POSSIBLE CAUSE OF EUROPEAN BLUEBERRY DISEASE IS RELATED TO NORTH AMERICAN MILKWEED YELLOWS PHYTOPLASMA

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SUMMARY

A phytoplasma lineage previously found in Germany and North America was identified in diseased plants of wild European blueberry (*Vaccinium myrtillus* L.) exhibiting symptoms of shoot proliferation in Lithuania. On the basis of RFLP and nucleotide sequence analyses of 16S rDNA, the phytoplasma (strain VAC-L) was classified in the group 16SrIII (X-disease phytoplasma group), subgroup F. Phylogenetic analysis based on 16SrRNA gene sequences confirmed that the VAC-L phytoplasma is closely related to phytoplasma strain VAC from diseased, cultivated blueberry in Germany and to North American milkweed yellows phytoplasma strain MW1, both of which are classified in subgroup III-F. The results extend the known geographic range of phytoplasmal diseases in blueberry, and raise questions concerning intercontinental movement of subgroup III-F strains and their potential to infect cultivated *Vaccinium* spp. in North America.

Key words: Detection, identification, phytopathogenic mollicutes, phylogeny, *Vaccinium*.

INTRODUCTION

Recent investigations have revealed a widespread occurrence and broad diversity of phytoplasmal diseases of plants in Lithuania. Phytoplasmas associated with diseases of forage legumes, vegetables, ornamentals, and forest trees have been identified and classified based on analyses of ribosomal (r) RNA gene sequences (Jomantiene *et al*., 2000, 2002a, 2002b; Staniulis *et al*., 2000; Valiunas *et al*., 2000, 2001a, 2001b, 2002). These phytoplasmas belong to several phylogenetically distinct groups, each representing at least a single species (Gundersen *et al*., 1994). Staniulis (1989) reported electron microscopy of a putative phytoplasma in the phloem of wild European blueberry plants (*Vaccinium myrtillus* L.) exhibiting symptoms of chlorosis, little leaf, and shoot proliferation in southern Lithuania, but the phytoplasma was not identified. Recently, we observed diseased plants of wild blueberry exhibiting symptoms of witches' broom in widespread forests in Lithuania. This is noteworthy because of the beneficial, anti-aging and other health related nutraceutical potentials of blueberries as an antioxidant and antibacterial agent (Kowalchuk 1976; Ames *et al*., 1993; Weiss *et al*., 2002; Joseph *et al*., 2003), and because in Lithuania wild *V. myrtillus* plants are a popular source of fruits for human consumption, and the species is a major component of pine forest biodiversity.

Although ultra thin section electron micrographs by Staniulis (1989) suggested the presence of an unidentified, putative phytoplasma in diseased blueberry in Lithuania, there has been no DNA-based study to establish the presence of a phytoplasma in Lithuanian wild blueberry and to determine its identity. Phytoplasmal diseases of blueberry have been reported in other regions of Europe (Blattny and Vana, 1974; de Leeuw, 1975; Tomenius and Ahamn, 1983), but their relationship to the putative phytoplasma in Lithuanian wild blueberry has remained unknown. The objectives of the present study were to determine the possible association of phytoplasma with disease in blueberry in Lithuania, and to determine the relatedness of the blueberry phytoplasma to known strains worldwide. The results indicated that diseased, wild blueberry in Lithuania is infected by a phytoplasma closely related to strains that had previously been found in Germany and North America. A preliminary report in abstract form has been published (Valiunas *et al*., 2002).

MATERIALS AND METHODS

Plant samples, polymerase chain reaction (PCR) conditions, and primers. Samples of leaf tissue were collected over a period of three years from 20 naturally infected European blueberry plants exhibiting symptoms of shoot proliferation (witches' broom) and chlorosis in a forest of Aukstaitija National Park and in forests of other regions of Lithuania. Nucleic acid for

