

NUCLEOTIDE SEQUENCE VARIATIONS IN THE HSP70 GENE OF OLIVE LEAF YELLOWING-ASSOCIATED VIRUS

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SUMMARY

Single strand conformation pattern (SSCP) analysis of the HSP70 homologue gene of thirty isolates of Olive leaf yellowing-associated virus (OLYaV) from different geographical origins showed the existence of wide variability within this virus. This variability was clearly confirmed when multiple alignments of the HSP70 nucleotide sequences of 13 isolates that had different SSCP patterns were analysed. All isolates clustered with OLYaV-type in a phylogenetic tree constructed with the available HSP70 sequences of several members of the family *Closteroviridae*. The level of variability in a fragment of 383 nucleotides of the HSP70 gene ranged from 1% to 23%. Three distinct groups of isolates were identified, each of which had an internal variability lower than 10%, but showed a higher divergence from members of the other two groups (from 15 to 23%). In RT-PCR assays, primers specific for HSP70, but not those specific for the RdRp or HSP90 cistrons, amplified isolates of all groups. Whether or not the molecular differences observed in a highly conserved portion of the *Closteroviridae* genome (HSP70) can be indicative of the existence of more than one species within the OLYaV population remains to be ascertained.

Key words: Olive, *Closteroviridae*, OLYaV, HSP70, genome variability, SSCP, sequence analysis.

INTRODUCTION

Olive leaf yellowing-associated virus (OLYaV), one of the 14 viruses known to infect olive in nature (Martelli, 1998; Cardoso *et al.*, 2005), occurs in many Mediterranean countries (Saponari *et al.*, 2002; Fadel *et al.*, 2005). It is currently classified as an unassigned virus in the family *Closteroviridae* (Martelli *et al.*, 2005). Studies are in progress on its molecular characterization but, so far, the information available is limited to three genes.

These are RNA-dependent RNA polymerase (RdRp), heat shock protein 70 homologue (HSP70), and heat shock protein 90 (HSP90) and they occupy nearly 5500 nucleotides of the genomic sequence (Elbeaino *et al.*, 2005).

A preliminary comparative analysis of the nucleotide sequences of the viral HSP70 genes, revealed a wide molecular variability among isolates (Fadel *et al.*, 2005). This finding was somewhat unexpected because the HSP70 expression product, a protein involved in cell-to-cell movement (Agranovsky *et al.*, 1998), has a highly conserved sequence among a number of members of the family *Closteroviridae* (Dolja *et al.*, 1994).

Therefore the molecular variability of the HSP70 gene of more OLYaV isolates obtained from different geographical origins was investigated by sequence analysis and single strand conformation polymorphism (SSCP).

MATERIALS AND METHODS

Virus sources. Virus sources were thirty OLYaV-infected olive accessions from different countries (Albania, Italy, Spain, Egypt, Tunisia, Lebanon, Syria, and USA) and locations (Table 1) that had been maintained in collections of the University of Bari and of the Mediterranean Agronomic Institute of Bari.

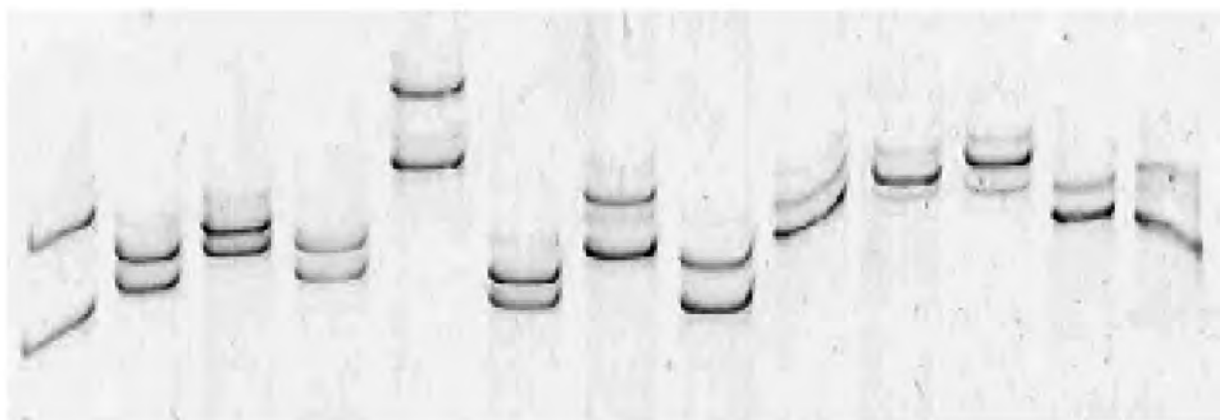
Total nucleic acid extraction. Total nucleic acids (TNAs) were extracted from *ca.* 100 mg of olive leaf veins or cortical scrapings according to Foissac *et al.* (2001) after grinding in 1 ml of 4.0 M guanidine isothiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc pH 5.2 and 2.5% (w/v) PVP-40.

