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Identification of Flavescence dorée-related phytoplasma infecting grapevines on the isle of Ischia (southern Italy)

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Summary

Among the grapevine diseases, Flavescence dorée (FD) is considered to be one of the greatest threats for grapevine cultivation throughout Europe. In Italy, where several major outbreaks of FD have occurred in the past, the disease is still mainly restricted to the northern regions and is under mandatory regulation. In 2011 the disease was recorded for the first time in southern Italy, in several vineyards located on the isle of Ischia (Campania). In order to determine the FD-related phytoplasma infecting these grapevines, molecular investigations were carried out on grapevine samples collected from several FD infected vineyards. The 16S rDNA/spacer region and the FD9 non-ribosomal fragment (*secY* gene) were investigated using a PCR/RFLP based method; a nucleotide sequence analysis of the FD9 amplified fragments was also carried out. The RFLP profiles obtained from all the analyzed samples showed the presence of FD-D phytoplasma type, on both 16S and *secY* genomic regions. Sequencing of the FD9 fragments showed a 99 % nucleotide sequence identity among the tested isolates and the various FD-D strains retrieved from the NCBI database.

K e y w o r d s : Flavescence dorée, grapevine, molecular characterization, *sec*Y gene, Italy.

Introduction

Of the grapevine diseases of phytoplasmal etiology, commonly denoted as Grapevine yellows (GY), Flavescence dorée (FD) is considered to be one of the greatest threats for grapevine cultivation in Europe. The FD-agent phytoplasma is efficiently transmitted by the insect vector *Scaphoideus titanus* Ball, and can cause devastating epidemics making it of quarantine importance. In Italy, several major outbreaks of FD have been reported in several regions in the north (Belli *et al*. 2010), and the disease is under mandatory regulation. Except for the occasional record in central Italy - Tuscany (Bertaccini *et al.* 2003), Marche (Credi *et al*. 2002) and Umbria (Natalini *et al.* 2005) - the presence of FD is still mainly restricted to the north, according with the natural spreading area of the insect vector.

In the other grape-growing areas of central and southern Italy, disease monitoring has never revealed the presence of FD phytoplasma in grapevine showing typical GY symptoms, and only Stolbur phytoplasma (16SrXII-A subgroup), the causal agent of Bois noir (BN), has been identified. The vector *S. titanus* was found in a few central (Latium and Abruzzo) and southern (Campania and Basilicata) regions (Viggiani 2002, 2004, Romanazzi *et al*. 2007, Bagnoli *et al*. 2008), where the insect probably arrived as eggs carried by grapevine propagation material, however its presence has never been associated with FD epidemics. Only in 2011 an outbreak of FD was recorded in southern Italy, in various vineyards located on the isle of Ischia (Campania region), where individuals of *S. titanus* were also identified (Griffo *et al*. 2011).

According to the most comprehensive and widely accepted phytoplasma classification system, FD is associated with two molecularly diverse phytoplasma types, FD-C and FD-D, both classified in the 16SrV (Elm yellow) ribosomal group, but assigned to the distinct subgroups 16SrV-C and –D respectively, on the basis of nucleotide differences found on 16S rRNA gene (Martini *et al.* 1999). The genomic region including the entire 16S rDNA and the spacer region between 16S and 23S rRNA genes has been specifically used to differentiate these two FD-related phytoplasmas by a polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) based method described in Martini *et al*. (1999).

This differentiation has also been confirmed on other more variable genomic regions. The ribosomal fragments encoding for the proteins L22 and S3 (*rpl*V and *rps*C genes) and the FD9 non-ribosomal fragments (*sec*Y gene) have been widely employed to characterize FD-related phytoplasma isolates from different geographical origins and to assess further molecular variability within the 16SrV-C and -D subgroups (Daire *et al*. 1997, Angelini *et al*. 2001, Martini *et al*. 2002, Arnaud *et al*. 2007, Botti and Bertaccini 2007, Quaglino *et al*. 2010).

With all these molecular approaches, the geographical distribution of FD phytoplasma strains that occur in grapevine growing areas of different European countries has been assessed. In Italy, both FD types are currently present in most of the viticulture areas where FD is common (Bertaccini *et al*. 2008), whereas the occasional finding of FD in central Italy has always been associated with the FD-C type.

