PARTIAL CHARACTERISATION OF A CLOSTEROVIRUS ASSOCIATED WITH A CHLOROTIC MOTTLING OF FIG

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SUMMARY

A double-stranded RNA (dsRNA) *ca.* 19 Kbp in size was obtained from tissue extracts of a fig plant (*Ficus carica*) with chlorotic mottling and vein clearing of the leaves. Leaf dips showed the presence of filamentous closterovirus-like particles with distinct cross banding and a length of about 1800 nm. No virus was recovered by mechanical inoculation to herbaceous hosts. However, virus aggregates were plentiful in thin-sectioned companion cells and differentiating sieve tubes of symptomatic leaves. Using denatured dsRNA preparations as template, a 620 bp cDNA fragment was amplified by RT-PCR using degenerate primers designed on the phosphate motifs 1 and 2 of the heat shock-protein 70 homologue (HSP70h) of members of the family *Closteroviridae.* Computer-assisted analysis of the sequenced 620 bp fragment showed it to be part of a closteroviral HSP70h gene having identity at the amino acid level with the comparable gene of different closteroviral species ranging from 34% to 48%. In a phylogenetic tree constructed with the amino acid sequences of part of the HSP70h of several members of the family *Closteroviridae,* the fig virus grouped with species of the genus *Closterovirus*. RT-PCR with specific primers designed on the HSP70h sequence detected the virus in 36 of 57 field-grown trees, most of which had mosaic symptoms. The present results show that fig hosts a putative closterovirus species for which the name of Fig leaf mottle-associated virus (FLMaV) is proposed.

Key words: *Ficus carica*, *Closteroviridae*, dsRNA, HSP 70h, sequencing, RT-PCR.

INTRODUCTION

Fig (*Ficus carica*) mosaic is an ubiquitous disease originally described from California (Condit and Horne, 1933), which is transmitted by grafting and, in nature, by the eryophid mite *Aceria ficus* (Flock and Wallace, 1955). Leaves of affected trees are more or less severely deformed and show a wide array of discolourations, i.e, various patterns of chlorotic mottling and blotching, vein banding, vein clearing, chlorotic-necrotic ringspots and line patterns.

The aetiology of the disease is still uncertain. Isometric and filamentous virus particles have been observed occasionally in thin-sectioned tissues of symptomatic leaves of fig accessions from Italy and England (Martelli *et al.*, 1993), putative potyviruses have been recorded from Croatia (Grbelja, 1983) and Spain (Serrano *et al.*, 2004), a putative carlavirus was reported from Japan (Doi, 1989), and double stranded RNAs (dsRNA) with a size ranging from 0.6 to 6.6 kb were recovered from infected trees in Portugal and Turkey (Nolasco and Sequeira, 1991; Açikgöz and Döken, 2003). However, cells of infected trees from California and 14 European, Mediterranenan, and Middle Eastern countries consistently contained intracytoplasmic enveloped spherical to ovoid bodies of undetermined nature, which are mite-transmitted and suggested to be the putative aetiological agents of the disease (Bradfute *et al.*, 1970; Plavsic and Milicic, 1980; Appiano, 1982; Appiano *et al.*, 1990; Martelli *et al.*, 1993).

In summer 2005, a fig tree of cv Canestrelle (accession N17) with chlorotic mottling and vein clearing of the leaves was observed in the province of Taranto (Apulia, southern Italy). Although these symptoms were within the range of those characterizing the mosaic disease, their severity, especially on young leaves, and the presence of filamentous virus-like particles in leaf dips, prompted specific investigations, the results of which are reported in the present paper.

MATERIALS AND METHODS

Mechanical transmission. Tissues from young strongly symptomatic leaves of accession N17 were macerated in a mortar in the presence of 0.05M phosphate buffer containing 2.5% nicotine and used for mechanical inoculation of celite-dusted leaves of *Chenopodium amaranticolor, C. quinoa, Nicotiana benthamiana, N. cavicola, N. occi-*

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dentalis, Gomphrena globosa, and *Cucumis sativus.* Inoculated plants were kept in a glasshouse at *ca.* 24°C and observed for symptom expression.

