Genetic Characterization and Classification of Two Phytoplasmas Associated with Spartium Witches'-Broom Disease

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ABSTRACT

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In plants of Spanish broom (*Spartium junceum*) affected by an etiologically uncertain witches'-broom disease, plant-pathogenic phytoplasmas were detected by fluorescence and scanning electron microscopy. Restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA, polymerase chain reaction (PCR)–amplified by means of universal phytoplasma primers, revealed two different phytoplasmas present in plants with similar symptoms. The less frequently detected phytoplasma had the same RFLP profile as elm yellows phytoplasmas, whereas the prevalent agent had an RFLP profile similar to that of phytoplasmas in the apple proliferation strain cluster. Sequence analysis of 16S rDNA confirmed that the prevalent Spartium witches'-broom phytoplasma is a member of the apple proliferation strain cluster, but this organism is distinctly different from other cluster members. With universal, group- and pathogen-specific rDNA primers, the causal agents were detected in most symptomatic plants. The plants testing negatively in direct PCR yielded an amplification product when the rDNA fragment obtained with universal primers was re-amplified with group- or pathogen-specific primers. The nested-PCR assay also revealed that most of the plants examined were doubly infected with the two phytoplasmas detected by direct PCR. In the infected plants, one of these organisms was predominant and readily detectable by direct PCR while the other occurred in low numbers and could only be identified by nested PCR.

Spartium junceum L. (Spanish broom) is a fabaceous shrub that is a component of the maquis and similar semiarid Mediterranean habitats. This rapidly growing plant is highly adaptable to various environmental conditions and is widespread in central and southern Italy including the Italian islands. *Spartium junceum* is of considerable ecological importance due to its role in decreasing soil erosion. The plant is also used as an ornamental and the flowers yield a yellow dye. In the past, the flexible rushlike twigs were used in viticulture and basketry.

In many or all Italian regions where *S. junceum* grows, plants are severely affected by a lethal witches'-broom disease that has not been described in detail. This disease was probably observed in Italy as early as the late nineteenth century, when the cause was attributed to the gall mite *Phytoptus spartii* (G. Can.) (8). More recently, Castagnoli (7) redescribed the mite as *Eryophyes spartii* (G. Can.) and also suspected this pest as the causal agent of the disease. However, she considered that other biotic factors may be involved. In a preliminary note, Ragozzino and Cristinzio (25) reported on the electron microscopic detection of phytoplasmas (previously termed mycoplasmalike organisms) (12) in symptomatic *S. junceum.*

Symptoms. Based on author's observations, the most characteristic symptom of the disease is witches'-broom, which develops from numerous axillary buds that give rise to excessive numbers of shoots with extremely shortened internodes (Fig. 1). The length of affected shoots is reduced to about one-tenth that of normal shoots, while their thickness is noticeably greater. The size of the leaflets is reduced, while their number is increased. Affected plant parts show yellowing symptoms that become more pronounced over time. In diseased plants the flowers have an erect habit and show malformations such as virescence and phyllody. Diseased plants fail to set fruits. The disease usually starts at one or a few branches and may spread over the whole plant within a few months. In the winter, severely affected plants show off-season growth resulting in the formation of new witches'-brooms. This young growth is susceptible to frost and may be killed during winter. Cuts made into the stems of plants showing off-season growth reveal phloem necrosis. Another typical symptom of severely affected plants is the lack of feeder roots. Other symptoms include stunting of infected plants, twisting and fasciation of twigs, and purple discoloration and rugosity of the bark, which also may have cracks. Affected plants die within one or a few years after appearance of the first symptoms.

Recently, considerable progress has been made by the use of DNA-based methods in detecting, identifying, and classifying phytoplasmas. In particular, by restriction site and sequence analysis of 16S rDNA, many phytoplasmas have been distinguished and phylogenetically classified (11,16,27,29). Two of the various phylogenetic clusters that were established are the apple proliferation (AP) strain cluster, which includes, among others, the apple proliferation (AP), pear decline (PD), and European stone fruit yellows (ESFY) agents, and the elm yellows (EY) strain cluster, which includes the EY agent and other phytoplasmas. Phytoplasma detection was improved by the development of procedures for the in vitro amplification of phytoplasmal DNA through the polymerase chain reaction (PCR) (2,18,26). In order to clearly elucidate the etiology of the Spartium witches'-broom disease, we employed both microscopic and PCR methods. Two phytoplasmas from diseased plants were identified, characterized, and classified using restriction fragment length polymorphism (RFLP) analysis of PCR-amplified rDNA and sequence analysis of the 16S rRNA gene.

