

## DETECTION AND IDENTIFICATION OF PHYTOPLASMAS INFECTING CULTIVATED AND WILD PLANTS IN LIGURIA (ITALIAN RIVIERA)

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### SUMMARY

Restriction length polymorphism analysis of PCR-amplified ribosomal DNA was used to detect and characterize phytoplasmas infecting 49 out of 56 symptomatic plants of 16 different botanical species. The survey was conducted on vegetable and ornamental crops as well as shrubs and trees growing outdoors or under protected conditions in a region of Liguria, northern Italy. Different primer pairs were used to drive direct or nested PCR reactions. Seven plants of four botanical species showed typical phytoplasma symptoms but failed to give amplification. *AluI*, *HhaI* and *MseI* restriction profiles of amplified fragments showed that phytoplasmas infecting Brussels' sprouts, cineraria, marguerite, dahlia, French hydrangea, lavender, lettuce, olive, pistachio, primrose, Spanish broom, strawflower and violet were uniform and indistinguishable from the European aster yellows reference strain. Thus, all these isolates could be considered typical members of the 16Sr-IB subgroup. Phytoplasmas with restriction patterns indistinguishable among them and from those of the Serbian pepper stolbur reference strain were identified in samples of jasmine, kiwi, lavender and violet. Some of the diseases examined were already described from Italy, but a phytoplasma different from those previously reported was identified in Spanish broom. Eight plant species (cineraria, dahlia, jasmine, kiwi, lavender, pistachio, strawflower and violet) had not previously been identified as natural hosts of phytoplasmas. With the exception of violet, harbouring the two phytoplasmas, all other hosts were infected by only one kind of phytoplasma.

### RIASSUNTO

**INDIVIDUAZIONE E CARATTERIZZAZIONE DI FITOPLASMI DI SPECIE COLTIVATE E SPONTANEE IN LIGURIA.** L'analisi del polimorfismo della lunghezza dei frammenti di restrizione del DNA ribosomiale amplificato

mediante reazione polimerasica a catena è stata impiegata per identificare i fitoplasmi presenti in 49 su 56 campioni prelevati da 16 differenti specie botaniche con sospetti sintomi di infezione da fitoplasma. L'indagine è stata condotta nella piana di Albenga, in Liguria, su specie ortive ed ornamentali coltivate in serra o pieno campo e su piante spontanee. Differenti coppie di iniziatori di reazione sono state impiegate per l'innescamento delle reazioni polimerasiche dirette o ripetute, ma in sette campioni di piante con sintomi tipici di fitoplasmosi, appartenenti a quattro diverse specie botaniche, non è stato amplificato in nessun caso DNA riferibile a fitoplasmi. Il confronto dei profili di restrizione ottenuti a seguito di digestione con gli enzimi *AluI*, *HhaI* ed *MseI* ha mostrato che i fitoplasmi presenti in 13 specie diverse (cavolini di Bruxelles, cineraria, dalia, elicriso, ginestra, lattuga, lavanda, margherita, olivo, ortensia, pistacchio, primula, e viola) erano geneticamente uniformi tra loro ed indistinguibili dall'isolato europeo del giallume dell'astro usato come riferimento, e che quindi tali fitoplasmi potevano essere considerati tutti membri tipici del sottogruppo filogenetico identificato come 16Sr-IB. Altri fitoplasmi, identificati in campioni di actinidia, gelsomino, lavanda e viola, hanno mostrato profili di restrizione fra loro indistinguibili ed identici a quelli dello stolbur del peperone isolato in Serbia usato come riferimento. Alcune delle specie esaminate erano già state segnalate in Italia come ospiti di fitoplasmi. Actinidia, cineraria, dalia, elicriso, gelsomino, lavanda, pistacchio e viola vengono segnalate per la prima volta quali ospiti naturali di fitoplasmi. Con la sola eccezione della viola, che è risultata infetta contemporaneamente da entrambi i fitoplasmi citati, tutte le altre specie si sono rivelate ospiti di uno solo dei due fitoplasmi.

*Key words:* 16S rDNA amplification, RFLP, characterization.