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use as template in PCR was extracted from fresh tissue using Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) according to manufacturer's instructions. Two pairs of oligonucleotides were used to prime PCRs. P1/P7 and R16F2n/R16R2 (R16F2n/R2) are phytoplasma universal primer pairs that prime amplification of sequences from the phytoplasma rRNA operon. In nested PCR, DNA amplified in PCR primed by P1/P7 was diluted 1:50 with sterile water and used as template in PCR primed by R16F2n/R2. All PCRs were carried out in final volume of 25 µl under conditions as previously described (Lee *et al*., 1993). PCRs were carried out for 35 cycles using the following parameters: 1 min (3 min for first cycle) denaturation at 94°C, annealing for 2 min at 55°, and primer extension for 3 min (10 min in final cycle) at 72°C. Resulting PCR products were analysed by electrophoresis through 1% agarose gel, stained with ethidium bromide, and DNA bands visualized using a UV transilluminator. DNA fragment size standard was 1 kb ladder (Life Technologies, Gaithersburg, MD).

RFLP analyses of amplified phytoplasma DNA. Products from nested PCR primed by R16F2n/R2 were analysed by single enzyme digestion, according to manufacturer's instructions, with *Alu*I (Fermentas AB, Vilnius, Lithuania), *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Mse*I, *Rsa*I, *Sau*3AI (New England Biolabs, Beverly, MA, USA). The RFLP profiles of digested DNA were analyzed by electrophoresis through 5% polyacrylamide gel, staining with ethidium bromide, and visualisation using a UV transilluminator. DNA fragment size standard was ØX174 RFI DNA *Hae*III digest (Life Technologies, Gaithersburg, MD, USA). RFLP patterns were compared with those previously published (Lee *et al*., 1998).

Nucleotide sequencing and putative restriction site analysis. Products of PCR primed by P1/P7 were cloned in *E. coli* using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and were sequenced using automated DNA sequencing. The nucleotide sequence determined in this study was deposited in the GenBank database. Other sequences used were obtained from GenBank (accession numbers given in Fig. 2). Putative restriction maps were constructed by the use of the DNASTAR program MapDraw option.

Phylogenetic analysis. 16S rRNA gene sequences (1.2 kbp in size, representing the sequence between annealing sites of primer pair F2n/R2) from six phytoplasma strains from Lithuania and 30 other phytoplasma strains representing 14 16S rRNA phytoplasma groups (*sensu* Lee *et al*., 1998), and *Acholeplasma laidlawii* and *A. palmae* were aligned using Clustal X version 1.63b (Thompson *et al*., 1997) and DNASTAR. Analyses were performed with the computer program Clustal X. A phylogenetic tree was constructed by the neighbor-joining method, and the tree was viewed by using TreeViewPPC (Page, 1996). *A. laidlawii* was selected as the outgroup to root the tree.

RESULTS AND DISCUSSION

A preliminary survey of populations of wild blueberry in different forests in Lithuania indicated that up to 10% of plants may be diseased, depending upon location. Plants exhibiting symptoms of shoot proliferation bore numerous dead branches and were devoid of fruits. A phytoplasma-characteristic 1.2 kb 16S rDNA product was amplified from DNA of all 20 diseased, but from no healthy, blueberry plants tested, using phytoplasma universal primer pairs P1/P7 and R16F2n/R2 in nested PCRs (data not shown), indicating that both diseased plants were infected by phytoplasma, termed phytoplasma strain VAC-L.

The RFLP patterns of phytoplasmal 16S rDNA amplified from all 20 diseased plants were characteristic of phytoplasmas belonging to group 16SrIII (X-disease phytoplasma group) (data not shown). The collective RFLP patterns indicated that the VAC-L phytoplasma was most closely related to milkweed yellows (MW1) phytoplasma reported (Griffiths *et al*., 1994) in North America. VAC-L and MW1 phytoplasmas were indistinguishable on the basis of collective RFLP patterns using eight restriction enzymes (Fig. 1). The collective patterns from analyses using *Mse*I and *Hha*I were identical for VAC-L and MW1 rDNAs and distinguished these phytoplasmas from other phytoplasmas classified in the group 16SrIII. Based on these results, VAC-L phytoplasma was classified in the group 16SrIII, subgroup F (III-F, milkweed yellows phytoplasma subgroup).