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To date, no information has been available regarding the FD-related phytoplasmas infecting grapevine in the outbreak on Ischia. In order to determine the FD types, molecular investigations were carried out on grapevine samples collected from FD-infected vineyards located on the island and the results are reported here. The 16S rDNA/spacer region and the FD9 non-ribosomal fragment were investigated by PCR/RFLP analysis. In addition, a nucleotide sequence analysis of the FD9 amplified fragments was carried out.

Material and Methods

Source of material and DNA extraction: Ten symptomatic grapevine samples belonging to local and international varieties were collected in September 2012 from four commercial vineyards located in two different places in the south-west of Ischia where the first GY symptoms (Fig. 1) were observed in 2009 and the presence of FD phytoplasma was ascertained in 2011 (Griffo *et al*. 2011). The geographical origin and variety of the collected samples are listed in the Table.

DNA from naturally FD-C and -D infected grapevines (Veneto, north Italy), EY1 (Elm yellow, 16SrV-A) from periwinkle, SAY (Aster yellow, 16SrI-B) from periwinkle (both kindly provided by the University of Milan, Italy) and STOL (Stolbur, 16SrXII-A) from tomato (CRA-PAV, Rome, Italy) were used as phytoplasma reference controls. To perform the molecular analyses, total nucleic acid (TNA) was preliminary extracted from each sample starting with 0.5 g of fresh leaf midribs powdered in liquid nitrogen, following the procedure described in Marzachì *et al*. (1999), with some modifications. DNA extracts were dissolved in 100 µl of sterile deionized water. For the PCR amplification, 2 µl of 10 fold diluted extracts were used.

FD detection and differentiation on 16S rDNA/16S-23S spacer region: To confirm the presence of FD in all collected samples and to verify the occurrence of other common phytoplasmas associated with GY symptoms (16SrI-B, 16SrXII-A), extracted DNAs from each sample were preliminary amplified in nested PCR with the primer pairs R16(V)F1/R1 and R16(I)F1/R1 (Lee *et al*. 1994) after a direct amplification with the universal primers P1 (Deng and Hiruki 1991) and P7 (Schneider *et al*. 1995). Reaction mixture and PCR conditions are detailed in Pasquini *et al*. (2001). Amplified fragments obtained from R16(V)F1/R1 nested PCR were then digested overnight at 37 °C with the *Bfa*I (Biolabs) restriction enzyme and submitted to RFLP analyses in order to distinguish FD (16SrV-C/D) from Elm yellow (EY) phytoplasma (16SrV-A).

Fig. 1: Typical GY symptoms on 'Shiraz' (left) and 'Biancolella' (right) from the monitored vineyards located on the isle of Ischia.

Table

Number and origin of the FD isolates molecularly characterized

Isolate no.	Vineyard no.	Variety	Vineyard age	Town
223V(*)	1	Grenache	16	Forio (Cuotto)
224V	1	Grenache	16	Forio (Cuotto)
225V	1	Grenache	16	Forio (Cuotto)
226V(*)	2	Biancolella	10	Forio (Campotese)
227V	2	Biancolella	10	Forio (Campotese)
228V	3	Biancolella	10	Forio (Campotese)
229V(*)	3	Biancolella	10	Forio (Campotese)
230V	3	Biancolella	10	Forio (Campotese)
231V	4	Shiraz	9	Forio (Campotese)
232V(*)	4	Shiraz	9	Forio (Campotese)

*FD isolates whose FD9 sequence was deposited in GeneBank.

To determine the FD type (FD-C or D), P1/P7 direct PCR-amplicons from all FD positive samples were further amplified in the same conditions with the primers $16R_{758f}$ (Gibb *et al*. 1995) and $M23SR_{1804r}$ (Padovan *et al*. 1995), which cover a fragment of 1,050 bp including the entire 16S rDNA, the spacer region between 16S and 23S rDNA up to the beginning of the 23S rDNA. The obtained amplicons were then submitted to RFLP analysis after overnight digestion with *Taq*I (Biolabs) endonuclease, following the protocol specified in the manufacturer's instructions.

