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Characterization and classification of phytoplasmas from wild and cultivated plants by RFLP and sequence analysis of ribosomal DNA

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Abstract

Restriction fragment length polymorphism and sequence analysis of PCR-amplified ribosomal DNA were used to identify and classify phytoplasmas associated with diseases of various wild and cultivated plants. The diseases examined were either not known before or the presumable causal agents were not yet identified and characterized or were only known from other geographic areas. New diseases examined were those causing virescence and phyllody of *Bunias orientalis* and *Cardaria draba*. Both were associated with strains of the aster yellows phytoplasma. The same type of aster yellows phytoplasma was also found to be associated with yellows and phyllody diseases of *Portulaca oleracea*, *Stellaria media*, *Daucus carota* ssp. *sativus*, and *Cyclamen persicum*. In German and French DNA samples from diseased *Trifolium repens*, the clover phyllody phytoplasma was identified, which could clearly be distinguished from other phytoplasmas of the aster yellows group. Strains of the stolbur phytoplasma were detected in big bud-affected tomatoes and almost exclusively in *Convolvulus arvensis*. In *Cirsium arvense* and *Picris echioides* two distinct phytoplasmas were identified which showed relationship to the sugarcane white leaf phytoplasma group but may represent a new group or subgroup. In *Conyza* (syn.: *Erigeron*) *canadensis* a phytoplasma of the X-disease group was detected. A strain from *Gossypium hirsutum* showed the same restriction profiles as the faba bean phyllody phytoplasma.

Abbreviations: rDNA – ribosomal DNA; RFLP – restriction fragment length polymorphism; PCR – polymerase chain reaction; AY – aster yellows; AP – apple proliferation; CIRP, cirsium phyllody; CPh – clover phyllody; FBP – faba bean phyllody; SCWL – sugarcane white leaf; WX – western X-disease.

Introduction

Phytoplasmas are associated with diseases of several hundred plant species (McCoy et al., 1989). Because phytoplasmas cannot be cultured under axenic conditions, their identities and taxonomic positions were unclear or uncertain until recently, when methods of molecular genetics were introduced into plant mycoplasmology. Then it became possible to reliably characterize, differentiate, and classify the phytoplasmas. Mainly based on restriction fragment length polymorphism (RFLP) and sequence analysis of ribosomal DNA (rDNA), various groups and subgroups could be distinguished (Lee et al., 1993; Schneider et al., 1993; Gundersen et al., 1994; Seemüller et al., 1994). Using this technology most or all phytoplasmas that infect

economically important crop plants have been phylogenetically classified. In contrast, only a minority of the phytoplasmas that were previously microscopically detected in many wild plant species were examined with molecular methods. This situation is unsatisfactory because wild plants may be alternative hosts of phytoplasmas that cause diseases in crop plants. Examples are phytoplasmas of the aster yellows (AY) group which are known or suspected to infect many vegetable crops, ornamentals, trees, and weeds (e.g. Frazier and Severin, 1945; Severin and Frazier, 1945; Severin and Freitag, 1945; Lee et al., 1993; Marcone and Ragozzino, 1995a; Berges et al., 1997).

During the last few years we observed several herbaceous plant species that showed symptoms typical for those associated with phytoplasma infections. Phytoplasma diseases of two of these plants, which include *Cardaria draba* (L.) Desv. (hoary cress) and *Bunias orientalis* L. (hill mustard), have, to our knowledge, never been described. Both species showed yellowing and pronounced virescence and phyllody symptoms (Figures 1A and B). Phytoplasmal diseases of the other plants have previously been described, mostly on the basis of symptomatology and microscopical examination. These plants include the following species: (1) *Cirsium arvense* (L.) Scop. (Canada thistle) showing yellowing, proliferating growth, small leaves, divided flower heads, virescence, and phyllody (Figure 1C) similar to those described by Begtrup and Thomsen (1975) and Staniulis and Genyte (1976); (2) *Stellaria media* (L.) Vill. (common chickweed) which grew poorly and showed phyllody symptoms as described by Begtrup and Thomsen (1975); (3) *Convolvulus arvensis* L. (field bindweed) showing symptoms of yellowing and stunting and/or proliferating and erect growth habit (Figure 1D) as described by Marchoux et al. (1970) and Maixner et al. (1995); *Lycopersicon esculentum* Mill. (tomato) showing typical stolbur symptoms (Valenta et al., 1961); (5) *Trifolium repens* L. (white clover) showing typical clover phyllody symptoms as described by Bos and Grancini (1965); (6) *Conyza* (syn.: *Erigeron*) *canadensis* (L.) Cronq. (horseweed) showing axillary shoot growth and dwarfed, yellowish green flower heads as described by Frazier and Severin (1945); (7) *Picris echioides* L. (bristly oxtongue) showing narrow and chlorotic leaves, upright growth habit, virescence, and stunting as described by Frazier and Severin (1945) and Arzone et al. (1995); (8) *Portulaca oleracea* L. (purslane) showing spindling upright growth, small leaves, elongated internodes and chlorosis as described by Fazier and Severin (1945); (9) *Daucus carota* L. ssp. *sativus* (Hoffm.) Arcang. (carrot) showing dense clusters of dwarfed, chlorotic, upright adventitious shoots as described by Severin and Frazier (1945), and (10) *Cyclamen persicum* Mill. (cyclamen) showing virescence and phyllody symptoms as described by Bertaccini (1990). In addition, an isolate of *Gossypium hirsutum* L. (cotton) affected by yellowing, stunting, virescence, and phyllody as described by Cousin et al. (1969b) was included in this study.