MATERIALS AND METHODS

Plant samples and reference phytoplasma strains. Sampling of diseased and nonsymptomatic *S. junceum* plants was carried out in central and southern Italy throughout 1994, in particular during the growing season. A total of 25 diseased plants were sampled from their natural habitats: 10 plants in the Campania region, eight plants in the Basilicata region, three plants in the Calabria region, and two plants each in the Apulia and Latium regions. In addition to these field-collected samples, the following phytoplasma strains, which were previously transmitted

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to periwinkle (*Catharanthus roseus* L.) and maintained in this host by periodic grafting, were included in this study for comparison: AAY, American aster yellows, collected by R. E. McCoy, Fort Lauderdale, FL, and obtained from R. Marwitz, Berlin; STOL, stolbur of pepper, collected by D. Sutic, Beograd (Serbia) and obtained from R. Marwitz; AT, apple proliferation, obtained from R. Marwitz; ASHY, ash yellows collected by W. A. Sinclair, Ithaca, NY; ULW, elm yellows, collected by G. Morvan, Avignon-Montfavet (France) and obtained from F. Dosba, Bordeaux (France); GVX, Green Valley strain of western X-disease, collected by A. H. Purcell, Berkeley, CA, and obtained from M. F. Clark, East Malling (UK); SUNHP, sunnhemp witches'-broom, collected in Thailand by E. Seemüller; BVK, from the leafhopper *Psammotettix cephalotes* (H.-S.), collected by W. Heintz, Dossenheim (Germany).

The origins of the strains maintained in periwinkle are described elsewhere (27). These strains represent all primary phylogenetic clusters established by Seemüller et al. (29) and their phylogenetic positions based on 16S rDNA sequences are depicted in Figure 2.

Fluorescence and scanning electron microscopy. Phytoplasmas were detected by fluorescence microscopy using the DAPI (4′-6-diamidino-2-phenylindole) method (28). For detection by scanning electron microscopy (SEM), specimens were taken from young shoots, stems, and roots, fixed in 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7, and postfixed in 1% osmium tetroxide in the same buffer. Following dehydration with an ethanol series and critical-point drying, the specimens were mounted on SEM specimen mounts with silver conducting paste. The specimens were then coated under vacuum with 3 to 5 nm of gold and examined with a Cambridge Stereoscan S250 Mk2 scanning electron microscope (Leo Electron Microscopy Ltd., Cambridge, UK).

DNA isolation. Phloem of nonsymptomatic and diseased *S. junceum* plants was prepared as aseptically as possible from approximately 30-mm-thick branches, stems, and roots by removing the outer bark with a knife and excising the layer of conductive tissue with a sterile scalpel. Young shoots including the leaves were taken from healthy and infected periwinkles. The DNA was extracted using a phytoplasma enrichment procedure (2,3).

Primers and PCR amplification. Both universal and specific primers derived from ribosomal sequences were used. Universal primers P1 (10) and P7 (14), which prime at the 5′ end of the 16S rRNA gene and in the 5′ region of the 23S rRNA gene, respectively, amplify a DNA fragment approximately 1,800 bp in length. Primer pair fB1/rULWS, which primes in the 5′ region of the 16S rRNA gene and in the 16S/23S rDNA intergenic region (spacer), specifically amplifies a fragment about 1,650 bp from the EY phytoplasma (14). Similarly, primer pair fAY/rEY (5′-GCA CGT AAT GGT GGG CAC TT-3′/5′-CGA AGT TAA GCC ACT GCT TTC-3′), which is directed to 16S rDNA sequences, specifically amplifies EY phytoplasma DNA (1). For specific detection of the prevalent phytoplasma associated with Spartium witches'-broom disease, reverse primer rSP was designed from the 16S/23S rDNA spacer sequence of this organism. This oligonucleotide, which primes just downstream of the conserved $tRNA^{Ile}$ gene and has the sequence 5′-GCT AAT TAG AAA TAT CAA CTA-3′, was paired with universal primers fU5 (18) or P1. For detection of the prevalent *S. junceum* pathogen, primer pair fO1/rO1, which specifically amplifies all fruit tree phytoplasmas of the AP strain cluster (18), was also employed. In addition, primer pairs fAT/rAS and fAT/rPRUS, which preferentially or specifically amplify the AP and ESFY phytoplasmas, respectively (14), were included in this study.

Amplifications were performed with a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, CT) in a 50-µl volume containing 125 µM of the four dNTPs, 0.5 µM each primer, 1 U of Replitherm DNA polymerase, 1× polymerase buffer (both Biozyme, Biozym Diagnostik, Hameln, Germany), 5 µl of template DNA (100 to 200 ng), and water. The mixture was overlaid with three drops of mineral oil and subjected to 35 cycles at the following incubations: 30 s denaturation at 95°C, 60 s annealing at 55°C (50°C with primer pair fB1/rULWS and 48°C with primer pairs P1/rSP and fU5/rSP), and 90 s extension at 72°C. Five microliters of the PCR products was analyzed by electrophoresis in a 1.5% horizontal agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) in the presence of ethidium bromide (0.5 µg/ml). DNA bands were visualized under UV light. In nested PCR, the first amplification was performed with primer pair P1/P7. The reaction was then diluted with sterile distilled water (1:40) and was re-amplified with appropriate primers under the conditions described above.