Total nucleic acid extraction. For total nucleic acids (TNAs) extraction from leaf veins or cortical scrapings of N17, 100 mg of tissue were macerated in 1 ml grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc and 2.5% w\v PVP-40), TNAs were recovered with a silica-capture procedure (Foissac *et al.*, 2001) and stored at -20°C until used.

Double-stranded RNA analysis. About 30 g of tissues from cortical scrapings, leaf veins, or petioles were ground in a mortar with liquid nitrogen. TNA were extracted with two phenol-chloroform treatments (Dodds, 1993). After CF11 cellulose chromatography, DNA and single-stranded RNA were digested according to Saldarelli *et al.*, (1994) by incubating for 30 min at 37°C. After an additional phenol-chloroform extraction, and centrifugation at 9,000g for 10 min, dsRNA was ethanol-precipitated from the supernatant and electrophoresed in 5% TAE polyacrylamide gel (Sambrook *et al.*, 1989).

Cloning and sequencing. Nuclease-treated dsRNA pellets were eluted, denatured by boiling at 95°C for 10 min followed by fast cooling, and then the addition of 0.5µl methyl mercuric hydroxide (100mM) and, after 10 min at room temperature, of 1 µl of 0.5M 2-mercaptoethanol. Reverse transcription was done using random hexamer primers and 200 U/µl of RTase from *Avian myeloblastosis virus* (Roche Applied Science, Basel, Switzerland) at 42°C for 1 h.

Aliquots of cDNA were PCR-amplified using a set of degenerate primers designed to amplify sequence in the closteroviral heat shock protein 70 homologue gene (HSP70h) (Tian *et al.*, 1996). Amplicons were electrophoresed in 6% polyacrylamide slab gels, ligated directly into pGEM-T Easy vector (Promega, Madison, USA) according to the manufacturer's instructions. The ligation product was used to transform *E. coli* DH5α cells. DNA from clones was analyzed by enzymatic digestion and subjected to automated sequencing (MWG Biotech, Ebersberg, Germany).

Computer-assisted analysis of nucleotides and protein sequence. Nucleotide and protein sequences were assembled using the Strider 1.1 Program (Marck, 1988). Protein sequences were aligned with the Clustal X program. Homology with known proteins from the Protein information resources (PIR, release 47.0) was determined using the FASTA (Pearson and Lipman, 1988) and BLASTA (Altschul *et al.*, 1990) programs. Phylogenetic trees were constructed and bootstrap analysis was made with the NEIGHBOR, SEQBOOT, PROTDIST and CONSENSE programs of the PHYLIP package (Felsenstein, 1989).

Electron microscopy. Dips in 2% aqueous uranyl acetate were made from leaf petioles of accession N17. For thin sectioning, tissue pieces from veins and mesophyll tissues of the discolored areas of young leaves were processed according to standard procedures at 4°C throughout (Martelli and Russo, 1984), i.e. fixation in 4% glutaraldehyde in 0.05M phosphate buffer for 2 h, post-fixation in 1% osmium tetroxide for 2 h, staining overnight in 2% aqueous uranyl acetate, dehydration in ethanol, and embedding in Spurr's medium. Thin sections were stained with lead citrate and viewed with a Philips Morgagni 282D electron microscope. Controls consisted of leaf tissues from a healthy fig seedling processed as above.

