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Molecular detection, classification and phylogenetic analysis of subgroup 16SrI-C phytoplasmas detected in diseased *Poa* and *Festuca* in Lithuania

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Phytoplasma strains were detected in two grass species, *Poa pratensis* L. (common meadow grass) and *Festuca arundinacea* Schreb. (tall fescue), exhibiting yellows disease symptoms in Lithuania. Analysis of amplified 16S rDNAs revealed that the phytoplasmas associated with these diseases, designated as poa stunt (PoaS) and festuca yellows (FesY), respectively, were '*Candidatus* Phytoplasma asteris'-related strains belonging to group 16SrI (aster yellows phytoplasma group) subgroup 16SrI-C (I-C, clover phyllody phytoplasma subgroup). Together with other data, the results strengthen the concept that subgroup I-C phytoplasma strains have a broad pathogenic potential. It is possible that *P. pratensis and F. arundinacea*, previously undescribed as phytoplasma hosts, play a role in the epidemiology of phytoplasmal diseases affecting cereal grasses like oat and rye in the region. The insect vectors of subgroup 16SrI-C phytoplasma strains in Lithuania and neighboring countries remain unknown, emphasizing the need for future investigations to understand how these phytoplasmas spread in the Baltic region.

Key words: *Mollicutes*, clover phyllody, PCR, RFLP, phylogeny, PoaS, FesY

INTRODUCTION

Diverse species of grasses grown in Lithuania are important for human and livestock nutrition. These species include oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.), and ryegrass (*Lolium multiflorum* Lam.) for human consumption, and tall fescue (*Festuca arundinacea* Schreb.) and common meadow grass (*Poa pratensis* L.) for animal feed. Research in recent years has revealed that these grass species are susceptible to diseases caused by phytoplasmas in Lithuania. Phytoplasmas are cell wall-less bacteria that are carried from plant to plant by phloem-feeding insects, mainly leafhoppers. Phytoplasmas are responsible worldwide for diseases of many plant species, including grasses [1, 2].

Phytoplasmas cannot be isolated in culture, and therefore, conventional methods by which most other bacteria can be detected and identified are not suited for use with phytoplasmas. Thus, modern molecular methods have been adopted for the detection, identification, phylogenetic analysis, and taxonomy of phytoplasmas [3, 4]. The use of such methods resulted in the discovery that several diseases of oats, barley, ryegrass, and *Triticosecale* cereal grasses in Lithuania are caused by phytoplasmas [5–8]. The biodiversity of phytoplasmas that cause diseases in grasses has been less studied than that of phytoplasmas affecting other plant groups, and relatively little is known about insect vectors of grass-infecting phytoplasmas [5, 6, 7, 9, 10]. The present work was initiated to expand knowledge concerning the biodiversity of phytoplasmas infecting grass species and to understand possible relationships of diseases in grass species used for animal feed to diseases in cereal grass species. A preliminary report of the results was published as part of a doctoral thesis [11] and in [12].

MATERIALS AND METHODS

Plant samples and DNA extraction. Leaf tissue samples were collected from naturally infected, symptomatic common meadow grass (*P. pratensis*) and tall fescue (*F. arundinacea*) in Vilnius and Kedainiai districts, respectively, in Lithuania. The veins of the leaves were excised, and DNAs for use as templates in polymerase chain reactions were extracted from the veins using a Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) according to manufacturer's instructions.

Polymerase chain reaction (PCR). P1/P7 [13, 14] and R16F2n/R16R2 [15] are phytoplasma universal oligonucleotide pairs that prime amplification of DNA 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 a template in PCR primed by R16F2n/R16R2. All PCRs were carried out in a final volume of 50 μl under conditions as previ-

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ously described [3]. 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 GeneRuler™ 1 kb DNA Ladder (MBI Fermentas).

RFLP analysis, nucleotide sequencing, and sequence analysis. Products from nested PCRs were analysed by single enzyme digestion according to manufacturer's instructions, with *Alu*I, *Mse*I, *Kpn*I, *Hha*I, and *Hae*III (MBI Fermentas). The RFLP profiles of digested DNA were analysed by electrophoresis through 5% polyacrylamide gel, followed by staining with ethidium bromide, and were visualized using a UV transilluminator. The DNA fragment size standard was ØX174 RFI DNA/*Bsu*RI (*Hae*III) digest size standard (MBI Fermentas). RFLP patterns were compared with those previously published [2, 16].