RFLP analysis of PCR products. Ten microliters of PCR products obtained with primer pair P1/P7 was subjected to RFLP analysis using the appropriate restriction endonucleases (*Alu*I and *Rsa*I) according to the manufacturer's instructions (MBI Fermentas, Vilnius, Lithuania). Five microliters of the digests was resolved on vertical 5% (wt/vol) polyacrylamide gels

Fig. 1. Witches'-broom on *Spartium junceum*.

in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). After electrophoresis, the DNA was stained with ethidium bromide and visualized under UV light. The molecular weights of the fragments were determined by comparison with the 1-kb DNA ladder (BRL-Life Technologies, Eggenstein, Germany).

DNA sequencing and data analysis. The PCR product obtained with primer pair P1/P7 from a *S. junceum* plant infected with the predominant phytoplasma was directly sequenced by cycle sequencing, using the fmol DNA sequencing system (Promega, Madison, WI) as described (29). The full-length sequence has been deposited in the EMBL Data Library (Heidelberg, Germany) under the accession number X92869. The sequence of the 16S rRNA gene of the *S. junceum* pathogen was aligned with 16S rDNA sequences of other previously characterized phytoplasmas obtained from the GenBank database using the Pileup program of the UWGCG software system (Genetic Computer Group, Madison, WI). This alignment was then analyzed by means of Phylogenetic Analysis Using Parsimony (PAUP) version 3.1.1 (Illinois Natural History Surver, Champaign). A phylogram was generated by performing bootstrap analysis of 100 replicates with 10 random additions of each replicate. Branch swapping was carried out using the tree bisection and reconnection method. Phylogenetically uninformative bases were not included in this analysis. *Bacillus subtilis* was used as an outgroup in all phylogenetic analyses.

RESULTS

Microscopic phytoplasma detection. Examination of cryotome-sectioned tissues stained with the fluorochrome DAPI revealed typical phytoplasma fluorescence in sieve tubes of diseased plants but not in those of healthy plants (data not shown). Phytoplasma numbers were higher in roots and several-year-old branches than in twigs. No phytoplasma fluorescence was found in young shoots, including the growth that resulted in the formation of witches'-brooms. The presence of phytoplasmas in diseased plants and their absence in healthy plants was confirmed by SEM examination. The morphology of the organisms was mostly spherical (Fig. 3). Evidence of multiplication by budding and binary fission was often observed. The diameter of the structures varied from 80 to 900 nm, with an average of about 400 nm.

Phytoplasma detection with universal primers and RFLP analysis of the PCR products. With the universal phytoplasma primer pair P1/P7, the target DNA was amplified from samples taken from 22 of the 25 symptomatic *S. junceum* plants that were collected. An amplification product was also obtained from all phytoplasma strains that were maintained in periwinkle. However, the primer pair did not amplify DNA from nonsymptomatic *S. junceum* or periwinkle plants (Table 1).

Two different restriction profiles were obtained when the rDNA amplified from templates from diseased *S. junceum* plants was digested with the frequently cutting restriction endonucleases *Alu*I and *Rsa*I (Fig. 4; Table 1). Following digestion with *Alu*I, 19 of the 22 plants testing positively with primers P1/P7 showed a uniform restriction profile different from that of all phytoplasmas that were included in this study for comparison. However, the *Alu*I RFLP profile of the phytoplasma from *S. junceum* had several bands in common with the profile of the AP phytoplasma (Fig. 4A). The relatedness of the phytoplasma from *S. junceum* with the AP agent was confirmed by digestion of the amplification products with *Rsa*I, which resulted in an identical pattern of the two organisms (Fig. 4B). The name Spartium witches'-broom (SPAR) phytoplasma is proposed for this AP phytoplasma-related organism. Three of the 22 *S. junceum*