### INTRODUCTION

Phytoplasmas are non-culturable wall-less prokaryotes (class *Mollicutes*) causing yellows and witches' broom type diseases distributed world wide in several

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hundred plant species (McCoy *et al.*, 1989; Lee and Davis, 1992). In the past, numerous reports on such disorders, some of which now clearly assigned to identified phytoplasmas, were based merely on symptom expression and pathogen/vector relationships. Recently, molecular methods, based on restriction fragment length polymorphism (RFLP) analysis of ribosomal genes (rDNA) specifically amplified by means of polymerase chain reaction (PCR) technology, have been used to detect and characterize phytoplasmas in a number of plant and insect hosts (Deng and Hiruki, 1991; Ahrens and Seemüller, 1992; Lee *et al.*, 1993; Lorenz *et al.*, 1995; Gundersen and Lee, 1996; Smart *et al.*, 1996). These results, together with comparisons of conserved phytoplasma DNA sequences have allowed differentiation of phytoplasmas into a number of groups or subclades, forming a molecular framework for classification schemes (Lee *et al.*, 1993; Gundersen *et al.*, 1994, 1996; Schneider *et al.*, 1997a; Davis and Sinclair, 1998; Seemüller *et al.*, 1998). Plasmid DNA sequences harboured in a wide range of phylogenetically different phytoplasmas have also been used for the efficient diagnosis of these pathogens (Goodwin *et al.*, 1994).

Numerous plant diseases, affecting different crops, have been associated in Italy with various phytoplasmas belonging to the 16S rDNA groups I (16Sr-I, aster yellows and related phytoplasmas), V (16Sr-V, elm yellows and related phytoplasmas), X (16Sr-X, apple proliferation and related phytoplasmas) and XII (16Sr-XII, stolbur and related phytoplasmas) (Lee *et al.*, 1993; Davis and Sinclair, 1998; Lee *et al.*, 1998; Seemüller *et al.*, 1998). While phytoplasmas causing serious diseases in fruit trees have frequently been reported throughout the peninsula and characterized to some extent at the molecular level (Poggi Pollini *et al.*, 1993, 1995; Lee *et al.*, 1995a, b; Malisano *et al.*, 1996; Marcone *et al.*, 1996 a, b, d; Poggi Pollini *et al.*, 1996), systematic surveys in weeds, shrubs, vegetable, ornamental and forage crops have been conducted using molecular technologies in central and southern Italy only (Marcone *et al.*, 1997b). Most of the phytoplasmas detected in this survey was found to belong to the 16Sr-I group, but in alfalfa (*Medicago sativa* L.) a putative new member of the faba bean phyllody group was detected, and tomato plants (*Lycopersicon esculentum* Mill.) were shown to harbour an organism indistinguishable from the typical stolbur phytoplasma (Marcone *et al.*, 1997b). From the same area, stolbur phytoplasmas have also been detected in field tomato plants (Albanese *et al.*, 1998) and field bindweed (*Convolvulus arvensis* L.) (Schneider *et al.*, 1997b). Phytoplasmas belonging mainly to the 16Sr-I group have been associated with diseases of ornamental [*Anemone coronaria* L., *Argyranthemum* (*Chrysanthemum*)

*frutescens* L., *Gladiolus* spp., *Hydrangea macrophylla* (Thunb.) Ser., *Ranunculus* hybrids] and horticultural crops (*Brassica oleracea capitata* L., *Cucurbita pepo* L., *Lactuca sativa* L.) in the Italian Riviera (Bertaccini *et al.*, 1990, 1992; Minucci *et al.*, 1994; Vibio *et al.*, 1994; 1995), and a representative of the 16Sr-V group has been detected in *Rubus fruticosus* L. (Lee *et al.*, 1995b).

The aim of this study was to assess by molecular analysis the association of specific phytoplasmas with yellows-type disorders in the agronomically important region of the Italian Riviera, in order to direct research on potential vectors and design protection strategies. We have examined plants showing phytoplasma like symptoms and consequently reinvestigated some already known phytoplasma diseases. Through 16S rDNA PCR and subsequent RFLP analysis we have identified the causal organisms involved. We have also detected phytoplasmas in some plants previously unrecorded as natural hosts, or known to be susceptible to phytoplasmas other than those we have identified.