RT-PCR. TNA extracts (8 to 10 µl) were mixed with 1 µl random hexamer primers, (Boehringer Mannheim, GbmH, Mannheim, Germany) (0.5 µg/µl), denatured at 95°C for 5 min and quickly chilled in ice. Reverse transcription reactions were done for 1h at 39°C by adding 4µl M-MLV buffer 5x (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂), 2µl of 10mM DTT, 0.5µl of 10mM dNTPs, and 200 units Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Bethesda Research Lab-

Table 1. Response of OLYaV isolates to RT-PCR with primers designed on sequences of the HSP70, HSP90, and RdRp cistrons.

Isolate*	Origin	Primers HSP70	Primers HSP90	Primers RdRp
1	Italy (Sicily)	+	+	+
2	Italy (Apulia)	+	-	-
3	Unknown	+	-	+
4	Italy (Apulia)	+	-	-
5*	Italy (Sicily)	+	+	+
6	Unknown	+	+	-
7	Italy (Apulia)	+	+	+
8	Italy (Sicily)	+	+	-
9*	Italy (Sicily)	+	+	+
10	Italy (Sicily)	+	+	+
11*	USA	+	-	-
12	Italy (Apulia)	+	-	+
13	USA	+	-	-
14*	Egypt	+	+	+
15* ^o	Tunisia	+	-	-
16*	Lebanon	+	-	-
17* ^o	Italy (Apulia)	+	+	+
18	Egypt	+	+	+
19	Italy (Sicily)	+	+	+
20	Italy (Apulia)	+	+	-
21	Albania	+	+	-
22	Italy (Apulia)	+	+	+
23	Italy (Apulia)	+	-	-
24	Spain	+	-	-
25	Italy (Apulia)	+	-	+
26	Syria	+	-	+
27	Syria	+	-	+
28	Syria	+	-	+
X16*	Lebanon	+	-	-
P5* ^o	Italy (Apulia)	+	-	-
OLYaV r.s*	Italy (Sicily)	+	+	+

* Isolates selected for sequencing the HSP70 fragment (383 bp)

^o Samples infected by two OLYaV isolates

Isolates: OLYaV rs P5 17 17 P5 X16 15 15 5 9 11 14 16
 Cloned DNA C H G E F D

Fig. 1. SSCP analysis in a 10% non-denaturing polyacrylamide gel showing different recombinant HSP70 DNA patterns of OLYaV isolates. Isolates 1, 5, 9, 11, 14, 16 and X16 showed single infection by OLYaV, while isolates P5 (Recombinant DNA: C and E), 15 (Recombinant DNA: D and F), and 17 (Recombinant DNA: G and H) gave more than one SSCP pattern, thus revealing infection by more than one variant of OLYaV.