Primer selection and virus survey. Primers (N17s: CGTGGCTGATGCAAAGTTTA; N17-a: GTTAACG-CATGCTTCCATGA) that amplified a segment of *ca.* 350 bp, were designed on the HSP70h sequence of isolate N17 and used for virus detection by RT-PCR in fig trees from commercial stands in the Apulian provinces of Bari and Taranto. TNAs were extracted from leaf vein tissues according to Foissac *et al.* (2001). After random cDNA synthesis, PCR was done using a 3 min heating step at 94°C followed by 33 cycles of 35 sec melting at 94°C, 30 sec annealing at 55°C, and 35 sec elongation at 72°C, with a final extension of 5 min at 72°C. The reaction products were resolved by electrophoresis in 6% polyacrylamide slab gels.

RESULTS AND DISCUSSION

Mechanical transmission. No virus was apparently recovered with any of several mechanical transmission trials. All inoculated hosts remained symptomless and no virus was transmitted from any of them by sub-inoculations to new sets of healthy plants.

dsRNA analysis. Electrophoretic analysis of extracts from three different tissues (cortical scrapings, leaf veins, or leaf petioles) from the N17 source, consistently yielded a dsRNA band, of *ca.*19 kb (Fig. 1), a size similar to those of dsRNAs extracted from plants infected by other members of the family *Closteroviridae* (Martelli *et al.*, 2005).

Identification of the HSP70h gene. A DNA fragment of *ca.* 620 bp was amplified from extracts from N17 leaf veins or petioles submitted to RT-PCR using Tian *et al.* (1996) degenerate primers (not shown). When the sequence of this fragment (accession number AM113547) was analysed by computer-assisted programmes, it was shown to encode a polypeptide of *ca.* 220 amino acids that was part of a closteroviral HSP70h protein. Pairwise amino acid alignment showed a 48%
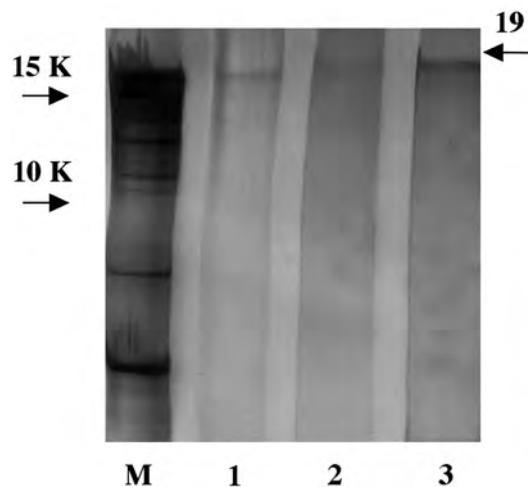
Fig. 1. Electrophoretic pattern of dsRNA extracted from cortical scrapings of fig isolate N17 (lane 3) compared with that from tissue infected with *Grapevine leafroll-associated virus 2* (GLRaV-2) (lane 1) and that from tissue infected with *Grapevine leafroll -associated virus 3* (GLRaV-3) (lane 2), used as controls. DNA ladder is in lane M.

identity with the HSP70 gene of *Beet yellows virus* (BYV) and *Beet yellow stunt disorder virus* (BYSDV), and a 45% identity with that of *Grapevine leafroll-associated virus-2* (GLRaV-2) and *Citrus tristeza virus* (CTV). An identity level of 34% was determined with representatives of the genus *Crinivirus* [*Cucumber yellow stunt disorder virus* (CYSDV), *Beet pseudo yellows virus* (BPYV), and *Sweet potato chlorotic stunt virus* (SPCSV)] and of the genus *Ampelovirus* [*Pineapple mealybug wilt-associated virus 2* (PMBWaV-2)]. In a phylogenetic tree constructed with some of the available HSP70h sequences, the N17 sequence clustered in a clade comprising closterovirus species (Fig. 2).