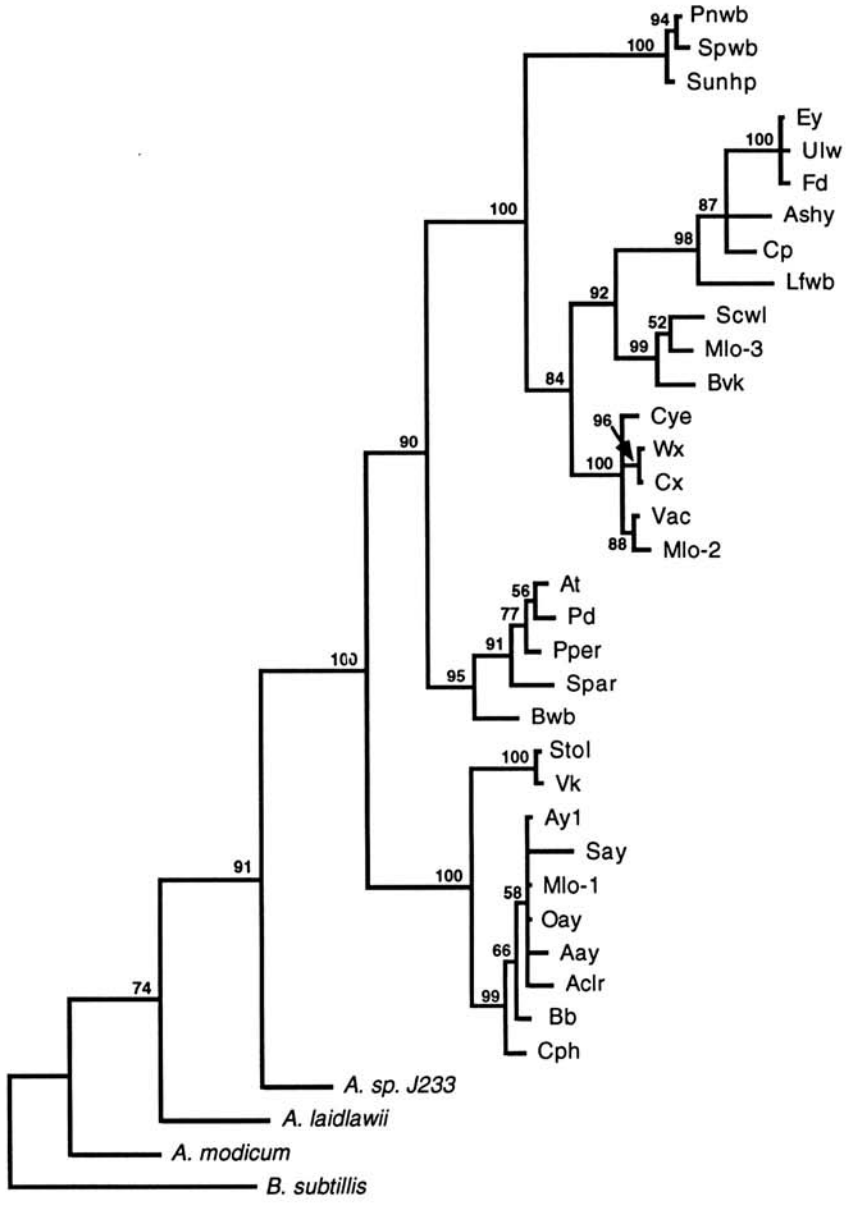

Fig. 2. Position of the Spartium witches'-broom (Spar) phytoplasma in a phylogram of 16S rDNA sequences generated using a PAUP bootstrap analysis (Illinois Natural History Survey, Champaign). The numbers along the branches indicate bootstrap confidence values. *Bacillus subtilis* was used as an outgroup and *Acholeplasma* spp. were included for comparison. Phytoplasma strain abbreviations (from top): Pnwb, peanut witches'-broom; Spwb, sweet potato witches'-broom; Sunhp, sunnhemp witches'-broom; Ey and Ulw, elm yellows; Fd, flavescence dorée of grapevine; Ashy, ash yellows; Cp, clover proliferation; Lfwb, loofah witches'-broom; Scwl, sugarcane white leaf; Mlo-3, rice yellow dwarf; Bvk, leafhopper-borne; Cye, clover yellow edge; Wx, western X-disease; Cx, Canadian X-disease; Vac, Vaccinium witches'-broom; Mlo-2, tsuwabuki witches'-broom; At, apple proliferation; Pd, pear decline; Pper, European stone fruit yellows; Bwb, buckthorn witches'-broom; Stol, stolbur; Vk, grapevine yellows (Vergilbungskrankheit); Ay1 through Cph, strains of the aster yellows strain cluster.

plants that tested positively upon P1/P7 amplification showed *Alu*I and *Rsa*I RFLP profiles identical to that of the EY phytoplasma (Fig. 4A, B).

Phylogenetic position of the SPAR phytoplasma. Nucleotide sequence analysis of the PCR-amplified 16S rRNA gene revealed that the SPAR phytoplasma is most closely related to the AP, PD, and ESFY phytoplasmas and is, thus, a member of the AP strain cluster. The buckthorn (*Rhamnus catharticus* L.) witches'-broom (BWB) phytoplasma, another member of the AP strain cluster, is more distantly related (Fig. 2). The SPAR phytoplasma shows a 16S rDNA sequence similarity of 97.1 to 97.2% with the three fruit tree phytoplasmas. Greater differences exist in the sequences of the 16S/23S rDNA spacer region. In this fragment of about 220 bp in length the SPAR phytoplasma differs from the AP and ESFY phytoplasmas in 13.9% of the positions and from the PD phytoplasma in 14.8% of the positions.