RFLP analysis was performed on the digested amplicons after separation of the fragments by electrophoresis on a 5 % polyacrylamide gel in TBE1X buffer. The RFLP profiles were visualized under UV light after staining in ethidium bromide.

FD differentiation on FD9 non-ribosomal fragment and sequencing: For specific amplification of the FD9 non-ribosomal DNA fragment, the primer pairs FD9f2 (Angelini *et al.* 2001)/FD9r (Dairé *et al*. 1997) and FD9f3/FD9r2 (Angelini *et al*. 2001) were used in direct and nested PCR, respectively, following the conditions reported in the literature (Angelini *et al*. 2001). Nested PCR amplicons (1,150 bp size) were then submitted to RFLP analysis and sequencing. For the RFLP analysis, amplicons were digested separately with *Alu*I (Biolabs) and *Mse*I (Biolabs) restriction enzymes, according to the manufacturer's instructions.

Nucleotide sequences analyses of the FD-9 amplified fragments from all tested isolates were performed after multiple-sequence alignments with CLUSTAL W (Thompson *et al*. 1994). Comparison with sequences retrieved from the GeneBank was performed using BLAST (Altschul *et al*. 1997) on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

FD detection and differentiation on 16S rDNA/16S-23S spacer region: Direct PCR with primers P1/P7 did not show any band except for the reference positive controls. Amplified fragments of an expected size (1,100 bp) were obtained from all tested samples in R16(V)F1/R1 nested PCR, whereas no bands were observed in R16(I)F1/R1 nested PCR, except for SAY and STOL reference controls. RFLP analysis of R16(V)F1/R1 amplicons after digestion with *Bfa*I restriction enzyme showed an identical profile to those of a 16SrV-C and D reference phytoplasmas in all the analyzed samples.

As expected on the basis of R16(V)F1/R1 nested PCR, amplicons of an appropriate size (1,050 bp) were also obtained from all samples when amplified with the universal primer pair $16R_{758f}$/$M23SR_{1804r}$ after direct amplification with P1/P7 primers. RFLP analysis performed on $16R_{758f}$/$M23SR_{1804r}$ amplicons after digestion with *Taq*I endonuclease showed the presence of a RFLP profile that refers to those of the 16SrV-D reference control in all the samples tested (Fig. 2).

FD differentiation on FD9 non-ribosomal fragment and sequencing: FD9 fragments of an expected size (1,150 bp) were obtained in nested PCR by employing the primer pair FD9f3/FD9r2 from all the analyzed samples (Fig. 3). RFLP analysis performed on these fragments after digestion with *Alu*I and *Mse*I endonucleases revealed the presence of one single type of RFLP profile for each considered enzyme, in all the tested samples, which always corresponded to the one exhibited by the 16SrV-D reference control (Fig. 4).

Nucleotide sequence analysis performed on the FD9f3/FD9r2 amplified fragments from all investigated isolates showed an identity percentage of 100 % with each other. When compared with nucleotide sequences from the NCBI database, a 99 % identity was found among these isolates and several FD-D strains including the FD-D strain from Slovenia, first detected in *Orientus ishidae* (HM367597), FD92 from France (AF458384), and FD-D from Italy (AY197685). Nucleotide sequences of FD9-fragments of four FD isolates (223V, 226V, 229V, 232V), representative of different tested varieties and vineyards (Table), were deposited in GeneBank with the following accession numbers: KC960449, KC960450, KC960451, KC960452.

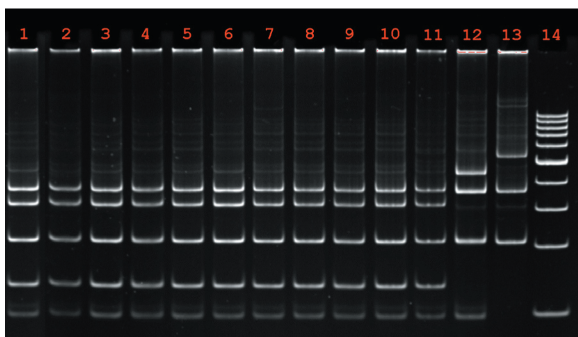

Fig. 2: Polyacrylamide gel (5 %) showing the RFLP profiles obtained from $16R_{758f}$ /$M23SR_{1804r}$-nested PCR amplicons after digestion with *Taq*I restriction enzyme. Lines 1-10: grapevine samples; line 11: FD-D positive control; line 12: FD-C positive control; line 13:EY1; line 14: DNA marker (size 100 bp).