DNA products of PCR primed by P1/P7 (from PoaS phytoplasma) and/or by R16F2n/R16R2 (from FesY phytoplasma) were cloned in *E. coli* using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and sequenced using automated DNA sequencing. Putative restriction maps were constructed by the use of the DNASTAR software MapDraw option. For calculation of sequence similarities, sequences were aligned by using the DNASTAR software MegAlign option.

Phylogenetic analysis. 16S rRNA gene sequences (1.2 kb in size, representing the R16F2n/R16R2 PCR product) from 27 phytoplasma strains and *Acholeplasma palmae* were aligned, for phylogenetic analysis, using Clustal X version 1.63b software [17]. A phylogenetic tree was constructed by the Neighbor–Joining method, and the tree was viewed by using TreeViewPPC [18]. *A. palmae* was selected as the outgroup to root the tree. GenBank accession numbers of the nucleotide sequences are given in Fig. 2. Bootstrapping was performed 1000 times for estimation of stability and support for the clades.

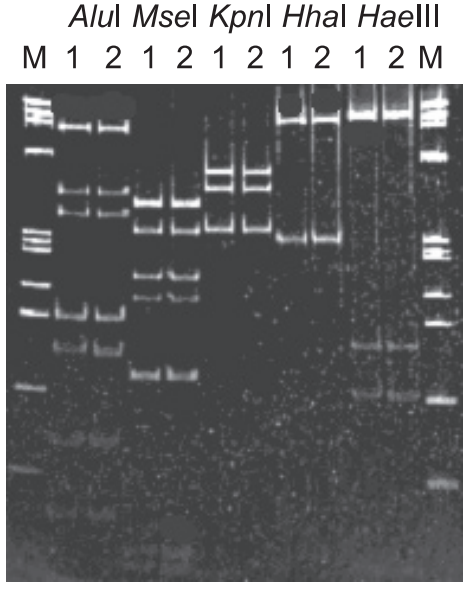

Fig. 1. RFLP patterns of 16S rDNA amplified in nested PCR primed by oligonucleotide pair R16F2n/R16R2 from poa stunt (PoaS) and festuca yellows (FesY) phytoplasmas. Results are from single enzyme digests using *Alu*I, *Mse*I, *Kpn*I, *Hha*I, and *Hae*III, respectively. 1 – PoaS; 2 – FesY; M, ØX174 RFI DNA/*Bsu*RI (*Hae*III) digest size standard (MBI Fermentas), fragment sizes: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp

RESULTS

Tall fescue (*Festuca arundinacea* Schreb.) and common meadow grass (*Poa pratensis* L.) are important as green crop for livestock nutrition. Diseased plants of *P. pratensis* exhibiting general stunting, yellowing of plants and proliferation symptoms were found in the Vilnius District of eastern Lithuania. Diseased plants of *F. arundinacea* exhibiting general yellows, stunting, proliferation of stems, and sterility and whitening of spikes were found in Dotnuva, district of Kedainiai, in central Lithuania. The diseases in the two grass species were termed poa stunt (PoaS) and festuca yellows (FesY), respectively.

Molecular detection and classification of phytoplasmas and analysis of 16S rDNA. In nested PCRs primed by phytoplasma universal primer pairs P1/P7 and R16F2n/R16R2, phytoplasma-characteristic 1.8 kb and 1.2 kb rDNA fragments were amplified from DNAs of all diseased but from no healthy plants tested, indicating that the diseased plants were infected by phytoplasmas (data not shown). The phytoplasmas detected in *P. pratensis* and *F. arundinacea* were termed strains PoaS and FesY, respectively.

The RFLP patterns of PCR-amplified PoaS and FesY 16S rDNAs (products of PCRs primed by primer pair R16F2n/R16R2) were characteristic of phytoplasmas belonging to subgroup 16SrI-C (clover phyllody (CPh) phytoplasma subgroup) (Fig. 1) according to the classification system of Lee et al. [2].