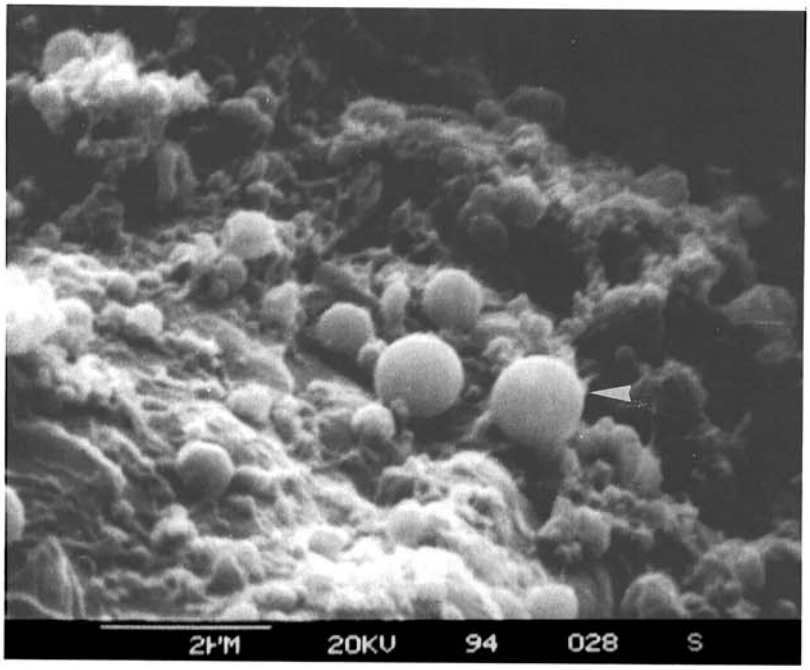

Fig. 3. Scanning electron micrograph showing phytoplasmas (arrow) in a sieve tube element of an infected *Spartium junceum* plant.

Table 1. Detection of phytoplasmas associated with Spartium witches'-broom disease by direct and nested polymerase chain reaction with various primers, and differentiation of the causal agents by restriction fragment length polymorphism (RFLP) analysis

Origin/ samples[a]	Primers used							RFLP pattern[b]
	P1/ P7	P1/ rSP	fO1/ rO1	fB1/ rULWS	fAY/ rEY	Nested fU5/rSP	Nested fB1/ rULWS	
C 1	+	+	+	–	–	+	–	SPAR[c]
C 2	+	+	+	–	–	+	+	SPAR
C 3	+	+	+	–	–	+	–	SPAR
C 4	–	–	–	–	–	+	–	–
B 5	+	+	+	–	–	+	–	SPAR
B 6	+	+	+	–	–	+	–	SPAR
Cl 7	+	+	+	–	–	+	–	SPAR
C 8	+	+	+	–	–	+	–	SPAR
C 9	+	+	+	–	–	+	+	SPAR
A 10	+	+	+	–	–	+	+	SPAR
A 11	+	+	+	–	–	+	–	SPAR
Cl 12	+	–	–	+	+	+	+	EY[d]
L 13	+	+	+	–	–	+	+	SPAR
B 14	+	+	+	–	–	+	+	SPAR
L 15	+	+	+	–	–	+	+	SPAR
B 16	+	+	+	–	–	+	+	SPAR
C 17	+	+	+	–	–	+	+	SPAR
C 18	+	–	–	+	+	+	+	EY
B 19	–	–	–	–	–	+	+	–
Cl 20	+	+	+	–	–	+	+	SPAR
C 21	+	+	+	–	–	+	+	SPAR
B 22	–	–	–	–	–	+	+	–
B 23	+	–	–	+	+	+	+	EY
B 24	+	+	+	–	–	+	+	SPAR
C 25	+	+	+	–	–	+	–	SPAR
Healthy	–	–	–	–	–	–	–	

[a] Collected in: A, Apulia; B, Basilicata; C; Campania; Cl, Calabria; L, Latium.
[b] After direct amplification with primers P1/P7.
[c] Spartium witches'-broom phytoplasma.
[d] Elm yellows phytoplasma.

Like all members of the AP strain cluster the SPAR phytoplasma has a unique *Pvu*I site following position 518 of the 16S rRNA gene that is lacking in phytoplasmas of other groups (all nucleotide positions given correspond to positions in the sequence of strain OAY of the aster yellows phytoplasma) (17). Within the AP strain cluster the SPAR phytoplasma differs from the three fruit tree phytoplasmas in the presence of two additional *Alu*I sites following positions 234 and 627. These sites are responsible for the differences in the *Alu*I restriction profiles described above. The SPAR phytoplasma can be further distinguished from the three fruit tree phytoplasmas by differences in other restriction sites that are suitable for characterizing the members of the AP strain cluster (18). The SPAR phytoplasma is lacking the *Ssp*I site following position 419, which is present in the AP phytoplasma but not in the PD and ESFY phytoplasmas. The SPAR phytoplasma also shows an *Sfe*I site following position 630, whereas the PD and ESFY phytoplasmas have an additional *Sfe*I site following position 998, and the AP phytoplasma shows an *Sfe*I site only at the latter position.

