotypes among vineyard sampling locations.

Vine denomination of origin (D.O.) zone / Localities-vineyard /Reference samples	Materials, Plant part, nematode	Geographic coordinates	CP haplotype ^b s													N ^c
			HA	HB	JA	JB	JC	MMA	MMB	MMC	MMD	MOA	MOB	MOC		
Montilla-Moriles D.O.																
Moriles-393-6.4.2 ^a (Córdoba, spring 2009)	Leaf (‘Macabeo’)	37°27'22.03"N, -4°36'45.49"W	-	-	-	-	-	-	-	-	-	4	5	-	9	
Moriles-393-6.4.2 ^a (Córdoba, spring 2009)	<i>X. index</i>	37°27'22.03"N, -4°36'45.49"W	-	-	-	-	-	-	-	-	-	-	5	-	5	
Moriles-393-10.13.1 ^a (Córdoba, spring 2009)	Leaf (‘Macabeo’)	37°27'21.21"N, - 4°36'46.65"W	-	-	-	-	-	-	-	-	-	-	-	8	8	
Moriles-393-10.13.1 ^a (Córdoba, spring 2009)	<i>X. index</i>	37°27'21.21"N, - 4°36'46.65"W	-	-	-	-	-	-	-	-	-	-	-	7	7	
Montemayor-116 (Córdoba, spring 2010)	Leaf (‘Shiraz’)	37°38'32.57" N, -4°41'57.65" W	-	-	-	-	-	-	11	-	-	-	-	-	11	
Montemayor-116 (Córdoba, spring 2010)	<i>X. index</i>	37°38'32.57" N, -4°41'57.65" W	-	-	-	-	-	-	12	-	-	-	-	-	12	
Montemayor-116 (Córdoba, spring 2010)	Root (‘1103P’)	37°38'32.57" N, -4°41'57.65" W	-	-	-	-	-	-	10	-	-	-	-	-	10	
Montemayor-116 (Córdoba, autumn 2010)	Stem (Shiraz’)	37°38'32.57" N, -4°41'57.65" W	-	-	-	-	-	-	-	6	1	-	-	-	7	
Montemayor-116 (Córdoba, autumn 2010)	<i>X. index</i>	37°38'32.57" N, -4°41'57.65" W	-	-	-	-	-	-	-	-	2	3	-	-	5	
Montemayor-116 (Córdoba, autumn 2010)	Root (‘1103P’)	37°38'32.57" N, -4°41'57.65" W	-	-	-	-	-	-	-	10	-	-	-	-	10	
Jerez-Xérès-Sherry & Manzanilla-Sanlúcar de Barrameda D.O.																
Jerez de la Frontera-429 (Cádiz, spring 2009)	Leaf (‘Palomino’)	36°42'15.95" N, -6°13'25.14" W	-	-	-	-	13	-	-	-	-	-	-	-	13	
Jerez de la Frontera-429 (Cádiz, spring 2009)	<i>X. index</i>	36°42'15.95" N, -6°13'25.14" W	-	-	1	11	-	-	-	-	-	-	-	-	12	
Condado de Huelva D.O.																
Rociana-474 (Huelva, spring 2009)	Leaf (‘Zalema’)	37°19'24.13" N, -6°36'48.96" W	-	11	-	-	-	-	-	-	-	-	-	-	11	
Rociana-474 (Huelva, spring 2009)	<i>X. index</i>	37°19'24.13" N, -6°36'48.96" W	6	4	-	-	-	-	-	-	-	-	-	-	10	
Rociana-474 (Huelva, spring 2009)	<i>X. italiae</i>	37°19'24.13" N, -6°36'48.96" W	-	5	-	-	-	-	-	-	-	-	-	-	5	
TOTAL			6	20	1	11	13	33	16	3	3	4	10	15	135	

^a Population sampled in two places with high infestation levels of *X. index* in the same commercial vineyard.

^b Haplotype references: HA= Huelva haplotype A; HB= Huelva haplotype B; JA= Jerez haplotype A; JB= Jerez haplotype B; JC= Jerez haplotype C; MMA= Montemayor haplotype A; MMB= Montemayor haplotype B; MMC= Montemayor haplotype C; MMD= Montemayor haplotype D; MOA= Moriles haplotype A; MOB= Moriles haplotype B; MOC= Moriles haplotype C.

^c N= number of clones analyzed per vineyard.

TABLE 3 Percentage nucleotide and amino acid identities for the coding region from the coat protein gene of *Grapevine fanleaf virus* among variants from the same sampling point.

	Amino acid identity (%)	Nucleotide identity (%)
Moriles-393-6.4.2^a	93.2-100 (0-11) ^b	86.7-99.7 (1-62)
Moriles-393-10.13.1^a	98.7-99.3 (1-2)	99.1-99.7 (1-4)
Montemayor-116	93.8-100 (0-10)	87.5-100 (1-62)
Jerez de la Frontera-429	95.7-100 (0-7)	92.8-99.7 (1-40)
Rociana-474	95.0-100 (0-8)	88.1-99.7 (1-58)

^a Population sampled in two places with high infestation levels in the same commercial vineyard.

^b Numbers within brackets refers to the numbers of amino acid or nucleotide differences between variants in the same sampling point.