The phytoplasmas detected in most of the plants described above have not been properly identified. The remaining plants were collected in areas where these species were never examined for the taxonomic positions of the presumable causal agents. Thus, the subject of the research described here was to characterize and classify the infecting phytoplasmas using molecular methods. Because most of the diseased plants examined were collected in or around fruit orchard, it was particularly interesting to determine whether the phytoplasmas investigated are related to those infecting pome and stone fruits.

Materials and methods

Sources of phytoplasmas

The following diseased plants were collected in the field: Three plants of each *C. draba* and *B. orientalis* in an agricultural area near Halle, central Germany; 8 carrot plants in northern Germany near Rostock; 10 plants of *C. arvense*, 25 plants of *C. arvensis*, 2 plants of *S. media*, and 3 plants of *T. repens* in or around apple and stone fruit orchards near Heidelberg, southwestern Germany; 5 plants of *P. echioides* and 3 plants of *C. arvensis* in an apple orchard, 4 other plants of *C. arvensis*, and 4 tomato plants in vegetable fields, all located in the Agri valley of southern Italy (Basilicata region); 2 plants of *C. arvensis* and 4 plants of *P. oleracea* in apricot orchards in the Caserta province (Campania region) of southern Italy; and 3 plants of *C. canadensis* next to an apple orchard affected by apple decline (Parish et al., 1992) near Wenatchee, Washington, USA. In addition, 2 cyclamens obtained from a German nursery, and a phytoplasma from infected cotton, which was originally collected in Upper Volta (Cousin et al., 1969b) and was then maintained in the greenhouse by periodic grafting to young cotton seedlings, were examined. DNA samples from phytoplasma-diseased sugarcane, *Crepis setosa*, and *P.*

Figure 1. Phytoplasma-infected plants that were examined: (A) *Cardaria draba* showing phyllody symptoms (right, healthy); (B) *Bunias orientalis* showing phyllody symptoms (left, healthy); (C) *Cirsium arvense* showing phyllody symptoms (left, healthy); (D) *Convolvulus arvensis* showing stunting and proliferation symptoms (left, healthy).

echioides, which were previously examined (Schneider et al., 1993; Marcone et al., 1997b), as well as healthy plants from all species examined were included in the work.

The following phytoplasma reference strains, which were maintained in periwinkle (*Catharanthus roseus*), were included in this study for comparison: AAY, American aster yellows, collected by R. E. McCoy, Fort Lauderdale, Florida; AT, apple proliferation (AP) from southwestern Germany, transmitted to periwinkle by R. Marwitz, Berlin, Germany; BVK, strain of the sugarcane white leaf (SCWL) group, obtained from the leafhopper *Psammotettix cephalotes* by W. Heintz, Dossenheim; FBP, faba bean phyllody (FBP), collected by M.-T. Cousin in the Sudan; GVX, Green Valley strain of western X-disease, collected by A. H. Purcell, Berkeley, California; KVG, clover phyllody (CPh), collected by R. Marwitz in Berlin, Germany; KVF, CPh from France, collected by M.-T. Cousin; KVM, clover phyllody, collected by G. Morvan, Avignon-Montfavet, France; and STOL, stolbur of pepper, collected by D. Sutic, Beograd, Serbia. These strains represent most of the major phylogenetic groups established by Schneider et al. (1993), Seemüller et al. (1994), and Schneider et al. (1995a).

DNA isolation, PCR amplification, and RFLP analysis

DNA from aerial parts of the plants examined was extracted using a phytoplasma enrichment procedure (Ahrens and Seemüller, 1992). For amplification of phytoplasmal rDNA, universal phytoplasma primers P1 and P7 were employed which prime at the 5′ end of the 16S rRNA gene and in the 5′ region of the 23S rRNA gene, respectively (Figure 5; Schneider et al., 1995b). The DNA fragment obtained was approximately 1800 bp in length. PCR and RFLP analysis of the amplification products was performed as described (Schneider et al., 1995a; Marcone et al. 1996).