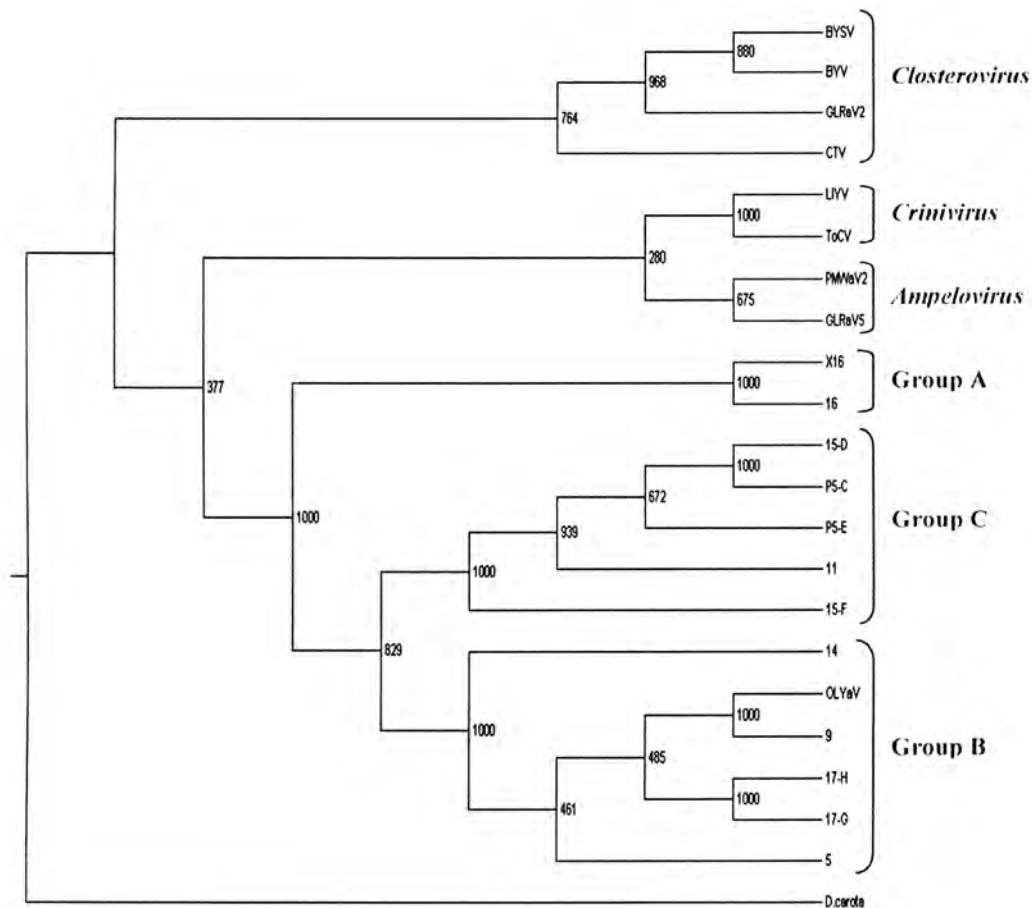


Fig. 2. Phylogenetic tree constructed with nucleotide sequences of the HSP70 genes of OLYaV isolates and those of other members of the family *Closteroviridae*. Nucleotide sequences were aligned by using Clustal X and neighbouring joining dendrograms were made by using Treeview. The alignments were bootstrapped 1000 times (Bootstrap values below 70% are not shown). The horizontal scale is proportional to the level of divergence while the vertical scale is arbitrary. Closteroviruses: *Citrus tristeza virus* (CTV, NC001661), *Beet yellow stunt virus* (BSV, U51931), *Beet yellows virus* (BYV, X734), *Grapevine leafroll-associated virus 2* (GLRaV-2, Y14131). Ampeloviruses: *Pineapple mealybug wilt associated-virus 2* (PMWaV-2, AF414119), *Grapevine leafroll-associated virus 5* (GLRaV-5, AF039552). Criniviruses: *Lettuce infectious yellows virus* (LiYV, U67448), *Tomato chlorosis virus* (ToCV, AJ968396). Outgroup: *Daucus carota* (HSP70 X60088). Olive leaf yellowing-associated virus (OLYaV, AJ440010) and the other OLYaV isolates under study are unassigned viruses in the *Closteroviridae* family (Groups A, B and C).

Table 3. Intra-specific variability of the HSP70 gene of different isolates of some closterovirus species.

Virus species	Virus isolates (accession number)	Variability level (%)
<i>Beet yellows virus</i> (BYV)	Reference isolate (NC001598)	---
	BYV (X73476)	0
	BYV-strain 4 (AF 190581)	10.1
	BYV Californian isolate (AF056575)	9.6
<i>Citrus tristeza virus</i> (CTV)	Reference isolate (U16304)	---
	CTV (AF01623)	7.1
	CTV (AAB046398)	12.5
	CTV (U56092)	12.7
<i>Grapevine leafroll-associated virus 2</i> (GLRaV-2)	Reference isolate (AJ748519)	---
	GLRaV-2 (AJ748517)	9.3
	GLRaV-2 (AJ748516)	10.8
	GLRaV-2 (AJ748515)	8.8

of the HSP90 and RdRp cistrons were used, only some of the accessions gave positive results (Table 1).

SSCP analysis of PCR amplicons from all isolates showed the presence of at least ten distinct types of SSCP pattern. Some PCR amplifications resulted in more complex SSCP products, suggesting the presence of mixed infections by different isolates. Further amplifications of the recombinant DNA of the transformed bacterial colonies were therefore made and the products were subjected to new SSCP analyses. In three cases, the presence of mixed infections was confirmed because two different SSCP patterns were observed for samples from single olive trees (Fig. 1, P5, 15, 17). Therefore sequence analyses were made of 13 OLYaV isolates, i.e. a representative isolate for each of the ten different SSCP pattern observed, and the three divergent variants detected in samples from mixedly infected plants.