## MATERIALS AND METHODS

### Plant samples and phytoplasma reference strains.

Protected (Table 1, G) or field plants (Table 1, O) were surveyed throughout 1998 at monthly intervals in the plain of Albenga and at San Remo (Italian Riviera). Samples of branches, buds when present and/or leaves were collected from symptomatic fruit trees, shrubs, ornamental or horticultural plants, along with corresponding parts of healthy-looking individuals. Differing numbers of samples (Table 1) from: *Actinidia deliciosa* (*chinensis*) Planch. (kiwi: K), *A. frutescens* (marguerite: D), *B. oleracea gemmifera* DC (Brussels' sprout: B), *Dahlia* spp. (dahlia: Dh), *Gerbera jamesoni hybrida* Bolus (gerbera: G), *Helichrysum bracteatum* Willd. (strawflower: S), *H. macrophylla* (French hydrangea: H), *Jasminum officinale* L. (jasmine: J), *L. sativa* (lettuce: Le), *Lavandula officinalis* Chaix (lavender: L), *Olea europea* L. (olive tree: O), *Pistacia terebinthus* L. (pistachio: P), *Primula* spp. (primrose: Pr), *Senecio cruentus* (Masson) DC (cineraria: C), *Spartium junceum* L. (Spanish broom: Sb) and *Viola odorata* L. (violet: V) showing symptoms of varying complexities (Table 1) were processed and analyzed as detailed below.

For comparison, isolates of European aster yellows (EAY; 16Sr-IB subgroup), Serbian strain from pepper of the stolbur phytoplasma (Stol; 16Sr-XII group) and clover phyllody (CPh; 16Sr-IC subgroup) phytoplasmas, kindly supplied by M.F. Clark (Horticulture Research International, East Malling, UK), were graft-propagated and maintained in periwinkle [*Catharanthus roseus* (L.)

**Table 1.** Number of samples collected outdoors (O) or in greenhouses (G) with phytoplasma-like disease symptoms in Liguria in 1998.

Host plants	Symptoms												
	Plant						Leaf						Flower
	Dwarfing	Proliferation	Decline	Small	Roll	Curl	Yellowing	Reddening	Vein necrosis	Virescence	Phyllody	Abnormal	
8 Brussels' sprouts (O)	-	+	-	-	-	-	-	-	-	-	-	-	
6 Cineraria (G)	+	+	-	+	-	-	+	-	-	+	+	-	
13 Common daisy (O, G)	-	+	+	+	-	-	+	-	-	+	+	+	
7 Dahlia (G)	+	+	-	+	-	-	-	-	-	-	-	+	
3 French hydrangea (O, G)	+	-	-	+	+	-	-	+	-	+	+	-	
2 Gerbera (G)	-	-	-	-	-	-	-	-	-	+	-	+	
1 Jasmine (O, G)	+	-	-	+	-	+	-	-	-	-	-	-	
1 Kiwifruit (O)	-	-	-	+	+	-	-	-	+	-	-	-	
2 Lavender (O)	+	-	+	+	-	-	+	-	+	-	-	-	
2 Lettuce (O)	-	+	+	-	+	-	+	+	+	-	-	-	
1 Olive tree (O)	-	+	-	-	-	-	-	-	-	-	-	-	
1 Pistachio (O)	-	+	+	+	-	-	-	-	-	-	-	-	
6 Primrose (G)	-	+	+	-	-	-	+	-	+	+	+	+	
1 Strawberry (O)	+	+	-	+	-	-	+	-	-	-	-	+	
1 Spanish broom (O)	-	+	-	-	-	-	+	-	-	-	-	-	
1 Violet (O)	-	+	-	+	-	-	-	-	-	+	-	-	

G. Don]. DNA from elm yellows phytoplasma (EY; 16Sr-V group), a gift from Dr. E. Seemüller (Biologische Bundesanstalt, Dossenheim, Germany) was also used in nested PCR experiments. The relative taxonomic positions within the known spectrum of phytoplasmas of the references isolates has been described (Davis and Sinclair, 1998; Lee *et al.*, 1998; Seemüller *et al.*, 1998).

**DNA isolation.** Midribs or other plant parts (typically 1.5 g) from symptomatic or healthy-looking fresh field collected samples, as well as from greenhouse-maintained phytoplasma-infected or healthy periwinkles were isolated by hand and kept on ice. After tissue disruption and phytoplasma DNA enrichment (Ahrens and Seemüller, 1992), DNA was extracted by a method slightly modified from Doyle and Doyle (1990). After a first precipitation with isopropyl alcohol, the DNA was rinsed with 70% ethanol, resuspended in 10 mM TE (Tris-HCl, pH 8.0, 1 mM EDTA) and precipitated a second time in the presence of 0.3 M Na acetate and 2 vol. ethanol. The final pellet, after a second rinse in 70% ethanol, was resuspended in sterile distilled water (typically 100 µl).