DNA sequencing and data analysis

The PCR products obtained with primer pair P1/P7 from a diseased Canada thistle and a white clover plant were directly sequenced by cycle sequencing, using the *fmol* DNA sequencing system (Promega) as described (Seemüller et al., 1994). The full-length sequences have been deposited in the EMBL Data Library (Heidelberg, Germany) under the accession numbers X83438 (thistle) and X83870 (clover). The two sequences of the 16S rRNA genes of the two pathogens were aligned with 16S rDNA sequences of other, previously characterized phytoplasmas obtained from the GenBank database (see Figure 4 for accession numbers) using the Pileup program of the UW-GCG software system (Genetics Computer Group, 1991). This alignment was then analysed using Phylogenetic Analysis Using Parsimony (PAUP) version 3.1.1 (Illinois Natural History Surver, Champaign). A phylogram was generated by performing bootstrap analysis using 10 replicates with ten 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 and discussion

PCR amplification of phytoplasmal DNA

Eleven of the 25 symptomatic bindweed plants collected near Heidelberg yielded an amplification product of the expected size following PCR with primers P1/P7. The phytoplasma concentrations in the other plants may have been too low for detection with these relatively insensitive primers or the symptoms were due to herbicide treatment instead of phytoplasma infection. All other diseased plants examined including the periwinkle-maintained reference strains were phytoplasma-positive when the DNA templates were amplified with these primers. No amplification product was obtained from any of the nonsymptomatic plants (data not shown).

Diseases associated with aster yellows phytoplasmas

PCR-amplified rDNA from all infected plants of *C. draba*, *B. orientalis*, *S. media*, *P. oleracea*, *D. carota* ssp. *sativus*, and *C. persicum* showed the same *Alu*I and *Rsa*I restriction profiles as reference strain AAY, which we consider to be a typical strain of the AY phytoplasma (Figures 2 and 3, Table 1). The isolates from both *P. oleracea* and *C. persicum* as well as strain AAY were also examined using *Sau*3AI, *Mse*I, *Hha*I, *Taq*I, *Hin*fI, *Hpa*II, and *Hae*III as additional restriction endonucleases. In this case, too, these phytoplasmas showed with each enzyme the same profile (see Marcone et al., 1997b, for AAY profiles).