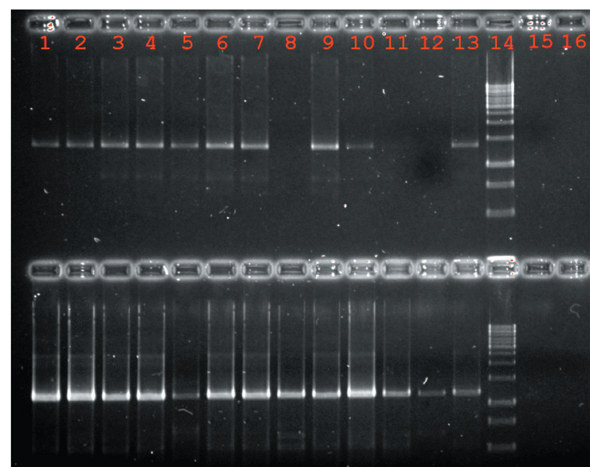

Fig. 3: Agarose gel showing the PCR amplicons obtained with the primer pairs FD9f2/FD9r (top) and FD9f3/FD9r2 (bottom) in direct and nested PCR respectively, from the analyzed samples. Lines 1-10: grapevine samples; line 11: EY1; line 12: FD-C positive control; line 13: FD-D positive control; line 14: DNA marker (size 1Kb); line 15-16: water controls.

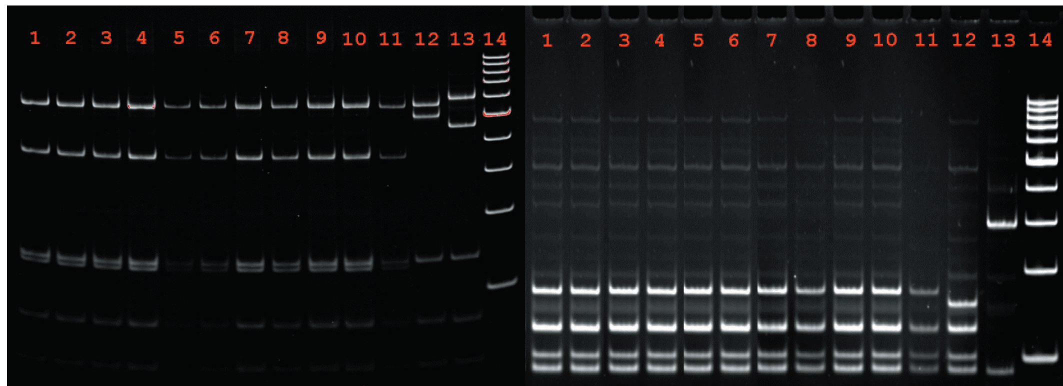

Fig. 4: Polyacrylamide gel (5 %) showing the RFLP profiles obtained from FD9f3/FD9r2-nested PCR amplicons after digestion with *Alu*I (left) and *Mse*I (right) restriction enzymes. Lines 1-10: grapevine samples; line 11: FD-D positive control; line 12: FD-C positive control; line 13: EY1; line 14: DNA marker (size 100 bp).

Conclusions

The presence of FD-related phytoplasma in symptomatic grapevines from the isle of Ischia, previously reported by GRIFFO *et al*. (2011), was confirmed in this molecular investigation. Analysis of the conserved 16S rRNA gene showed the presence of an FD-related phytoplasma in all the tested samples and clearly ruled out possible infection by Stolbur phytoplasma associated with BN, the other major widespread GY disease in all vine-growing areas of Italy.

Molecular characterization of the identified FD isolates performed on the 16S rRNA/spacer region highlighted the presence of an FD-D phytoplasma type (16SrV-D subgroup) in all symptomatic grapevines, irrespectively of their origin and variety. The same result was obtained by molecular characterization based on PCR/RFLP and sequence analysis performed on *sec*Y, which is more variable than the 16S rRNA gene. RFLP analysis of the FD9-amplified fragments digested with *Mse*I and *Alu*I endonucleases revealed an identical profile in all the tested isolates, which always corresponded to the FD-D reference strain (MARTINI *et al.* 1999), thus indicating that there was no variability among them in this genomic region.