Sequencing and phylogenetic analysis. Cloned cDNA fragments (383 bp) amplified by HSP70-specific primers, encoded a polypeptide of *ca.* 127 amino acids. The nucleotide sequences of the HSP70 gene of the 13 OLYaV isolates that had been subjected to SSCP analysis and those of the comparable genes of members of the family *Closteroviridae* were used for constructing a phylogenetic tree. In this tree, all OLYaV sequences, including that of the reference strain (Sabanadzovic *et al.*, 1999; GenBank accession No AJ440010), grouped in a separate cluster, clearly distinct from the clades containing members of the three genera of the family (Martelli *et al.*, 2005) (Fig. 2).

Range of variation in the HSP70 gene. Nucleotide sequence variability in the RT-PCR amplicons of the HSP70 gene (383 nt) of the 13 OLYaV isolates under study was investigated using the GeneDoc program (Nicholas *et al.*, 1997).

Comparison of these sequences disclosed the presence of at least three groups of isolates (Table 2): A, comprising two Lebanese isolates (X16 and 16), that showed a very low internal nucleotide variability (1.6%) but a divergence from all other isolates by 19.1% or more; B, comprising six isolates (OLYaV reference strain, 9, 17-H, 17-G, 5, and 14) with a mean nucleotide variability of 6.3%, and never higher than 8.1%. Nucleotide sequence divergence of these isolates from those of group A ranged from 19.8 to 22.0%, whereas the divergence from group C isolates ranged from 14.4% to 22.8%; C, comprising five isolates (11, 15-F, P5-E, 15-D and P5-C), with a mean internal nucleotide variability of 6.9%, never higher than 9.4%. One of the isolates in group C present in mixed infection in a Tunisian accession (15-D) had a high nucleotide homology (99.2%) with the Italian isolate P5-E.

As shown in Fig. 3, comparison of the predicted amino acid sequences of HSP70 proteins revealed sig-

nificant divergences between the three groups of variants. In particular: (i) the Lebanese isolates of group A (X16 and 16) showed the presence of a conserved amino acid sequence (RVKGFN), that was absent from group B and C isolates; (ii) group B isolates were substantially homogeneous, showing an amino acids variation pattern distinct from that of the other groups; (iii) group C comprised two distinct subgroups, that included isolates 15-D and P5-E and isolates P5-C, 11 and 15-F, respectively.

DISCUSSION

A group of 30 OLYaV isolates of different geographical origins was analyzed by RT-PCR using primers specific to three different genomic areas (RdRp, HSP70, and HSP90), and the SSCP profiles of amplified HSP70 fragments were determined. When more detailed SSCP and sequence analyses of the HSP70 gene of 13 representative isolates were done, the existence of a marked molecular variability within the virus population was found, which led to the identification of at least three distinct groups of isolates.

Group A comprised two Lebanese isolates, very similar to one another (1.6% nucleotide sequence variability), but highly divergent from all other isolate groups (19.1% to 23.4%). Group B comprised the OLYaV reference strain and five additional isolates, all of Italian origin (Sicily and Apulia), except for one from Egypt (isolate 14-B). In this group, the internal genetic divergence never exceeded 8%. By contrast, each member of this group had a nucleotide sequence divergence from all other isolates examined that ranged from 14.4% to 22.8%. Group C showed an internal genetic variability not exceeding 9.4%, and a divergence from the isolates of the other two groups that ranged from 14.4% to 23.3%. Group C was geographically heterogeneous as it included two Tunisian, two Apulian, and one American isolate.

These differences were reflected in the phylogenetic tree constructed with HSP70 nucleotide sequences, where the three groups of isolates clustered each in a separate branch (Fig. 2).

Genetic variability was not restricted to the HSP70 gene because when specific primers designed on the RdRp and HSP90 cistrons were used on accessions infected by the same 30 isolates under study, only some of them were amplified (Table 1). Interestingly, both sets of primers detected all isolates of group B (OLYaV reference strain, 9, 17G, 17H, 5 and 14), but none of those of the other two groups, further supporting the observed inter-group divergence.