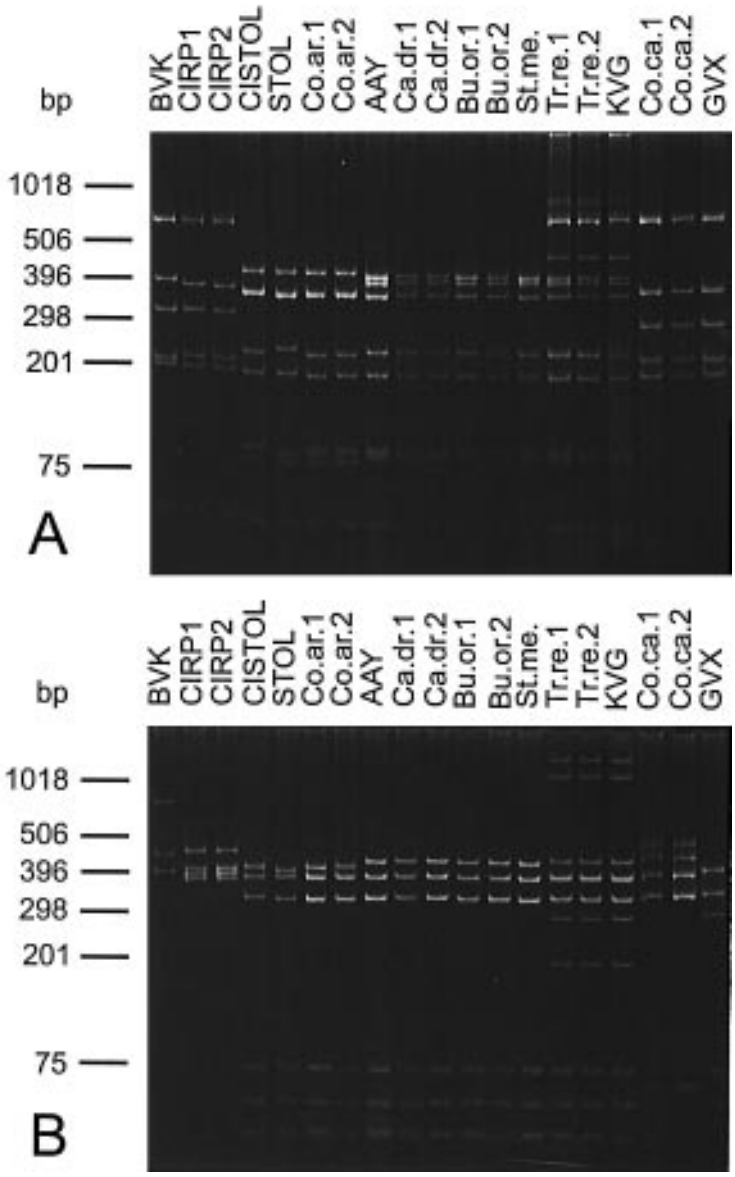

Figure 2. *Alu*I (A) and *Rsa*I (B) restriction profiles of ribosomal DNA amplified with primers P1/P7 from periwinkle-maintained phytoplasma reference strains and from template DNA extracted from diseased plants. BVK, strain of sugarcane white leaf group; CIRP1 and 2, *Cirsium arvense*; CISTOL, *C. arvense* infected with a stolbur phytoplasma; STOL, stolbur; Co.ar.1 and 2, *Convolvulus arvensis*; AAY, aster yellows; Ca.dr.1 through St.me, *Cardaria draba*, *Bunias orientalis*, and *Stellaria media*, respectively; Tr.re.1 and 2, *Trifolium repens*; KVG, clover phyllody; Co.ca.1 and 2, *Conyza canadensis*; GVX, western X-disease.

The detection of phytoplasmal diseases of *C. draba* and *B. orientalis* are new records. For *S. media*, *P. oleracea*, and *D. carota* ssp. *sativus* our findings confirm results of transmission experiments by Frazier and Severin (1945) and Severin and Frazier (1945), who observed the same symptoms on these plants following vector inoculation with the AY agent. Although the symptoms we observed in cyclamen were similar to those described by Bertaccini (1990), the associated phytoplasmas may not be the same. In her hybridization experiments, AY-specific DNA probes did not hybridize to DNA from diseased plants.

Table 1. RFLP patterns of rDNA amplified with primers P1/P7 from the phytoplamas examined (see text and Fig. 2 for phytoplasma abbreviations)

Plant species or reference strain	Geographic origin	No. of plants	Restriction profiles *Alu*I	*Rsa*I
Reference strain AAY	Florida, USA	1	AAY	AAY
Cardaria draba	Central Germany	3	AAY	AAY
Bunias orientalis	Central Germany	3	AAY	AAY
Stellaria media	Southwestern Germany	2	AAY	AAY
Daucus carota ssp. *sativus*	Northern Germany	8	AAY	AAY
Cyclamen persicum	Western Germany	1	AAY	AAY
Portulaca oleracea	Southern Italy (Campania)	4	AAY	AAY
Reference strain KVG	Central Germany	1	CPh	CPh
Trifolium repens	Southwestern Germany	3	CPh	CPh
Reference strain STOL	Serbia	1	STOL	STOL
Lycopersicon esculentum	Southern Italy (Basilicata)	4	STOL	STOL
Convolvulus arvensis	Southern Italy	9	STOL	STOL
Convolvulus arvensis	Southwestern Germany	10	STOL*	STOL*
Cirsium arvense	Southwestern Germany	1	STOL*	STOL*
Reference strain AT	Southwestern Germany	1	AP	AP
Convolvulus arvensis	Southwestern Germany	1	AP	AP
Reference strain BVK	Southwestern Germany	1	BVK	BVK
Cirsium arvense	Southwestern Germany	9	CIRP	CIRP
Crepis setosa refer. strain	Southern Italy (Basilicata)	1	Cr.se.	Cr.se.
Picris echioides	Southern Italy (Basilicata)	5	Cr.se.	Cr.se
Reference strain GVX	California	1	WX	WX
Conyza canadensis	Washington, USA	3	WX	Co.ca.
Reference strain FBP	Sudan	1	FBP	FBP
Gossypium hirsutum	Upper Volta	1	FBP	FBP

* Profile differs slightly from profile of reference strain STOL.

Clover phyllody

The *Alu*I and *Rsa*I restriction profiles obtained with DNA from diseased white clover plants sampled in Germany and from the periwinkle-maintained strains KVF, KVG, and KVM, which also originate from white clover, were similar (Figure 2, Table 1). Both the *Alu*I and *Rsa*I profiles of these phytoplasmas are unique within the diverse AY group, of which a Canadian strain of the CPh phytoplasma is considered to represent a subgroup (Lee et al., 1993; Gundersen et al., 1994). Sequence analysis of 16S rDNA of CPh strain KVG revealed that it is very closely related to the Canadian strain (99.5% sequence similarity). Although the CPh strains also show a high 16S rDNA sequence homology with other strains of the AY group (99.0% similarity between strains KVG and AAY), their distinction on the subgroup level appears to be justified from both the positions in the phylogenetic dendrogram and the differences in the RFLP profiles (Figures 2 and 4). The differences in the RFLP profiles are most likely due to the fact that the two rRNA operons, which obviously occur in all phytoplasmas (Schneider and Seemüller, 1994), differ from each other. It appears that one operon of the CPh phytoplasma is lacking the *Alu*I site at position 645 of the 16S rRNA gene which results in the pronounced 765-bp fragment on top of the profile. This site is present in both genes which have been sequenced. It is also likely, that in one operon the *Alu*I site in the conserved tRNA gene in the 16S-23S rDNA spacer region is missing (Figure 5). This would result in the 500-bp fragment which typical AY strains are lacking. Interoperon RFLP variation is known to occur in the phormium yellow leaf phytoplasma (Liefting et al., 1996) and probably also in AY strains from poplars (Berges et al., 1997).