FD-D phytoplasma type, associated with the first outbreaks recorded in the Veneto region (northern Italy) in the 1990s is considered to be the most virulent strain and is widespread in the grape growing areas of northern Italy (BERTACCINI *et al*. 2008). Outside these geographical areas, only FD-C has been detected to date and this is the first finding of FD type D in Italian vineyards a long way from the northern grape-producing areas. Considering the geographical isolation of the vineyards located on the isle of Ischia from the rest of the country, the introduction of both FD-D phytoplasma and its vector can be probably explained by long-distance movement.

The ability of FD-D to be transmitted by *S. titanus* has been experimentally demonstrated, as for FD-C (MORI *et al*. 2002). Although no data are available yet on the infectivity of the *S. titanus* population identified on Ischia, the simultaneous presence of an aggressive FD-D phytoplasma type and the specific insect vector highly increases the risk of epidemics, posing a serious threat for the local viticulture.

In 2011, when FD was first recorded, infected plants were promptly eradicated, adhering to Italian phytosanitary legislation. However, in 2012 new infected plants were found in other areas and eradication of the whole vineyards seems necessary in order to contain the epidemic.

As this new outbreak is restricted to an island, the other grape-growing areas of southern Italy, which are currently FD-free, do not appear to be directly threatened. However, this clear example of the long-distance propagation of a disease, reinforces the important role played by human trading activities in spreading pathogens and insects.

References

ALTSCHUL, S. F.; MADDEN, T. L.; SCHAFFER, A. A.; ZHANG, J.; ZHANG, Z.; MILLER, W.; LIPMAN, D. J.; 1997: Gapped BLAST (Basic Local Alignment Search Tool) and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. **25,** 3389-3402.

ANGELINI, E.; CLAIR, D.; BORGO, M.; BERTACCINI, A.; BOUDON-PADIEU, E.; 2001: Flavescence dorée in France and Italy. Occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. Vitis **40**, 79-86.

ARNAUD, G.; MALEMBIC-MAHER, S.; SALAR, P.; BONNET, P.; MAIXNER, M.; MARCONE, C.; BOUDON-PADIEU, E.; FOISSAC, X.; 2007: Multilocus sequence typing confirms the close genetic inter-relatedness between three distinct Flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. Appl. Environ. Microbiol. **73,** 4001-4010.

BAGNOLI, B.; FERRETTI, L.; TRIVELLONE, V.; NUCCITELLI, L.; PASQUINI, G.; 2008: Occurrence of *Scaphoideus titanus* in Latium region. Petria **18**, 304-308.

BELLI, G.; BIANCO, P.A.; CONTI, M.; 2010: Grapevine yellows in Italy: past, present and future. Journal of Plant Pathology, **92**, 303-326.

BERTACCINI, A.; ANGELINI, E.; BIANCO, P. A.; BOTTI, S.; CASATI, P.; DURANTE, G.; FILIPPIN, L.; MARZACHÌ, C.; PACIFICO, D.; PALTRINIERI, S.; QUAGLINO, F.; 2008: Molecular characterization of "Flavescence dorée" strains detected in Italy from 2004 to 2008. Petria **18**, 268-271.

BERTACCINI, A.; BOTTI, S.; TONOLA, A.; MILANO, C.; BRACCINI, P.; SFALAGNA, A.; 2003: Identificazione di fitoplasmi di Flavescenza dorata in vigneti della Toscana. L'informatore Agr. **59**, 65-67.

BOTTI, S.; BERTACCINI, A.; 2007: Grapevine yellows in northern Italy: molecular identification of Flavescence dorée phytoplasma strains and of Bois Noir phytoplasmas. J. Appl. Microbiol. **103**, 2325-2330.

Credi, R.; Terlizzi, F.; Stimilli, F.; Nardi, G.; Lagnese, R.; 2002: Flavescenza dorata della vite nelle Marche. L'informatore Agr. **58**, 61-63.

Daire, X.; Clair, D.; Larrue, J. ; Boudon-Padieu, E.; 1997 : Detection and differentiation of grapevine yellows phytoplasmas belonging to elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. European Journal of Plant Pathol. **103**, 507-514.