**Direct PCR amplification.** Several universal primer pairs derived from highly conserved ribosomal sequences, designed to amplify fragments of different specific lengths were used in direct PCR assays: R16F2/R2 (Lee *et al.*, 1993; 1225 bp), U5/U3 (Lorenz *et al.*, 1995; 862 bp) and R16mF2/R1 (Gundersen and Lee, 1996; 1434 bp). Primer pair CK6/CK8 (Goodwin *et al.*, 1994; 235 bp), that amplify a wide range of phytoplasma specific extrachromosomal sequences, were also used. Amplification reactions were programmed as described in the original papers.

Reaction products were analyzed by electrophoresis in 1% agarose gels buffered in 0.5x TBE (TBE buffer: 90 mM Tris(hydroxymethyl)aminomethane, 90 mM boric acid, 3 mM ethylenediaminetetraacetate Na salt, pH 8.3) along with 1 Kb DNA size marker (Gibco BRL, Gaithersburg, MD) and visualized by UV light after staining with ethidium bromide.

**Nested-PCR amplification.** DNAs from symptomatic plants which did not respond positively in direct PCR experiments with any primer pair used or that yielded products unsuitable for further characterization by RFLP, were subjected to nested PCRs. PCR products initially amplified with the universal primer pairs R16F2/R2 (Lee *et al.*, 1993) were diluted 1:40 and 2 µl aliquots used as templates in reactions primed by one of the following group-specific pairs: R16(I)F1/R1 R16(III)F2/R1, and R16(V)F1/R1 (Lee *et al.*, 1994; approximately 1.100, 800 and 1.100 bp, respectively).

DNAs from symptomatic plants which did not yield amplified products in any nested assay, were used as templates in further direct and nested PCRs using primer pairs designed to amplify a wide range of phylogenetically different phytoplasmas: R16mF2/R1 and R16F2n/R2, respectively (Gundersen and Lee, 1996). Amplification products were analyzed in agarose gels as above.

**RFLP analysis.** Amplified 16S rDNA fragments (typically 8 µl aliquots) were separately digested at 37°C for 2-16 h with 1.5-2 units of one of the following restriction endonucleases: *MseI* (GIBCO BRL), *HbaI* (Promega Corporation, Madison, WI) or *AluI* (New England Biolabs, Beverly, MA). Restriction fragments were resolved in 5% polyacrylamide gels buffered in TBE and visualized as mentioned. Size marker was 50-1000 bp PCR ladder (Amersham Italia, Milan, Italy).

## RESULTS

**Field surveys.** All plants with suspected phytoplasma diseases (Table 1) showed symptoms similar to those previously described for the same hosts in Italy (Bertaccini *et al.*, 1990, 1992; Minucci *et al.*, 1994; Vibio *et al.*, 1994, 1995; Poggi Pollini *et al.*, 1996; Marcone *et al.*, 1997a, b). Table 1 summarizes the range of symptoms observed. Shoot proliferation and dwarfing were the most prevalent symptoms on the whole plant, both in protected or outdoor crops. Symptoms generally became more pronounced towards the end of the growing season, *i.e.* soon before flowering or harvest. However, in trees, shrubs or flowers cultivated or spontaneously growing outdoors, symptoms tended to appear in full summer suggesting that infection might have occurred in early spring. Olive instead, showed symptoms at the beginning of spring.

**Phytoplasma detection by PCR.** Target DNAs were never amplified from asymptomatic plants collected in the field or from healthy greenhouse-grown periwinkles in any direct or nested PCR experiments (not shown).

Table 2 summarizes PCR results obtained with DNAs from symptomatic samples with different primer pairs. A relatively large number of symptomatic samples (41/56) possessed a phytoplasma DNA titre high enough to yield an amplified fragment of the expected size, at least with one of the primer pairs used in direct PCRs. Twenty five such samples, amplified using R16F2/R2 primer pair, were then directly analyzed by RFLP. Two DNA samples yielded too little product to allow reliable RFLP analysis (+/- in Table 2: Pr3 and 6). The remaining fourteen samples yielded a phytoplasma specific fragment with one or more of the other universal primer pairs used.

**Table 2.** Direct and group-specific nested PCR detection of phytoplasmas in outdoor or greenhouse samples. For abbreviations and nested PCR primer pairs see text. Nt: not tested.