The CPh phytoplasma derived from white clover sampled in France and Germany, which we identified, is genetically distinctly different from the phytoplasmas detected in Italy in this plant (Osler et al., 1994)

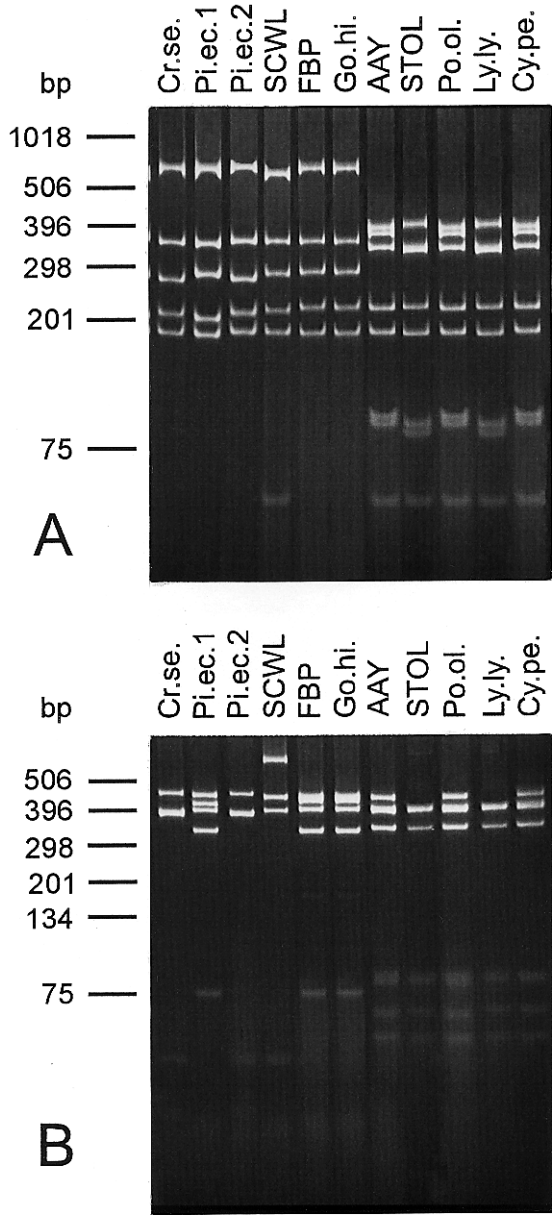

Figure 3. *Alu*I (A) and *Rsa*I (B) restriction profiles of ribosomal DNA amplified with primers P1/P7 from phytoplasma reference strains and from template DNA extracted from diseased plants. Cr.se., reference strain from *Crepis setosa*; Pi.ec.1, reference strain from *Picris echioides*; Pi.ec.2, *P. echioides* (phytoplasma identified in this study); SCWL, sugarcane white leaf; FBP, faba bean phyllody; Go.hi., *Gossypium hirsutum*; AAY, aster yellows; STOL, stolbur; Po.ol., *Portulaca oleracea*; Ly.ly., *Lycopersicon esculentum*; Cy.pe., *Cyclamen persicum*.

and from the phytoplasmas associated with clover proliferation and clover yellow edge in North America (Gundersen et al., 1994; Lee et al., 1994).

Field bindweed (C. arvensis) *and tomato*

All phytoplasmas detected in bindweed, which were collected in the Basilicata and Campania regions of southern Italy, were identical and showed the same *Alu*I, *Rsa*I, *Sau*3AI, *Mse*I, *Hha*I, *Taq*I, *Hin*fI, *Hpa*II, and *Hae*III profiles as reference strain STOL (Table 1, Figure 2; see also Marcone et al., 1997b, for STOL profiles). Profiles identical to those of stolbur reference strain STOL were also obtained with all DNA samples from stolbur-affected tomato plants, which were grown at two of the sites where diseased bindweed was collected in southern Italy (Table 1, Figure 3). The phytoplasmas identified in all but one isolate from bindweed collected in the Heidelberg area of southwestern Germany showed the same *Alu*I and *Rsa*I profiles, which differed slightly from those of reference strain STOL (Table 1, Figure 2). The significance of these small differences is not clear. More pronouced differences to reference strain STOL were found in bindweed samples previously collected in the Campania region (Marcone et al., 1997b). In addition, two different stolbur phytoplasma strains, which also differed from reference strain STOL, were identified in stolbur-affected tomatoes in Sardinia, Italy (Minucci and Boccardo, 1995). These results show a genetic diversity within stolbur phytoplasma strains which indicate that this group may consist of several taxonomic entities.