Phytoplasma detection using specific primers. SPAR-specific primer pair P1/rSP amplified the target DNA from the samples taken from the 19 *S. junceum* plants that showed the RFLP profiles of this phytoplasma (Fig. 5; Table 1). The SPAR phytoplasma was detected in the same plants with group-specific primer pair fO1/rO1 (Table 1). However, no amplification product was obtained from these plants with AP and ESFY phytoplasma–specific primer pairs fAT/rAS and fAT/rPRUS (data not shown). Sequence analysis revealed that the 16S rDNA–derived primers fO1/rO1 and fAT are fully homologous with the corresponding sequence of the SPAR phytoplasma while the spacer-derived primers rAS and rPRUS differ significantly from the corresponding SPAR phytoplasma sequences.

Primer pairs P1/rSP and fO1/rO1 did not amplify the target DNA from the three plants showing the RFLP profiles of the EY phytoplasma. However, phytoplasma infection was identified in these plants with EY-specific primer pairs fB1/rULWS and fAY/rEY (Fig. 5; Table 1). No visible amplification product was obtained with these primers from the samples showing

the restriction profile of the SPAR phytoplasma. With the exception of the homologous strain ULW of primers fB1, rULWS, and rEY, primer pairs P1/rSP, fB1/rULWS, and fAY/rEY did not amplify DNA from samples taken from healthy *S. junceum* or periwinkle plants nor from the various periwinkle-maintained phytoplasma strains tested, which include strain AT of the AP phytoplasma. No amplification product was obtained with either specific primer pair from the three symptomatic plants that tested negatively with primers P1/P7 (Table 1). Similarly, three of 12 samples taken from a single diseased plant that showed the EY restriction profile and that were recognized with primer pairs fB1/rULWS and fAY/rEY tested negatively in all PCR amplifications described above (data not shown).

Phytoplasma detection by nested-PCR assays. Primer pairs fU5/rSP and fB1/rULWS were employed in nested-PCR to re-amplify the products obtained with primer pair P1/P7 from all samples examined (Fig. 6; Table 1). The target DNA was amplified by means of SPAR-specific primer pair fU5/rSP from template DNA of all symptomatic plants including those that tested negatively in the examinations described above and those that showed the restriction profile of the EY phytoplasma. However, four of the 12 samples taken from the single plant showing the EY restriction profile tested negatively with these primers (data not shown). Primer pair fB1/rULWS amplified the target DNA from samples taken from 16 symptomatic plants, including the three plants previously identified as infected with the phytoplasma resembling the EY agent. This phytoplasma was also detected in two of the three plants that tested negatively in direct PCR and 11 plants that showed the restriction profile of the SPAR phytoplasma. In nested PCR, primer pairs fU5/rSP and fB1/rULWS did not initiate amplification in templates from healthy *S. junceum* and periwinkle plants or from the periwinkle-maintained phytoplasma strains except strain ULW.

DISCUSSION

The etiology of the witches'-broom disease of *S. junceum* has not been clearly established. Although gall mites induce malformation in many plants (6), it is not very likely that *E. spartii* causes this widespread disease, which is characterized by extensive witches'-broom formation and decline. In contrast, phytoplasmas are known to cause many witches'-broom diseases that lead to the decline of woody plants. Also, deterioration of feeder roots and phloem necrosis, which were observed in our work in diseased *S. junceum* plants, are often associated with phytoplasma diseases (20) and are not induced by gall mites. In conjunction with our finding that all diseased plants were infected with phytoplasmas, it appears that these pathogens are the major cause of the disease.

We identified two different phytoplasmas within *S. junceum* by amplifying template DNA from diseased plants. One of the organisms detected, the SPAR phytoplasma that was identified most often, is a member of the AP strain cluster. However, this organism is distinctly different from the other cluster members, as revealed by rDNA sequence analysis and by the presence or absence of restriction sites that are suitable markers for the differentiation of the phytoplasmas of the AP strain cluster (18). The second organism, which was less frequently detected in diseased plants, is a member of the EY strain cluster that showed the same *Alu*I and *Rsa*I restriction patterns as the EY phytoplasma. However, identical restriction patterns do not necessarily indicate that organisms are very closely related or identical. For instance, the EY phytoplasma and the phytoplasma causing rubus stunt show identical *Alu*I and *Rsa*I restriction profiles but differ significantly from each other when compared by Southern blot hybridization (23). In the areas where the *S. junceum* samples were collected, both the EY and the rubus stunt phytoplasmas were identified (21,22).