Amplified fragments (1.8 kbp in size) of two rRNA operons from PoaS were cloned. The nucleotide sequences of the cloned DNA fragments representing the two ribosomal RNA (rrn) operons of PoaS phytoplasma were determined, and the sequence data were deposited in the GenBank database under accession nos. DQ640501 (PoaS rrnA) and DQ640502 (PoaS rrnB). Similarly, 16S rDNA fragments (1.2 kbp in size) representing the two rrn operons amplified from FesY in PCR primed by R16F2n/R16R2 were cloned, sequenced, and the sequence data deposited in the GenBank under accession nos. DQ640503 (FesY rrnA) and DQ640504 (FesY rrnB). The putative restriction site maps of PoaS and FesY 16S rDNAs were in excellent agreement with results from the enzymatic RFLP analysis (data not shown).

Alignments of nucleotide sequences revealed that the 1.8 kb PoaS rrnA sequence differed from the corresponding sequence of CPh rrnA by one base in the 16S rDNA region; PoaS rrnA and CPh rrnA shared 99.9% sequence similarity. The rrnB sequences from PoaS and CPh phytoplasmas were mutually identical. The 1.2 kbp rrnA and rrnB sequences of FesY phytoplasma shared sequence similarities of 99.7% and 99.9%, respectively, with rrnA and rrnB of CPh phytoplasma.

Phylogenetic relationships. A phylogenetic tree of phytoplasma subgroup 16SrI-C strains and other phytoplasma strains was constructed on the basis of 16S rRNA gene sequences to visualize relationships of the *P. pratensis* and *F. arundinacea* phytoplasmas with other strains (Fig. 2). The branching order of the tree confirmed that PoaS and FesY phytoplasmas were related to CPh phytoplasma, supported their classification in phytoplasma subgroup 16SrI-C, and showed that they were most closely related to '*Candidatus* Phytoplasma asteris' among currently recognized phytoplasma species. All 16S rDNA sequences derived from phytoplasma strains in subgroup 16SrI-C formed a