The detection of a stolbur phytoplasmas in bindweed is confirming work by Fos et al. (1992) and Maixner et al. (1995), who identified stolbur strains in this plant. There is also earlier evidence that stolbur phytoplasmas infect bindweed (e. g. Cousin et al., 1969a, Marchoux et al., 1970).

In a single bindweed plant sampled in the Heidelberg area, a phytoplasma showing the same *Alu*I and *Rsa*I profiles as the AP agent was detected (Table 1, Figure 6). This isolate was clearly identified as AP phytoplasma by digestion of the P1/P7 product with *Ssp*I restriction enzyme (Lorenz et al., 1995). Although this is the first report of the occurrence of AP agent in a naturally infected herbaceous plant, further work is needed to evaluate the significance of bindweed as an alternative host of this phytoplasma.

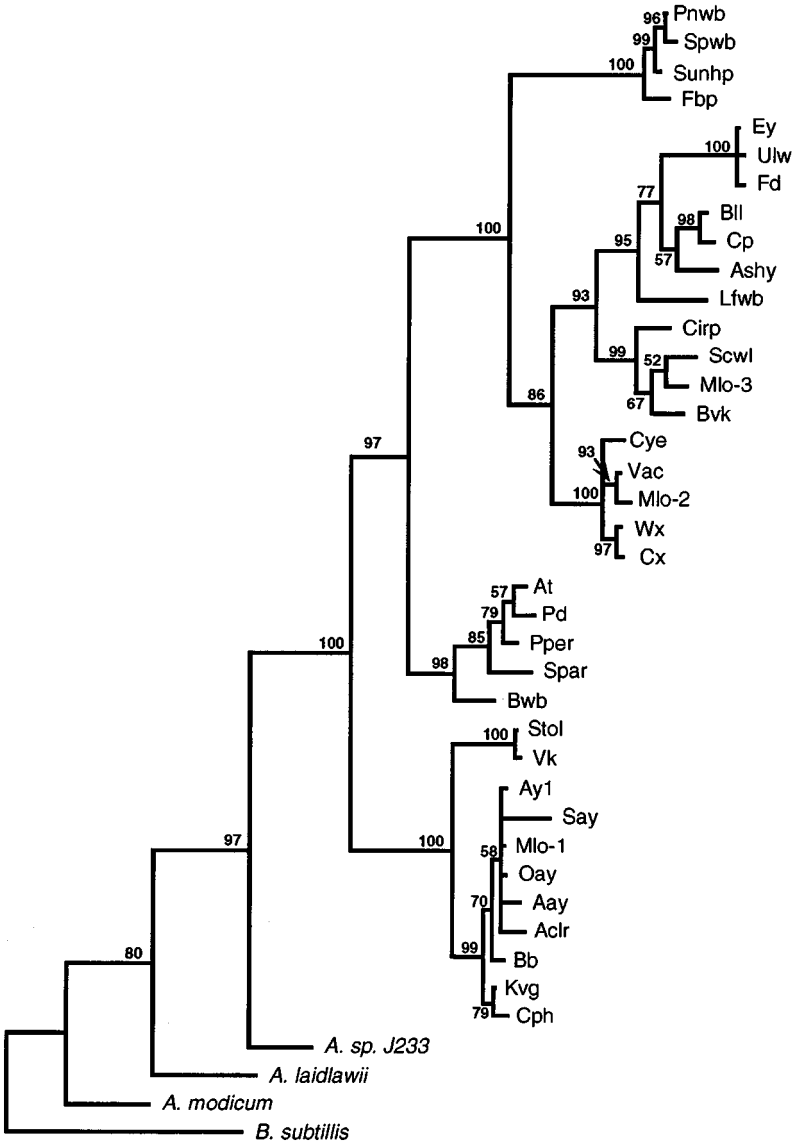

Figure 4. Positions of the cirsium phyllody (Cirp) and clover phyllody (Kvg) phytoplasmas examined in a phylogram of 16S rDNA sequences generated using a PAUP bootstrap analysis. The numbers along the branches indicate bootstrap confidence values. *Bacillus subtilis* was used as an outgroup and *Acholeplasma* spp. were included for comparison. Phytoplasma strain abbreviations and accession numbers (from top): Pnwb, peanut witches'-broom (L33765); Spwb, sweet potato witches'-broom (L33770); Sunhp, sunnhemp witches'-broom (X76433); Fbp, faba bean phyllody (X83432); Ey and Ulw, elm yellows (L33763 and X68376); Fd, flavescence dorée (X76560); Bll, brinjal little leaf (X83431); Cp, clover proliferation (L33761); Ashy, ash yellows (X68339); Lfwb, loofah witches'-broom (L33764); Scwl, sugarcane white leaf (X76432); Mlo-3, rice yellow dwarf (D12581); Bvk, leafhopper-borne (X76429); Cye, clover yellow edge (L33766); Wx, western X-disease (L04682); Cx, Canadian X-disease (L33733); Vac, vaccinium witches'-broom (X76430); Mlo-2, tsuwabuki witches'-broom (D12580); At, apple proliferation (X68375); Pd, pear decline (X76425); Pper, European stone fruit yellows (X68374); Spar, spartium witches'-broom (X92869); Bwb, buckthorn witches'-broom (X76431); Stol and Vk, stolbur (X76427 and X76428); Ay1 through Bb, strains of the aster yellows group ((L33767, M86340, D12580, M30970, X68373, X68383, L33733); Cph, clover phyllody fom Canada (L33762).

Canada thistle (C. arvense)