A 1.8 kbp rDNA sequence, amplified from VAC-L phytoplasma in P1/P7-primed PCR, was cloned and sequenced and the nucleotide sequence deposited in the GenBank database under accession no. AY034090. The putative restriction site maps of the VAC-L 16S rDNA were in excellent agreement with results from the enzymatic RFLP analysis (data not shown). Alignment of the VAC-L and MW1 16S rDNAs revealed that the sequences differed at only five base positions, a finding consistent with the concept that VAC-L and MW1 may be variant strains of the same species. The nucleotide sequence of 16S rDNA from a vaccinium witches' broom (VAC) phytoplasma in Germany was reported previously (Schneider *et al*., 1993; Seemüller *et al*., 1994). We wished to compare that sequence (GenBank accession no. X76430) with the 16S rDNA sequence from VAC-L phytoplasma. Sequence X76430 contains a number of undetermined bases (N's) and putative miscalled bases, presumably due to polymerase or sequenc-

ing errors, in highly conserved base positions; excluding these base positions, the VAC-L and VAC 16S rDNAs were indistinguishable within sequencing error. These results are consistent with other work indicating that VAC phytoplasma is also a member of subgroup III-F (Paltrinieri *et al*., 2000).

On the basis of analysis of 16S rDNA sequences, we constructed a phylogenetic tree indicating relationships among previously known phytoplasmas and the new phytoplasma detected in blueberry (VAC-L phytoplasma) (Fig. 2). The branching order of the tree confirmed that VAC-L, VAC, and MW1 are closely related.

The molecular detection and characterization of a phytoplasma associated with a proliferation disease of wild blueberry has implications for the ecology of the Baltic region. Although blueberry is not cultivated in Lithuania, it occurs widely in forests of the country. Being one of the dominant species in pine forests, *V. myrtillus* is an important component of the biodiversity in pine forest ecosystems. Based on the widespread occurrence of phytoplasma-diseased wild blueberry, we suggest that the VAC-L phytoplasma is having a significant impact, reducing the competitive advantage of wild blueberry.

In other regions, phytoplasmas have been reported in association with several diseases of *Vaccinium* spp., including blueberry stunt (Chen, 1971; Hartman *et al*., 1972; Tozzi *et al*., 1993) and cranberry false blossom (Chen, 1971; Xu and Chen, 1996) in North America; and vaccinium witches' broom of blueberry (Bos, 1960; Kegler *et al*., 1973; Blattny and Vana, 1974; de Leeuw, 1975) and little leaf disease of lingonberry and wild *V. myrtillus* blueberry (Tomenius and Ahman, 1983) in Europe. Phytoplasmas have also been detected, by DAPI staining, in symptomless plants of blueberry (Schaper and Converse, 1985). Phytoplasmas associated with some of the blueberry diseases have been classified in the widely used system of classification based on RFLP patterns of 16S rDNA (Lee *et al*., 1998). For example, cranberry false blossom phytoplasma in North America is closely related to clover yellow edge (CYE) phytoplasma, a member of group 16SrIII, subgroup B (III-B) (Xu and Chen, 1996); blueberry stunt (BBS1, BBS3) phytoplasma in North America is a member of group 16SrI, subgroup E (I-E) (Tozzi *et al*., 1993); and vaccinium witches' broom (VAC) phytoplasma in Europe is a member of subgroup III-F (Paltrinieri *et al*., 2000). Although apparently prevalent in wild blueberry in Lithuania (this communication) and present in cultivated blueberry (*V. corymbosum*) in Germany (Paltrinieri *et al*., 2000), subgroup III-F is not known in any other host plant in Europe.

The other phytoplasma belonging to this subgroup is milkweed yellows phytoplasma (MW1), previously reported in the United States (Griffiths *et al*., 1994). Milkweed (*Asclepias syriaca*) is the only known plant host of subgroup III-F phytoplasma strains in North America. The close relationship of VAC-L, VAC, and MW1 phytoplasmas, as indicated by the present RFLP and phylogenetic analyses, is consistent with their possible status as strains of the same species. These observations raise the question of possible intercontinental movement of subgroup III-F phytoplasma strains. The insect vector(s) that transmit MW1 phytoplasma in the United States and VAC-L phytoplasma in Lithuania are unknown, but the observed level of diseased wild *V. myrtillus* indicates that the VAC-L phytoplasma is being spread by a local insect vector in Lithuania. Taken together, the available data are consistent with the hypothesis that wild blueberry serves as a source of phytoplasma inoculum for infections of cultivated blueberry in Europe.