Electron microscopy. Filamentous particles with distinct cross banding and a length of up to 1500 nm, were readily seen in leaf dips from symptomatic N17 source (Fig. 3C), but not in dips from symptomless fig seedlings used as controls. Cytopathological effects similar to those induced by closteroviruses (Martelli and Russo, 1984), were observed in phloem parenchyma and com-
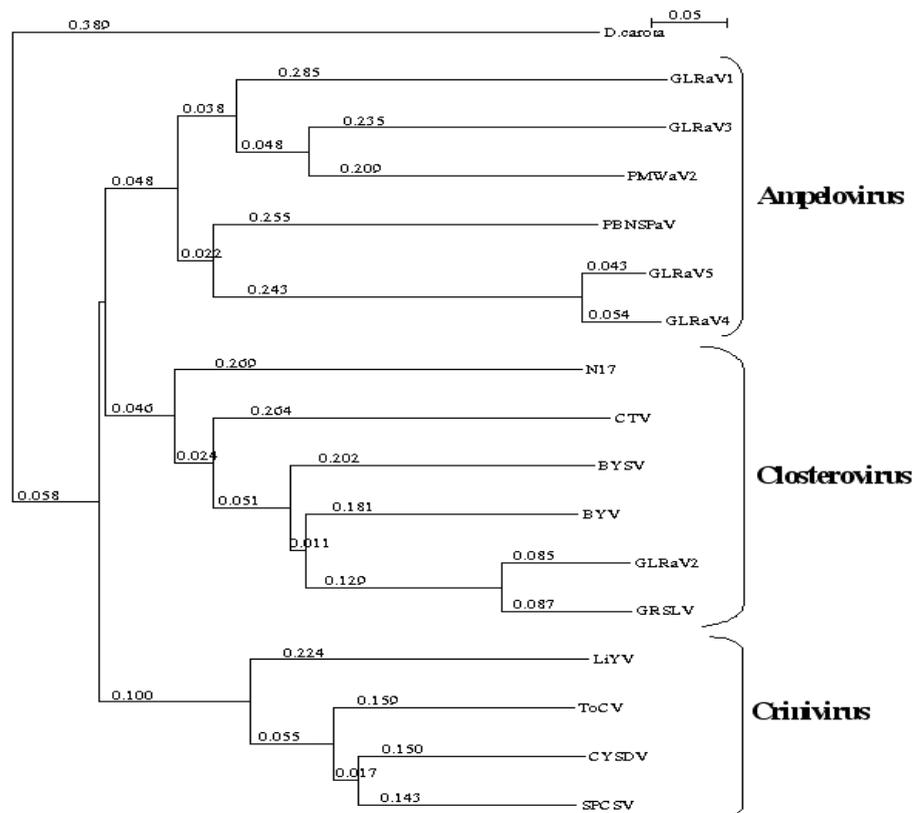