Using PCR and primers derived from rDNA sequences, we identified phytoplasmas in all diseased *S. junceum* plants. However, in three of the plants examined, the infection was detected only when the products obtained in the first PCR amplification with universal phytoplasma primers were re-amplified using group- or pathogen-specific primers. This indicates that the phytoplasmas occurred in very low numbers or that the pathogens were unevenly distributed in the plant. If they were present in low numbers and they caused the symptoms, the organisms were still able to induce disease. Very low phyto-

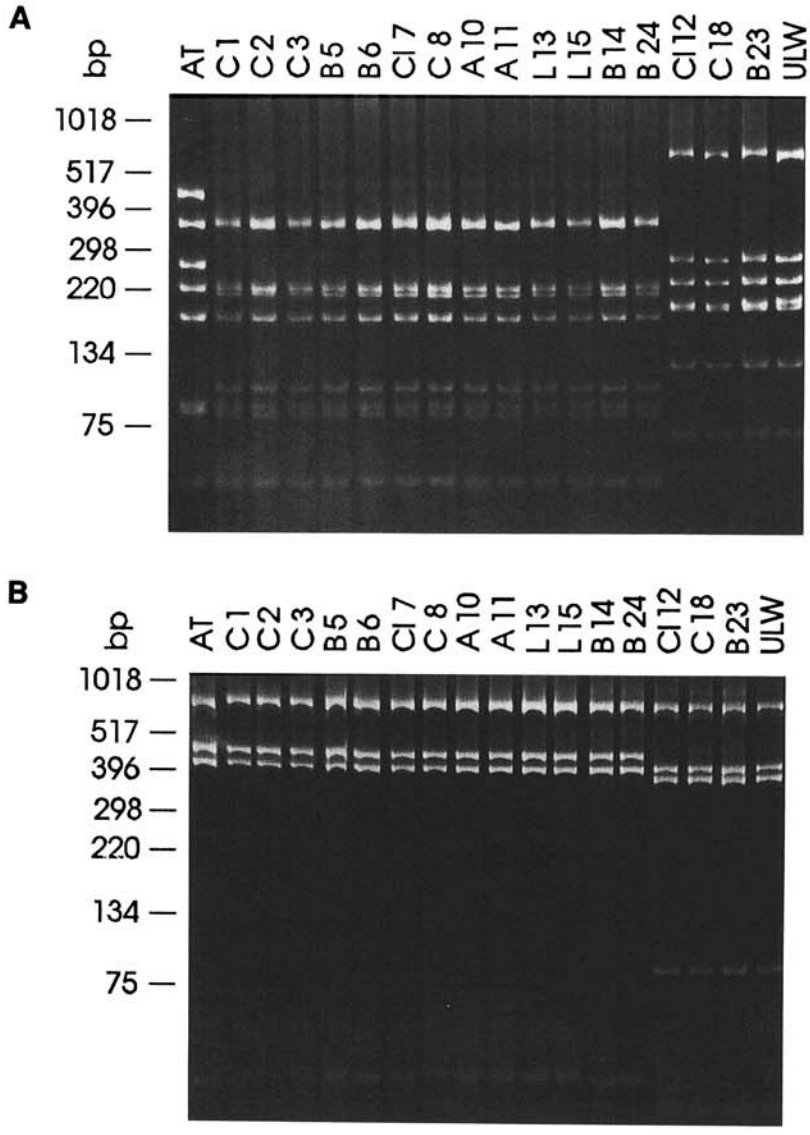

Fig. 4. **(A)** *Alu*I and **(B)** *Rsa*I restriction profiles of ribosomal phytoplasma DNA amplified by polymerase chain reaction using universal primer pair P1/P7. The target DNA obtained from diseased *Spartium junceum* samples (C1 through B23) show a restriction profile either related to that of the apple proliferation (AT) agent or identical to that of the elm yellows (ULW) phytoplasma.

plasma concentrations, which were not or hardly detectable by direct PCR, have been observed in diseased oaks (3) and grapevine (4). In these cases detection was made possible or improved by hybridization of the amplification products with oligonucleotide probes.

The highly sensitive nested-PCR assay was first described by Lee et al. (15) for phytoplasma detection. These authors successfully employed his approach to identify mixed infections, in which the secondary phytoplasmas occurred in very low concentrations. A similar situation was discovered in our work. Most of the plants examined were infected by two distinct phytoplasmas, of which one was predominant and readily detectable by direct PCR with universal or specific primers. In contrast, the second phytoplasma in a given plant was present in a very low titer that could not be detected by direct PCR amplification, but could by nested-PCR assays.