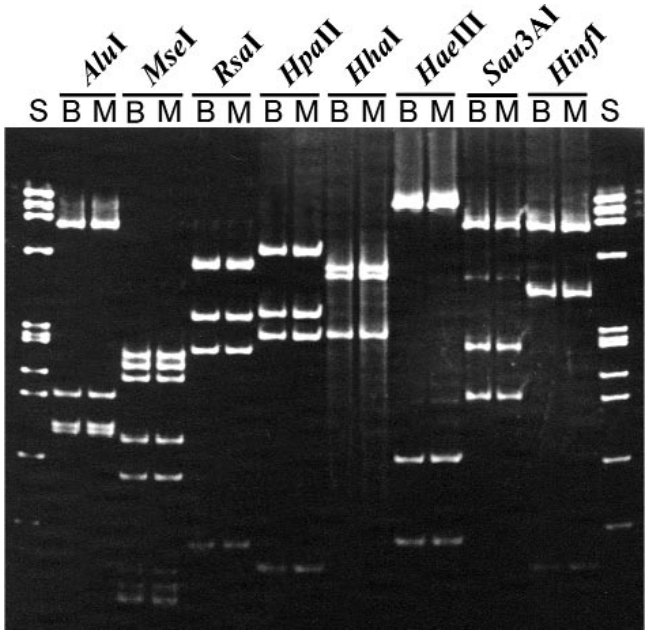

Fig. 1. RFLP analysis of 16S rDNA amplified in nested PCR primed by oligonucleotide pair R16F2n/R2 from phytoplasma strains. First round of PCR was primed by P1/P7 followed by re-amplification of target DNA in nested PCR primed by R16F2n/R2. DNA products from the second, nested PCR were digested with restriction endonucleases *Alu*I, *Mse*I, *Rsa*I, *Hpa*II, *Hha*I, *Hae*III, *Sau*3AI, and *Hin*fI. S, Phi X174 *Hae*III digest size standard. B, rDNA from blueberry phytoplasma strain VAC-L. M, rDNA from strain milkweed yellows phytoplasma strain MW1.