Deng, S.; Hiruki, C.; 1991: Amplification of 16S rRNA genes from culturable and non culturable Mollicutes. J. Microbiol. Methods **14**, 53-61.

Gibb, K. S.; Padovan, A. C.; Mogen, B. D.; 1995: Studies on sweet potato little-leaf phytoplasma detected in sweet potato and other plant species growing in Northern Australia. Phytopathology **85**, 169-174.

Griffo, R.; Benchi, D.; Bifulco, A.; Pesapane, G.; 2011: Flavescenza dorata anche in Campania. L'informatore Agr. **44**, 67.

Lee, I. M.; Gundersen, D. E.; Hammond, R. W.; Davis, R. E.; 1994: Use of mycoplasma like organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology **84**, 559-566.

Martini, M.; Botti, S.; Marcone, C.; Marzachì, C.; Casati, P.; Bianco, P.A.; Benedetti, R.; Bertaccini, A.; 2002: Genetic variability among Flavescence dorée from different origins in Italy and France. Mol. Cell. Probes **16**, 197-208.

Martini, M.; Murari, E.; Mori, N.; Bertaccini, A.; 1999: Identification and epidemic distribution of two *Flavescence dorée*-related phytoplasmas in Veneto (Italy). Plant Dis. **83**, 925-930.

Marzachì, C.; Alma, A.; d'Aquilio, M.; Minuto, G.; Boccardo, G.; 1999:Detection and identification of phytoplasmas infecting cultivated and wild plants in Liguria (Italian Riviera). J. Plant Pathol. **81**, 127-136.

Mori, N.; Martini, M.; Bressan, A.; Guadagnini, M.; Girolami, V.; Bertaccini, A.; 2002: Experimental transmission by *Scaphoideus titanus* Ball of two molecularly distinct Flavescence dorée type phytoplasmas. Vitis **41**, 99-102.

Natalini, G.; Santinelli, C.; Porcacchia, C.; 2005: Bilancio Fitosanitario 2004 – Umbria. Inform. Fitopatol. **25**, 49.

Padovan, A. C.; Gibb, K. S.; Bertaccini, A.; Vibio, M.; Bonfiglioli, R. G.; Magarey, P. A.; Sears, B. B.; 1995: Molecular detection of Australian grapevine yellows phytoplasma and comparison with grapevine yellows phytoplasmas from Italy. Aust. J. Grape Wine Res. **1**, 25-31.

Pasquini, G.; Angelini, E.; Benedetti, R.; Bertaccini, A.; Bertotto, L.; Bianco, P. A.; Faggioli, F.; Martini, M.; Marzachì, C.; Barba, M.; 2001: Armonizzazione della diagnosi della Flavescenza dorata della vite (FD): risultati di una prova comparativa, 921-940. In: Atti Progetto POM A32 (vol II), Norme fitosanitarie e commercializzazione delle produzioni vivaistiche', Locorotondo (BA-Italy), 4-7 dicembre 2001.

Quaglino, F.; Casati, P.; Bianco, P.A.; 2010: Distinct *rps*C single nucleotide polymorphism lineages of Flavescence dorée subgroup 16SrV-D phytoplasma co-infect *Vitis vinifera* L. Folia Microbiol. **55**, 251-257.

Romanazzi, G.; Murolo, S.; D'Ascenzo, D.; Di Giovanni, R.; 2007: Nuove acquisizioni sulla diffusione dei giallumi della vite in Abruzzo. Italus Hortus **14**, 253-256.

Schneider, B.; Seemüller, E.; Smart, D.; Kirkpatrick, B. C.; 1995: Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: S. Razin, J. G. Tully (Eds): Molecular and diagnostic procedures in mycoplasmology, vol. 1, 369-380. Academic Press, San Diego (CA-USA).

Thompson, J. D.; Higgins, D. G.; Gibson, T. J.; 1994: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. **22**, 4673-4680.

Viggiani, G.; 2002: Il vettore della Flavescenza dorata trovato in Basilicata. L'Informatore Agr. **58**, 59.

Viggiani, G.; 2004: Il vettore della Flavescenza dorata anche in Campania. L'informatore Agr. **60**, 98.

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