Fig. 2. Phylogenetic tree constructed with amino acid sequences of phosphate motifs 1 and 2 of the HSP70h protein of isolate N17 (AM113547) and of members of the family *Closteroviridae*. Criniviruses: *Lettuce infectious yellows virus* (LiYV, U67448), *Tomato chlorosis virus* (ToCV, AJ968396) *Cucurbit yellow stunt disorder virus* (CYSDV, AJ223619), *Sweet potato chlorotic stunt virus* (SPCSV, NP689401). Ampeloviruses: *Grapevine leafroll-associated virus-1* (GLRaV-1, AF195822), *Grapevine leafroll-associated virus-3* (GLRaV-3, AF037268), *Grapevine leafroll-associated virus-4* (GLRaV-4, AF039553), *Grapevine leafroll-associated virus-5* (GLRaV-5, AF039552), *Pineapple mealybug wilt associated-virus 2* (PMWaV2, AF414119), *Plum bark necrosis and stem pitting-associated virus* (PBNSPaV, AF195501). Closteroviruses: *Citrus tristeza virus* (CTV, NC001661), *Beet yellow stunt virus* (BYSV, U51931), *Beet yellows virus* (BYV, X73476), *Grapevine leafroll-associated virus 2* (GLRaV-2, AF039202). HSP70 of *Daucus carota* (X60088) was used as an outgroup.
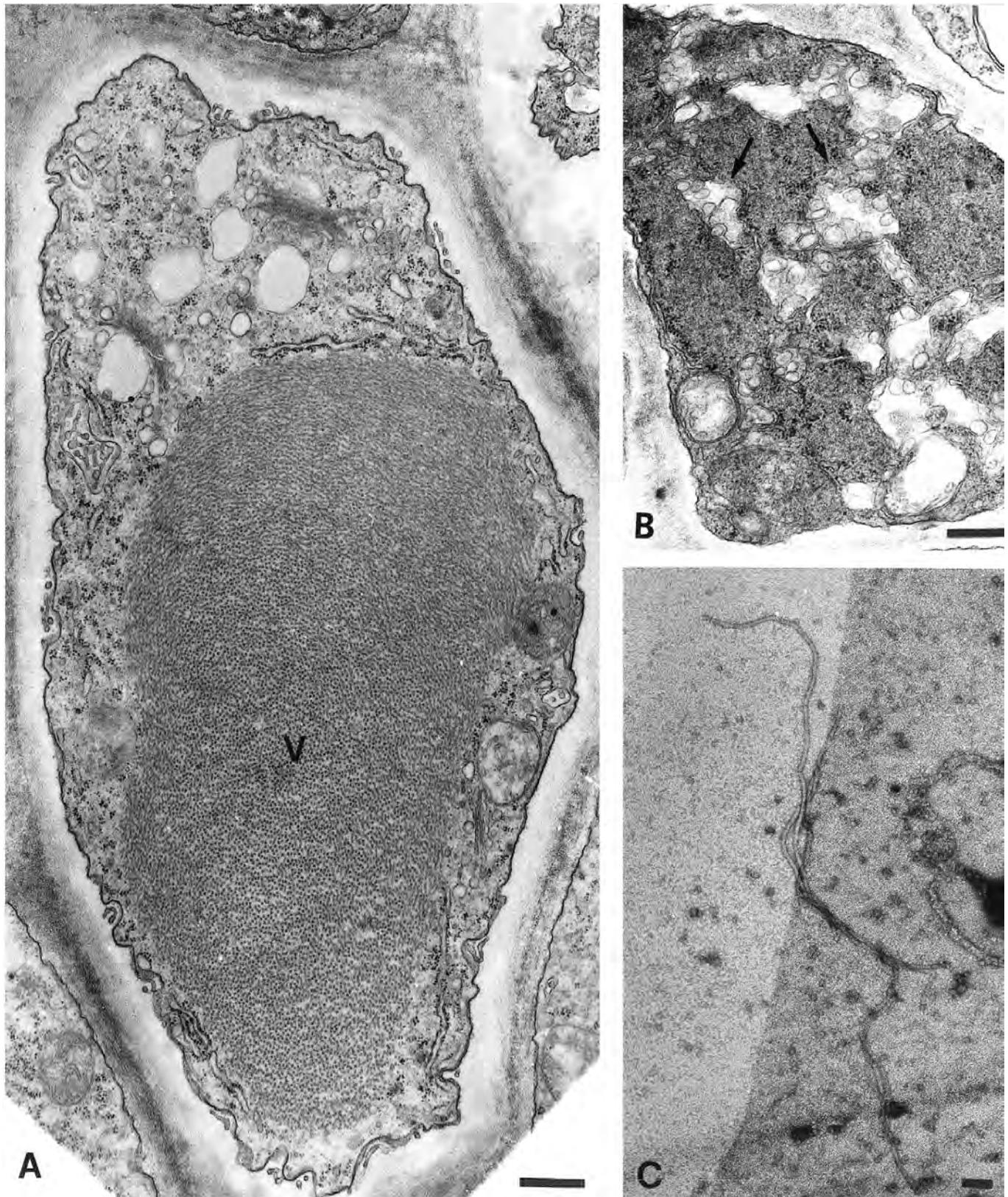
Fig. 3. Cytopathology of leaf tissues of fig infected by isolate N17. **A)** Phloem companion cell with a massive aggregated of virus particles (V). Bar = 500 nm. **B)** Clusters of membranous vesicles (arrows) in the cytoplasm of a phloem companion cell. Bar = 500 nm. **C)** Closterovirus-like particles in a dip from a symptomatic leaf of *F. carica* accession N17. Bar = 100 nm.
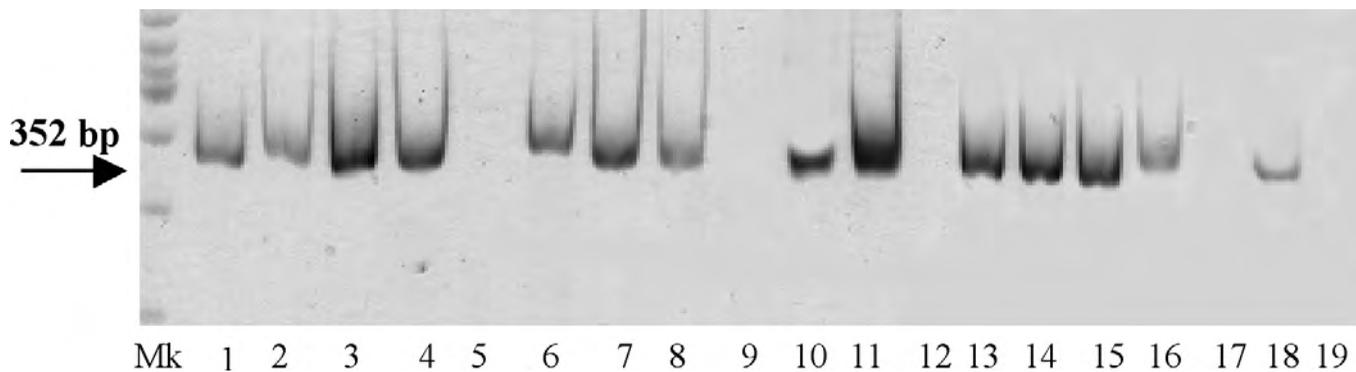
Fig. 4. RT-PCR products amplified from total nucleic acid extracts of leaves from field-grown fig trees using N17-specific primers. M, markers, positive control (isolate N17) in lane 3, water control in lane 19.