The level of genetic variability among groups of OLYaV isolates seems to be much higher than that registered for isolates of other members of the family *Closteroviridae*, as shown by the fact that the inter-isolate

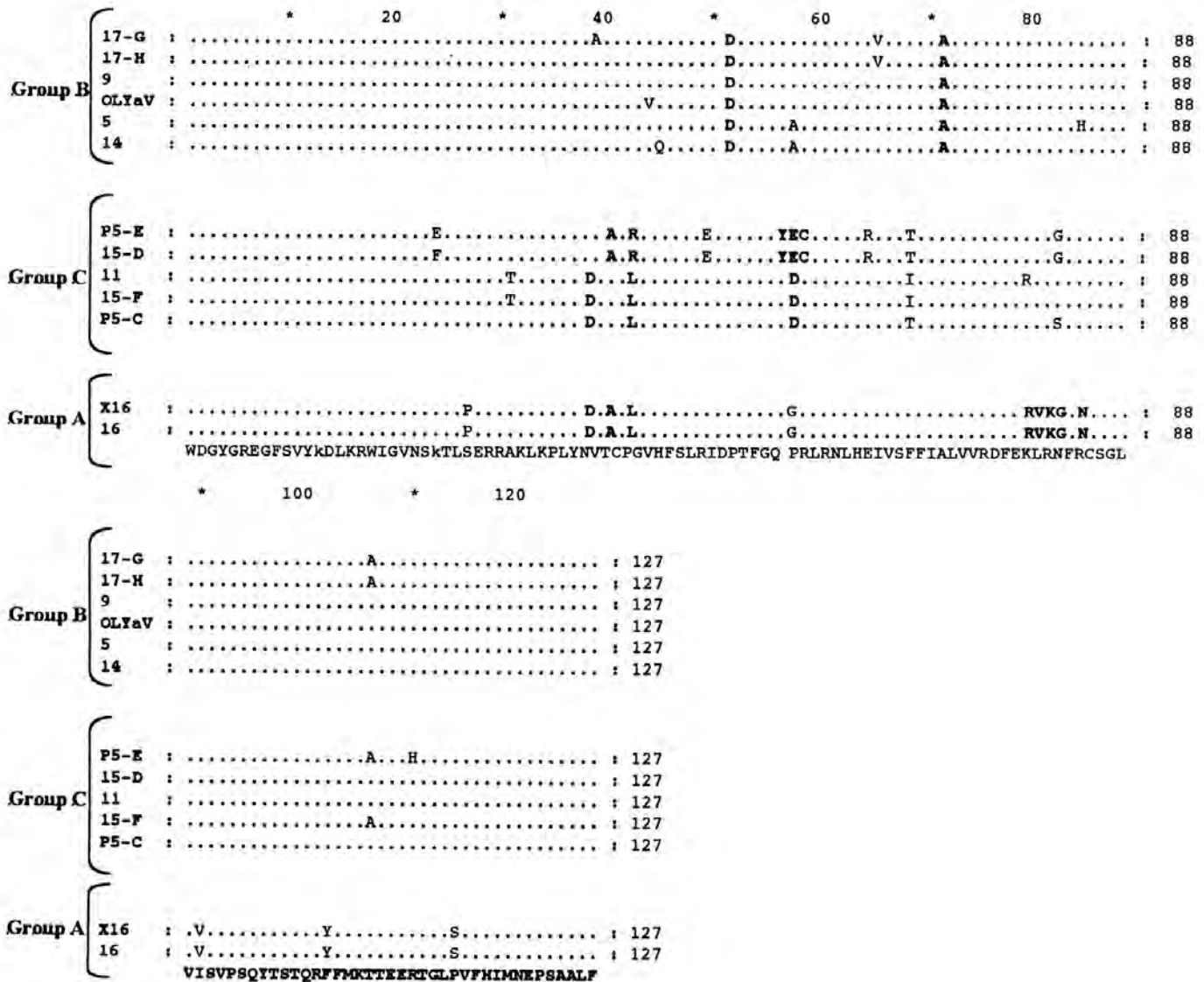


Fig. 3. Multiple sequence alignment generated by Clustal X of HSP70 amino acid sequences of the 13 OLYaV isolates. Dots indicate identical amino acids, letters differences in amino acid type and position.

variability in the HSP70 nucleotide sequence of *Beet yellows virus* (BYV), *Citrus tristeza virus* (CTV), and *Grapevine leafroll-associated virus 2* (GLRaV-2) does not exceed 10.1%, 12.7%, and 10.8%, respectively (Table 3).

The high genetic variability in the HSP70 protein of OLYaV isolates was confirmed by wide range of SSCP patterns observed in this study. However, the variability of SSCP patterns among OLYaV isolates belonging to the same molecular group makes this technique, if used alone, of little use for the allocation of different virus isolates to a group.