Host			Direct PCR			Nested PCR		
			R16F2/R2	U3/U5	CK6/CK8	16Sr-I	16Sr-III	16Sr-V
Brussels' sprouts	B	1	+	nt	nt	nt	nt	nt
		2	+	nt	nt	nt	nt	nt
		3	+	nt	nt	nt	nt	nt
		4	+	nt	nt	nt	nt	nt
		5	+	nt	nt	nt	nt	nt
		6	+	nt	nt	nt	nt	nt
		7	+	nt	nt	nt	nt	nt
		8	+	nt	nt	nt	nt	nt
Cineraria	C	1	+	nt	nt	nt	nt	nt
		2	-	-	-	+	-	-
		3	+	nt	nt	nt	nt	nt
		4	-	-	-	+	-	-
		5	+	nt	nt	nt	nt	nt
		6	+	nt	nt	nt	nt	nt
Marguerite	D	1	+	nt	nt	nt	nt	nt
		2	+	nt	nt	nt	nt	nt
		3	+	nt	nt	nt	nt	nt
		4	-	-	+/-	+	-	-
		5	-	+	nt	+	-	-
		6	-	-	-	-	-	-
		7	-	+	nt	+	-	-
		8	-	+	nt	+	-	-
		9	+	nt	nt	nt	nt	nt
		10	+	nt	nt	nt	nt	nt
		11	+	nt	nt	nt	nt	nt
		12	+	nt	nt	nt	nt	nt
		13	+	nt	nt	nt	nt	nt
Dhalia	Dh	1	-	-	+/-	+	-	-
		2	-	-	-	-	-	-
		3	-	-	-	-	-	-
		4	-	-	-	-	-	-
		5	-	-	-	-	-	-
		6	+	nt	nt	nt	nt	nt
Gerbera	G	7	+	nt	nt	nt	nt	nt
		1	-	-	-	-	-	-
Hydrangea	H	2	-	-	+/-	-	-	-
		1	-	-	-	+	-	-
Jasminum	J	2	-	+/-	nt	+	-	-
		1	-	-	-	+	-	-
		3	-	-	-	-	-	-
Kiwi	K	1	-	-	-	+	-	-
Lavender	L	1	-	-	-	+	-	-
		2	-	+/-	-	+	-	-
Lettuce	Le	1	-	-	-	+	-	-
		2	-	-	-	+	-	-
Olive	O	1	-	+	nt	+	-	-
Pistachio	P	1	+	nt	nt	nt	nt	nt
Primrose	Pr	1	+	nt	nt	nt	nt	nt
		2	+	nt	nt	nt	nt	nt
		3	+/-	+	nt	+	-	-
		4	-	+	nt	+	-	-
		5	-	+	nt	+	-	-
		6	+/-	nt	nt	+	-	-
Strawflower	S	1	-	+	-	+	-	-
Spanish broom	Sb	1	-	+/-	nt	+	-	-
Violet	V	1	-	+/-	nt	+	-	-

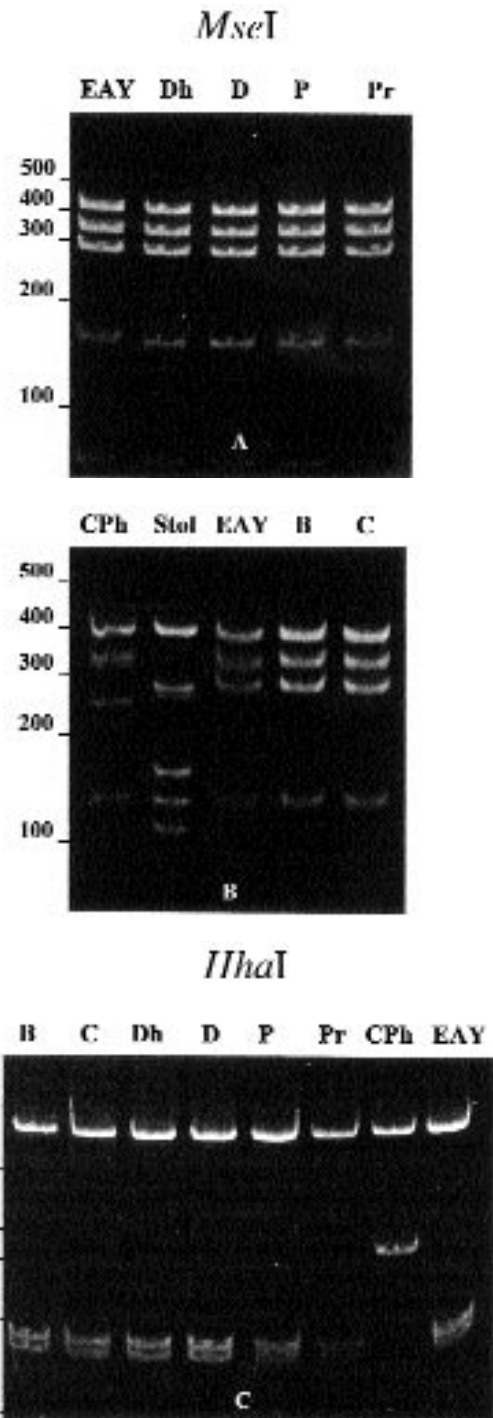
Amplicons that, due to their small size or low quantity, were not fully suitable for subsequent reliable differentiation by RFLP, were used as templates in nested PCR assays using different group-specific primer pairs. All these samples, except G2, yielded a phytoplasma-specific fragment of the expected size only when using the 16Sr-I group-specific primer pair (Table 2).