panion cells. These contained cytoplasmic accumulations of membranous vesicles with fibrillar material (Fig. 3B) and virus particles that were either intermingled with the vesicles or, more often, formed aggregates that could occupy a great deal of the cell lumen (Fig. 3A). Notwithstanding a very thorough search, no evidence was found of the presence in mesophyll cells of the membrane-bound bodies reported as being typically associated with fig mosaic (see Martelli *et al.*, 1993).

Field survey. In a preliminary field survey for assessing the incidence of isolate N17 in commercial fig stands, 57 samples (37 from symptomatic and 20 from symptomless trees) were collected from Apulian orchards and analyzed by RT-PCR, using virus-specific primers. Amplicons of the expected size (Fig. 4) were obtained from 28 of 37 trees showing mosaic-like symptoms, but also from 9 of 20 trees with no apparent symptoms.

Results of this study confirm that a number of taxonomically different viruses occur in *F. carica* trees showing symptoms in the range of those reported for the ill-defined "fig mosaic disease". They also provide the first evidence that fig can be a host for a virus of the family *Closteroviridae* which, based on molecular information, was tentatively identified as a putative species in the genus *Closterovirus*. The presence of this virus in both symptomatic and symptomless plants may depend on the differential response to infection of the germplasm being evaluated or, more likely, by the fact isolate N17 is not the aetiological agent of the classical "fig mosaic disease". Therefore, the name of Fig leaf mottle-associated virus (FLMaV) is proposed, as suggested by the symptoms shown by the tree in which the virus was found.

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