In 9 of the 10 thistles examined, a phytoplasma was detected that showed a uniform RFLP profile with *Alu*I and *Rsa*I (Table 1, Figure 2). These profiles as well as those obtained with *Sau*3AI, *Mse*I, and *Taq*I (not shown) were different from all known profiles of other

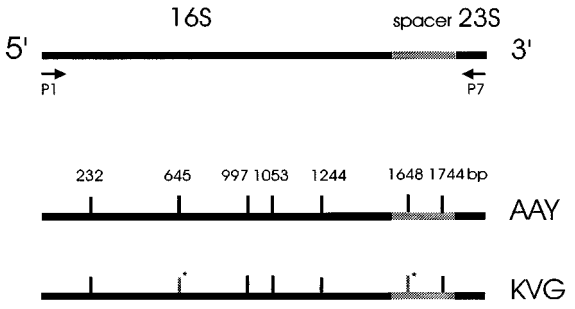

Figure 5. *Alu*I restriction map of PCR-amplified ribosomal DNA fragment of aster yellows strain AAY and putative restriction map of strain KVG of the clover phyllody phytoplasma. The restriction sites marked with an asterix appear to be missing in one of the two ribosomal operons of the clover phyllody phytoplasma. The fragments were amplified with primers P1/P7, whose priming positions are shown on top.

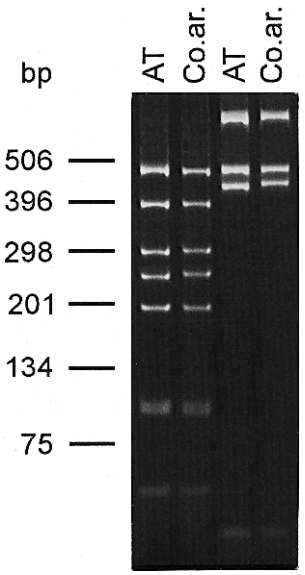

Figure 6. *Alu*I (left two lanes) and *Rsa*I (right two lanes) restriction profiles of ribosomal DNA amplified with primers P1/P7 from strain AT of the apple proliferation phytoplasma and a phytoplasma identified in *Convolvulus arvensis*.

phytoplasmas but showed similarities with reference strain BVK and the SCWL phytoplasma as well as the phytoplasmas causing yellows diseases of *Knautia arvensis*, *Crepis setosa*, and *Echium vulgare*, which were tentatively assigned to the SCWL group (Marcone et al., 1997b). The RFLP patterns obtained with *Hha*I, *Hin*fI, *Hpa*II, and *Hae*III were identical to one or more phytoplasmas of the SCWL group (not shown). These results indicate that the thistle agent is a distinct pathogen, for which the name cirsium phyllody (CIRP) phytoplasma is proposed. Consistent with the RFLP data, sequence analysis revealed that the CIRP phytoplasma is most closely related to the members of the SCWL group (Figure 4). However, it shows a sequence similarity of only 96.9% and 96.7% to the SCWL phytoplasma and strain BVK, respectively. Considering the phylogenetic distances in the existing phytoplasma classification (Seemüller et al., 1994; Schneider et al., 1995a), the CIRP phytoplasma may represent a different lineage and may not be classified with the established members of the SCWL group. Thus, additional 16S rDNA sequencing data from other related phytoplasmas are needed, before an appropriate classification is possible.

In one of the thistles examined, which was symptomatically similar to those infected by the CIRP phytoplasma, a stolbur phytoplasma was detected that was similar to those strains identified in German bindweed (Table 1, Figure 2).