Plants doubly infected with phytoplasmas have been identified in other work (4, 15,24). It is conceivable that this phenomenon is more widespread than currently known and may be particularly common in perennial plants, whose long life spans provide vast opportunities to be visited and inoculated by vectors infested with various phytoplasma pathogens. The inoculated phytoplasmas may develop in the plant differently, depending on host suitability or, perhaps, order of inoculation. It might be more difficult for a phytoplasma to multiply and spread in a plant host if the phloem is already colonized by another phytoplasma. This may explain why in some *S. junceum* plants the SPAR phytoplasma was the predominant pathogen and the EY agent–related organism the secondary phytoplasma, while in others the contrary was true. Although only the SPAR phytoplasma was detectable in all symptomatic plants examined, the fact that each phytoplasma was predominant in some of the plants may indicate that both could be primary pathogens in *S. junceum* and induce similar symptoms. Both pathogens may be able to induce witches'-brooms, because the closely related agents of AP, EY, and rubus stunt are known to cause this symptom in some or all of their hosts (5,13,21). A well-known example of various phytoplasmas inducing similar symptoms in the same plant occurs in grapevine, which may become infected by the flavescence dorée and stolbur agents as well as by other phytoplasmas (4,9,19, 24).

Both primers derived from 16S rDNA sequences and the 16S/23S spacer region were used in this work to detect the SPAR phytoplasma. All primers derived from 16S rDNA sequences proved suitable for amplification of SPAR phytoplasma DNA. However, primers developed on the basis of spacer sequences were considerably more specific. Those designed for specific detection of fruit phytoplasmas (rAS, rPRUS) did not amplify DNA of the SPAR phytoplasma although primer rAS shows some cross amplification among the fruit tree pathogens (14). In contrast, primer rSP designed for specific detection of the SPAR phytoplasma did not amplify DNA of the fruit phytoplasmas. The higher specificity of spacer-derived primers is due to the high variability of the nucleotide sequences flanking the conserved $tRNA^{Ile}$ gene that is present in all phytoplasmas (14). Primers based on the sequences flanking the tRNA gene were designed for specific detection of phytoplasma members of most phylogenetic clusters that were established. In many cases they enable detection of a narrow range of phytoplasmas (14).

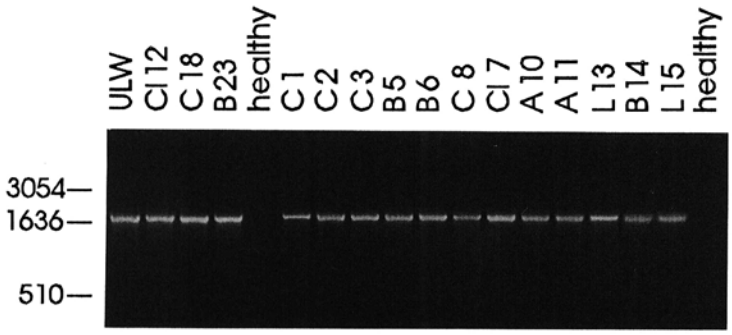

Fig. 5. Polymerase chain reaction amplification of ribosomal DNA fragments from template DNA extracted from diseased and healthy *Spartium junceum* shrubs or an elm yellows–infected periwinkle plant (ULW). Samples ULW through healthy (left) were amplified using elm yellows–specific primers fB1/rULWS, and samples C1 through healthy (right) were amplified with Spartium witches'-broom–specific primer pair P1/rSP.

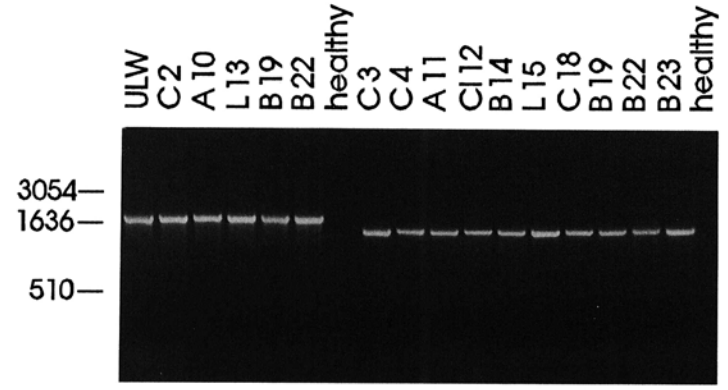

Fig. 6. Detection by nested polymerase chain reaction (PCR) assays of phytoplasma infections in *Spartium junceum* shrubs that tested negatively by direct PCR and of mixed infections. First amplification was performed using universal phytoplasma primers P1/P7. Second amplification was carried out using either elm yellows–specific primers fB1/rULWS (elm yellows strain ULW through healthy [left]) or Spartium witches'-broom–specific primer pair rU5/rSP (C3 through healthy [right]).

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