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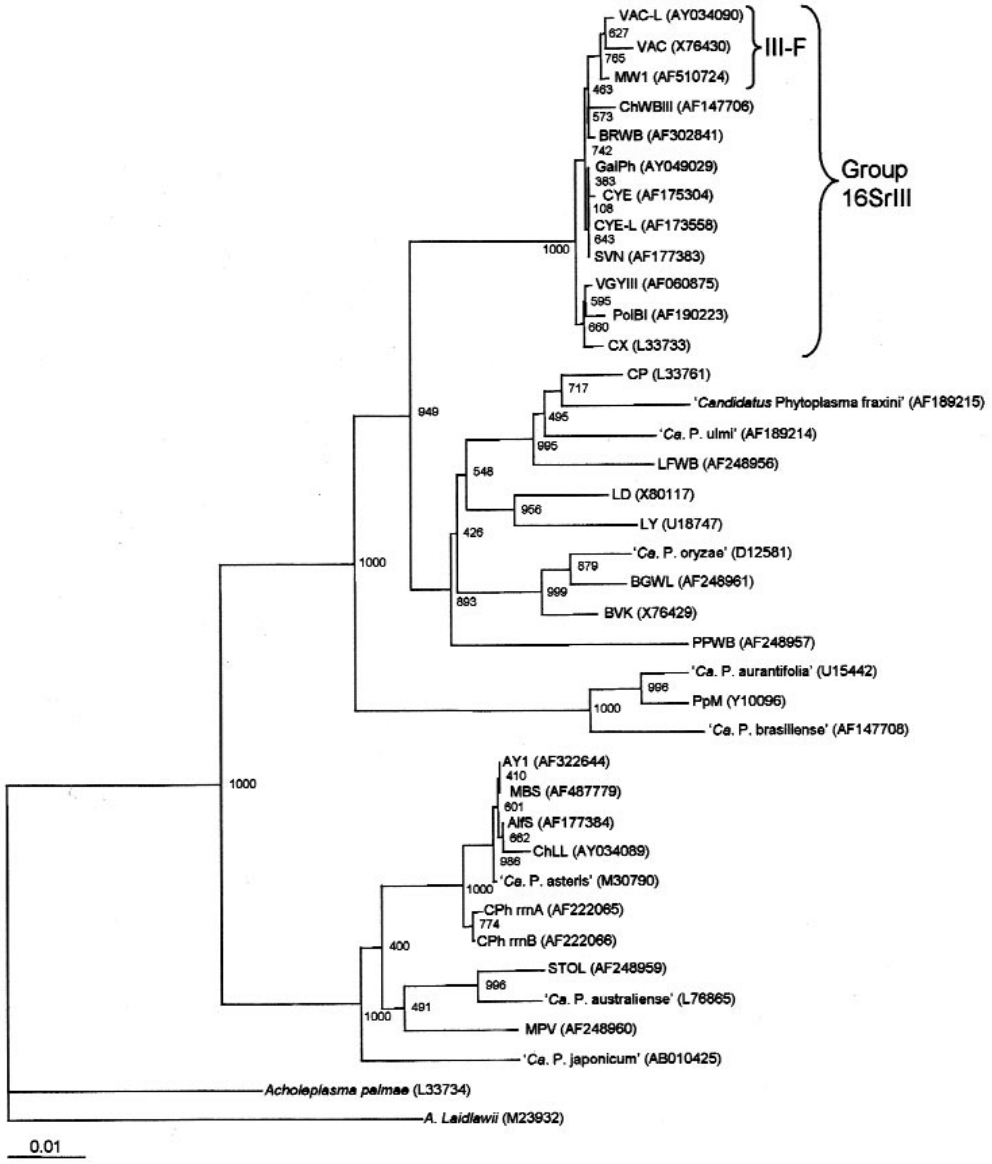

Fig. 2. Phylogenetic tree constructed by the Neighbor-Joining method of 16S rRNA gene sequences from 32 phytoplasmas and *Acholeplasma laidlawii* and *Acholeplasma palmae*, employing *Acholeplasma laidlawii* as the outgroup. VAC-L, blueberry phytoplasma Lithuania; VAC, Vaccinium witches' broom; MW1, milkweed yellows; ChWB, chayote witches' broom; BRWB, black raspberry witches' broom; GaiPh, gaillardia phyllody; CYE, clover yellow edge Canada; CYE-L, clover yellow edge Lithuania; SVN, soybean veinal necrosis; VGYIII, Virginia grapevine yellows; PoiBI, poinsettia free-branching; CX, Canada X-disease; CP, clover proliferation; '*Candidatus* Phytoplasma fraxini' (ash yellows); '*Ca*. P. ulmi (elm yellows); LfWB, loofah witches' broom; LD, coconut lethal disease; LY, coconut lethal yellows; '*Ca*. P. oryzae' (rice yellow dwarf); BGWL, Bermudagrass white leaf; BVK, phytoplasma from *C. roseus* in Germany; PPWB, pigeon pea witches' broom; '*Ca*. P. aurantifolia' (witches' broom disease of lime); PpM, papaya mosaic; '*Ca*. P. brasiliense' (hibiscus witches' broom); AY1, aster yellows; MBS, maize bushy stunt; AlfS, alfalfa stunt; ChLL, cherry little leaf; 'Ca. P. asteris' (Michigan aster yellows); CPh rrnA, clover phyllody rRNA operon A; CPh rrnB, clover phyllody rRNA operon B; STOL, stolbur; '*Ca*. P. australiense' (Australian grapevine yellows); MPV, Mexican periwinkle virescence; '*Ca.* P. japonicum'. GenBank accession numbers are given in parentheses. III-F, subgroup F in the group 16SrIII. Group 16SrIII, X-disease phytoplasma group.

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