Fifteen DNA samples from different botanical species did not respond to any of the primer pairs tested in direct PCR assays and were therefore tested with group-specific primers in nested assays. Eight of them yielded the expected phytoplasma-specific fragment using the group I-specific primer pair (Table 2), and were therefore amenable to further RFLP analysis. None of the extracts was amplified by groups III or V-specific primer pairs (Table 2), except DNAs from available reference isolates.

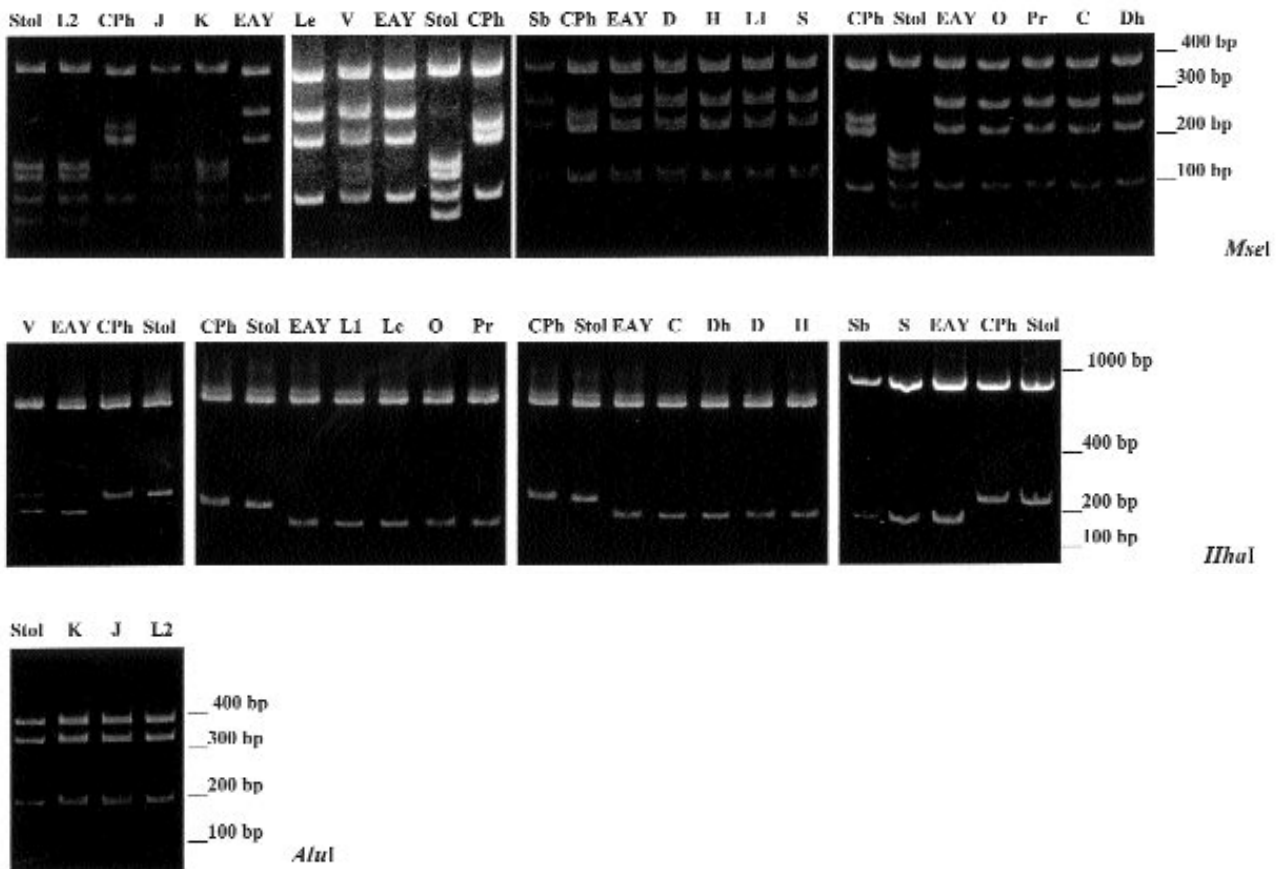
Seven samples, negative in all direct and/or group-specific nested PCR assays, plus the one that tested slightly positive only with the extrachromosomal primer pair CK6/CK8 (Table 2: G2) did not yield a phytoplasma-specific fragment in either direct PCR using R16mF2/R1 primer pairs or in subsequent nested assays using universal primer pair R16F2n/R2 (not shown). Soluble inhibitors were present only in extracts of kiwi and jasmine; however a 1:5 dilution was sufficient to restore amplification of control DNAs mixed with such samples.