Although it is more likely that these groups of isolates represent molecular variants of OLYaV rather than different virus species, further sequencing work, in particular of the viral CP gene will be needed before an answer to this question can be obtained.

REFERENCES

Agranovsky A.A., Folimonov A.S., Folimonova S., Morozov S., Schieman J., Lesemann D.H., Atabekov J.G., 1998. Beet yellows closterovirus HSP70-like protein mediates the cell-to-cell movement of a potexvirus transport-deficient mutant and a hordeivirus-based chimeric virus. *Journal of General Virology* **79**: 889-895.

Altschul S.F., Stephen F., Gish W., Miller W., Myers E.W., Lipman D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Cardoso J.M., Felix M.R., Clara M.I., Oliveira S., 2005. The complete genome sequence of a new necrovirus isolated from *Olea europea* L. *Archives of Virology* **150**: 815-823.

Dolja V.K., Karasev., Koonin E.V., 1994. Molecular biology and evolution of closteroviruses: Sophisticated build-up of large RNA genomes. *Annual Review of Phytopathology* **32**: 261-285.

- Elbeaino T., Minafra A., Saponari M., Savino V., Martelli G.P., 2005. Further characterisation of Olive leaf yellowing-associated virus. *Journal of Plant Pathology* **87**:223-228.
- Fadel C., Digiario M., Choueiri E., Elbeaino T., Saponari M., Savino V., Martelli G.P., 2005. Sanitary Status of olive viruses in Lebanon. *Bulletin OEPP/EPPO Bulletin* **35**: 33-36.
- Felsenstein J., 1989. PHYLIP-phylogeny inference package (version 3.5). *Cladistics* **5**: 164-166.
- Foissac X., Svanella-Dumas L., Gentit P., Dulucq M.J., Candresse T., 2001. Polyvalent detection of fruit tree trichocapillo- and foveaviruses by nested RT-PCR using degenerate and inosine containing primers (DOP RT-PCR). *Acta Horticulturae* **550**: 37-43.
- Marck C., 1988. "DNA Strider": a "C" programme for the fast analysis of DNA and protein sequences on the Apple Macintosh family computers. *Nucleic Acids Research* **16**: 1829-1836.
- Markoff A., Savov A., Vladimirov V., Bodganova N., Kemeny I., Ganey V., 1997. Optimization of single strand conformation polymorphism analysis in the presence of polyethylene glycol. *Clinical Chemistry* **43**: 30-33.
- Martins-Lopes P., Zhang H., Koebner R., 2001. Detection of single nucleotide mutations in wheat using single strand conformation polymorphism gels. *Plant Molecular Biology Reporter* **19**: 159-162.
- Martelli G.P., 1998. Enfermedades infecciosas y certificación del olivo: panorama general. *Phytoma España* **102**: 180-186.
- Martelli G.P., Agranovsky A.A., Bar-Joseph M., Boscia D., Candresse T., Coutts R.H.A., Dolja V.V., Falk B.W., Gonsalves D., Hu J.S., Jelkman W., Karasev A.V., Minafra A., Namba S., Vetten H.J., Wisler G.C., Yoshikawa N., 2005. The family *Closteroviridae*. In: Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (eds.). *Virus Taxonomy. Eight Report of the International Committee on Taxonomy of Viruses*, pp. 1077-1087. Elsevier-Academic Press, Amsterdam, The Netherlands.
- Nicholas K.B., Nicholas H.B., Deerfield D.W., 1997. GeneDoc: Analysis and visualization of genetic variation, *Emble News* **4**: 14.
- Pearson W.R., Lipman D.J., 1988. Improved tools for biological sequence comparison. *Proceeding of the National Academy of Science USA* **85**: 2444-2448.
- Sabanadzovic S., Abou-Ghanem N., La Notte P., Savino V., Scarito G., Martelli G.P., 1999. Partial molecular characterisation and RT-PCR detection of a putative closterovirus associated with leaf yellowing. *Journal of Plant Pathology* **81**: 37-45.
- Saitou N., Nei M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolution* **4**: 406-425.
- Sambrook L., Fritsch E.F., Maniatis T.A., 1989. *Molecular cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, New York, USA.
- Saponari M., Alkowni R., Driouech N., Hassan M., Grieco F., Pantaleo V., Di Terlizzi B., Digiario M., Savino V., Martelli G.P., 2002. Detection of olive-infecting viruses in the Mediterranean basin. *Acta Horticulturae* **586**: 787-790.
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.

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