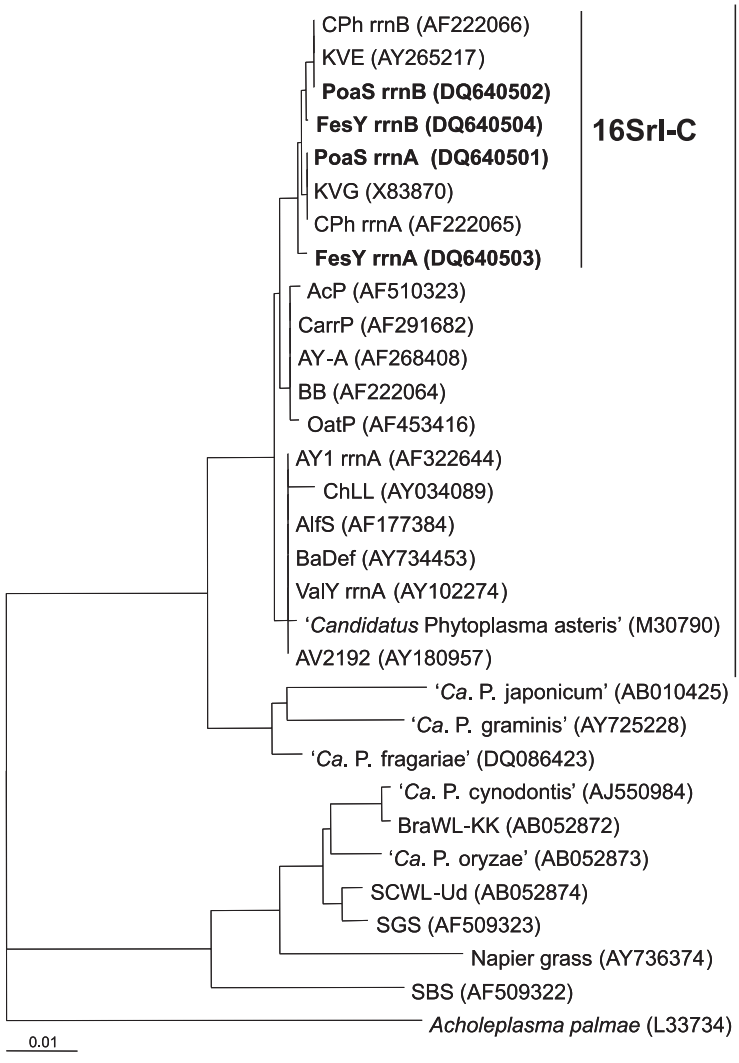

Fig. 2. Phylogenetic tree constructed by the Neighbor–Joining method from 16S rDNAs of 27 phytoplasma strains, including phytoplasma subgroup 16SrI-C strains, and *Acholeplasma palmae*, employing *A. palmae* as the outgroup. GenBank accession numbers of 16S rDNA sequences are in parentheses. Group 16SrI and subgroup 16SrI-C strains are indicated

distinct branch in the group 16SrI clade, underscoring their distinctness from other strains in group 16SrI.

DISCUSSION

The finding of subgroup 16SrI-C phytoplasmas in *P. pratensis* and *F. arundinacea* (this study) expands the known biodiversity of phytoplasmas known to infect grass species. Phytoplasmas belonging to several distinct species-level lineages have previously been reported in cereal plants and other grasses in which they can cause significant harvest losses [10]. For example, several phytoplasmas have been reported in oats, barley, ryegrass, and *Triticosecale* cereal grasses and smooth brome grass in Lithuania [5–8, 19]. Annual blue grass white leaf (ABGWL) phytoplasma was identified as a subgroup 16SrXIV-A phytoplasma from *Poa annua* in Italy [2, 9]. Bermuda grass white leaf (BGWL) disease of *Cynodon* sp. found in Southeastern Asia and Africa is caused by a phytoplasma of subgroup 16SrXIV-A [2, 9]. Phytoplasmas detected in other grasses (rice, sugarcane, Bermuda grass, and others) in Asia belong to subgroups 16SrXI-A, 16SrXI-B, 16SrVI-A, 16SrII-C, and group 16SrI [2, 9, 10, 20]. Prior to the present work, phytoplasmas had not been reported in *P. pratensis* or *F. arundinacea*. The results of this study underscore the concept [20] that strains of subgroup 16SrI-C have a broad pathogenic potential, since they infect dicotyledonous and monocotyledonous, woody and herbaceous plant species [22–24, this study]. Factors affecting the extent of the phytoplasmal diseases among such different plants are not understood.

It is possible that *P. pratensis and F. arundinacea*, previously undescribed as phytoplasma hosts, play a role in the epidemiology of phytoplasmal diseases affecting cereal grasses like oat and rye, and possibly other plant species, in the region. In particular, the insect vectors of subgroup 16SrI-C phytoplasma strains in Lithuania and neighboring countries remain unknown, emphasizing the need for future investigations to understand how these phytoplasmas spread in the Baltic region.

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Poa IR *Festuca* AUGALUS LIETUVOJE PAŽEIDŽIANČIŲ 16SrI-C POGRUPIO FITOPLAZMŲ MOLEKULINIS APTIKIMAS, KLASIFIKACIJA IR FILOGENETINĖ ANALIZĖ

S a n t r a u k a

Poa pratensis L. (pievinė miglė) ir *Festuca arundinacea* Screb. (nendrinis eraičinas) su geltligių požymiais buvo rasti Vilniaus ir Kėdainių rajonuose. Pagausintų 16S rDNR analizė rodo, kad fitoplazmų kamienai PoaS (poa stunt) ir FesY (festuca yellows) yra gimininginga '*Candidatus* Phytoplasma asteris' ir priklauso 16SrI (aster yellows) fitoplazmų grupei, 16SrI-C (I-C, clover phyllody) fitoplazmų pogrupiui. Šie augalai nebuvo anksčiau aprašyti kaip fitoplazmų šeimininkai. Remiantis ir kitų tyrėjų duomenimis, galima daryti prielaidą, kad I-C pogrupio fitoplazmų kamienai turi didelį patogeniškumo potencialą. 16SrI-C pogrupio fitoplazmų vabzdžiai-vektoriai Lietuvoje ir kaimyninėse šalyse yra nežinomi, todėl ateityje reikalingi išsamesni tyrimai.