**RFLP analysis of PCR products.** After separate restrictions (Fig. 1, panels A, B, C), RFLP profiles of 16S rDNA sequences amplified by direct PCR with primer pair R16F2/R2 indicated that the plants involved were infected by phytoplasmas of the 16Sr-IB subgroup (Lee *et al.*, 1993; Seemüller *et al.*, 1998).

RFLP profiles of 16S rDNA sequences amplified by nested PCRs with the 16Sr-I group-specific primer pair further indicated that most of the positive samples were infected by phytoplasmas of the 16Sr-IB subgroup (Fig. 2). However, amplified fragments from kiwi, jasmine and one lavender plant (Table 2; L2) gave RFLP patterns indistinguishable from those of the Serbian stolbur reference isolate strain (Fig. 2). In the case of violet DNA only, the presence of more complex patterns suggested co-infection by two phytoplasmas: a predominant 16Sr-IB subgroup member and a second phytoplasma, of the 16Sr-XII group (Davis and Sinclair, 1998; Lee *et al.*, 1998; Seemüller *et al.*, 1988) (Fig. 2).



**Fig. 1.** *Mse*I (panels A, B) and *Hba*I (panel C) restriction profiles of 16S rDNA amplified using the R16F2/R2 primer pair (Lee *et al.*, 1993) from DNAs of naturally infected plants and from periwinkles carrying reference isolates. Digests were fractionated in 5% polyacrylamide gels. Molecular sizes are indicated on the left. Abbreviations: EAY: European aster yellows; CPh: clover phyllody; Stol: stolbur; B: Brussels' sprout; C: cineraria; D: daisy; Dh: dahlia; P: pistachio; Pr: primrose.



**Fig. 2.** RFLP profiles of 16S rDNA amplified by nested PCR using R16(I)F1/R1 primer pair (Lee *et al.*, 1994) following a pre-amplification step with the universal RIGF2/R2 primer pair (Lee *et al.*, 1993). Products were digested with restriction endonucleases *Mse*I, *Alu*I and *Hha*I and processed as in Fig. 1. Abbreviations (see also Fig. 1): J: jasmine; K: kiwi; H: French hydrangea; L: lavender; Le: lettuce; O: olive; S: strawflower; Sb: Spanish broom.

## DISCUSSION

The differing numbers of samples for each botanical species brought to our attention probably reflected either the crop's economic importance and/or the frequency of symptom appearance within the area considered. Symptoms were generally more easily recognizable in well cared for protected crops than in less well tended or open fields, where it may be difficult to distinguish phytoplasma infections from other conditions. In any case, as widely recognized and confirmed also by our results, diagnosis based on symptomatology alone is unreliable.

Absence of amplification from symptomatic samples was not because of the presence of enzyme inhibitors in the extracts, but could have been due to either titres in phytoplasma DNA below detection limits even using very sensitive techniques, or the symptoms might have

been provoked by other agents, such as herbicide or hormone treatments. One of the two gerbera plants examined (G2) responded weakly only to the extrachromosomal primer pair. This may be because plasmid sequences are ubiquitously present in high copy numbers in phylogenetically different phytoplasmas (Goodwin *et al.*, 1994). Alternatively, the phytoplasmas sporadically detected by electron microscopy in different gerbera species with virescence symptoms from Italy (McCoy *et al.*, 1989) might belong to an unknown phylogenetic group not detectable by any of the other primers used.