Bristly oxtongue (P. echioides)

The *P. echioides* plants, which were collected in the Basilicata region, showed all the same rDNA profiles with *Alu*I, *Rsa*I, *Sau*3AI, *Mse*I, *Hha*I, *Taq*I, *Hin*fI, *Hpa*II, and *Hae*III restriction endonucleases (Figure 3, Table 1). These profiles were identical to those of the phytoplasmas previously identified in *Crepis setosa* and *Echium vulgare* by Marcone et al. (1997b). As described in the previous section, these phytoplasmas were tentatively classified as members of the SCWL group. However, phytoplasmas detected in other *P. echioides* plants, which in previous work were collected in the Campania and Basilicata regions and were symptomatically similar to the plants collected in this study in Basilicata, showed different profiles (Figure 3, Pi.ec.1) and were assigned to the FBP group (Marcone et al., 1997b). In a study based on symptomatology on yellows-affected *P. echioides* plants collected in the USA, Frazier and Severin (1945) reported that a similar disease was caused by an AY phytoplasma.

Horseweed (C. canadensis)

DNA samples from all horseweed plants examined showed the same *Alu*I and *Rsa*I restriction profiles. Of these, the *Alu*I profiles were identical to that of reference strain GVX of the western X-disease phytoplasma, whereas some fragments of the *Rsa*I profile differed from those of the reference strain (Table 1, Figure 2). In particular the *Alu*I profiles indicate that the horseweed-infecting phytoplasma is a member of

the X-disease group. This finding is partially confirming results obtained by Lee et al. (1994) who identified phytoplasmas of both the AY and X-disease group in diseased Maryland horseweed. AY phytoplasmas as cause of a horseweed disease were also reported by Frazier and Severin (1945). However, their diagnosis was solely based on symptoms, which depend not only from the pathogen but also from the host plant.

Cotton

The phytoplasma identified in cotton showed the same *Alu*I and *Rsa*I restriction profiles as the FBP phytoplasma (Figure 3, Table 1) and is, thus, a member of the FBP group, which was established by Schneider et al. (1995a).

Conclusions

This work to identify and classify the causal agents of several phytoplasmoses was primarily based on RFLP analysis of PCR-amplified rDNA, using mainly the two frequently cutting restriction enzymes *Alu*I and *Rsa*I. This approach proved suitable for phytoplasma differentiation and preliminary classification (Schneider et al., 1993). Other work has shown that the use of additional enzymes allows a more detailed differentiation. For example, strains of the AY, AP, and elm yellows groups could further be differentiated in this way (Lee et al., 1993; Marcone et al., 1997a; Kison et al., 1997). RFLP-based phytoplasma classification using only a few enzymes for analysis does not always coincide with the classification based on full-length sequences of the conserved 16S rRNA gene. It could be shown that a group that was established on differences in RFLP patterns was not justified whereas in another case a phytoplasma that showed only slight differences in the RFLP profile was significantly different according to 16S rDNA sequence analysis (Seemüller et al., 1994). These results show that the sequence of a large molecule such as the 16S rRNA gene reflects phylogenetic distance more accurately than restriction patterns which depend on significantly fewer genetic characters. Thus, it can be concluded that characterization and classification of most of the phytoplasmas examined can be improved by RFLP analysis using additional enzymes and by sequence analysis.

It is well established that different phytoplasmas may cause similar symptoms in a given host plant. Examples are grapevine infected by the flavescence dorée or bois noir phytoplasmas (Daire et al., 1993), *Spartium junceum* infected by phytoplasmas from the AP or elm yellows groups (Marcone et al., 1996), and tomatoes affected by the big bud syndrome which can be associated with either the stolbur agent or by phytoplasmas from the AY, FBP, elm yellows, and X-disease groups (Lee et al., 1993; Marcone and Ragozzino, 1995b; Schneider et al., 1993, 1995a; Boudon-Padieu et al., 1996). A similar situation may be true for the plants examined in our work. It is possible that the plants studied by other researchers were infected by other phytoplasmas than we detected, even if the plants showed similar symptoms. This may in particular be the case for *P. echioides*, in which we detected phytoplasmas that were different from those supposed to induce the disease described by Frazier and Severin (1945). However, the diagnosis by these authors was solely based on symptomatology which, as we know now, is not a suitable means to identify phytoplasmas. In contrast, the reports of Frazier and Severin (1945) and Severin and Frazier (1945) that the diseases of *S. media*, *P. oleracea* and carrot are associated with the AY phytoplasmas agree with our results. In these cases, phytoplasma identification was based on vector specificity.

Although most of the plants examined were collected in pome and stone fruit orchards, fruit tree phytoplasmas of the AP group (Seemüller et al., 1994) were very rarely detected. The only exception is the identification of the AP phytoplasma in a bindweed plant. This is the first report on the occurrence of this organism in a herbaceous plant. Work is in progress to determine the epidemiological significance of this finding by examining larger numbers of samples and including other geographical areas where AP occurs, too.

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