We obtained positive results with samples from eight botanical species previously not reported to be susceptible to phytoplasma infection. Cinerarias, dahlias, one of the lavenders, pistachio, strawflower and violet were affected by phytoplasmas belonging to subgroup 16Sr-IB (Lee *et al.*, 1993; Davis and Sinclair, 1998; Lee *et al.*,

1998; Seemüller *et al.*, 1998). Kiwi, jasmine, a second lavender and the same violet carried phytoplasmas indistinguishable from one another and from the pepper reference isolate, belonging to the stolbur or 16Sr-XII group (Davis and Sinclair, 1998; Seemüller *et al.*, 1998). All other plants listed in Table 1 and already known to be phytoplasma susceptible were also infected by members of the 16Sr-IB subgroup.

As anticipated on the basis of earlier reports dealing with ornamental and horticultural crops of the same region (Bertaccini *et al.*, 1990, 1992; Minucci *et al.*, 1994; Vibio *et al.*, 1994), we found that most of the phytoplasmas detected were members of the 16Sr-IB subgroup. The Spanish broom plant, host of an EAY-related phytoplasma (Conti *et al.*, 1997), was harbouring a member of this subgroup, whereas in southern Italy phytoplasmas of the 16Sr-X and -V groups have been associated with witches'-broom symptoms in this species (Marcone *et al.*, 1996c). *Cineraria* has been listed (McCoy *et al.*, 1989) as an experimental host of CPh phytoplasma (16Sr-IC subgroup), but it has not been previously reported as a natural host, and we found that a member of the 16Sr-IB subgroup was associated with the symptoms described.

Olive trees with yellowing, leaf chlorosis and malformation but not proliferation have been reported to be infected by a 16Sr-V group phytoplasma from a different region of northern Italy (Poggi Pollini *et al.*, 1996), and from central Italy a witches'-broom-like disorder was associated with a non-EAY-related phytoplasma (Del Serrone *et al.*, 1996). We found an olive tree harbouring a member of the 16Sr-IB subgroup, so olives appear to be susceptible in nature to phytoplasmas belonging to different groups. Multiple infections by phytoplasmas of the 16Sr-IC subgroup and occasionally the 16Sr-III group and -IB subgroup have also been reported (Danielli *et al.*, 1996).

Our detection of stolbur is the first report of natural phytoplasma infection in kiwi. The two lavender plants exhibiting similar symptoms were shown to be infected by two phylogenetically different phytoplasmas (16Sr-IB subgroup and stolbur group); it is well established that similar symptoms may be caused by different phytoplasmas in a given host plant, and this is just a further example. Violet was co-infected by a 16Sr-IB subgroup member and a stolbur with an apparent weaker titre. All members of the stolbur group we detected in different species appeared to be genetically uniform and indistinguishable from the Serbian reference isolate, whereas from other regions of Italy, differing isolates have been described from field bindweed (Marcone *et al.*, 1997a; Schneider *et al.*, 1997b). Mixed infection by different AY-related phytoplasmas have earlier been re-

ported from the same region of Liguria in naturally infected lettuce (Minucci *et al.*, 1994), whereas multiple phytoplasma infections appear to be more common in perennial fruit trees, shrubs or grapevines (Lee *et al.*, 1995a; Bianco *et al.*, 1996; Danielli *et al.*, 1996; Marcone *et al.*, 1996c).

Phytoplasmas of the 16Sr-I group appear to be the commonest among weeds, shrubs, vegetables, ornamentals and forage crops in various parts of Italy (Bertaccini *et al.*, 1990, 1992; Minucci *et al.*, 1994; Vibio *et al.*, 1994, 1995; Firrao *et al.*, 1996; Marcone *et al.*, 1997b). Occasionally, members of 16Sr-III, -V, -X, sugarcane white leaf, faba bean phyllody groups, and, more often stolburs have been detected (Bertaccini *et al.*, 1996; Marcone *et al.*, 1996, 1997; Minucci and Boccardo 1997; Schneider *et al.*, 1997b; Albanese *et al.*, 1998; Paltrinieri *et al.*, 1998). This may well be a consequence of the fact that the 16Sr-I group members are vectored in nature by several species of polyphagous leafhoppers (Deltocephalinae) which may play a very active role in spreading infections (Guglielmone *et al.*, 1994; Bosco *et al.*, 1997). Our results show that a large variety of crops may carry hopper borne phytoplasmas which represent an increasing phytopathological problem in Liguria. The constant presence in the same area of potential inoculum sources, at least for 16Sr-IB and -XII group phytoplasmas, in both protected and outdoor crops may also be an important factor in disease spread.

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