SUMMARY

Symptoms of shoot proliferation and abnormally small leaves were observed in diseased sour cherry (Cerasus vulgaris Mill., syn. Prunus cerasus L.) affected by cherry little leaf (ChLL) disease on the Neringa peninsula, Lithuania. Amplification of phytoplasma characteristic 16S rDNA from the diseased cherry indicated infection by a phytoplasma. On the basis of restriction fragment length polymorphism (RFLP) and nucleotide sequence analysis of 16S rDNA amplified by PCR, the ChLL phytoplasma was classified in group 16SrI (aster yellows phytoplasma group), new subgroup 16SrI-Q. Results from phylogenetic analysis of 16S rRNA gene sequences indicated that ChLL phytoplasma was related to ‘Candidatus Phytoplasma asteris’ and may represent a distinct phytoplasma lineage.

Key words: Cherry, detection, identification, phytopathogenic mollicutes, phylogeny.

INTRODUCTION

Phytoplasmas are plant pathogenic, wall-less unculturable bacteria classified in the class Mollicutes, that cause diseases resulting in harvest losses. Phloem-feeding insects, mainly leathoppers, transmit phytoplasmas from plant to plant (Davis and Lee, 2000). Twenty eight phytoplasma groups (16Sr groups), and more than 50 subgroups have been identified on the basis of RFLP analysis of 16S ribosomal (r)DNA (Lee et al., 1998; Marcone et al., 2000; Davis and Dally, 2001; Jomantiene et al., 2002a; Wei et al., 2007). Thus far, four 16Sr groups (16SrI, 16SrIII, 16SrV, and 16SrXII) and twelve subgroups have been reported in Lithuania (Jomantiene et al., 2000, 2002a, 2002b; Staniulis et al., 2000; Valiunas et al., 2001a, 2001b, 2004; Wei et al., 2007).

MATERIALS AND METHODS

Plant samples, DNA extraction and PCR. Samples of leaf tissue from one apparently healthy (this tree was negative for the presence of phytoplasma) and one naturally infected sour cherry exhibiting symptoms of shoot proliferation and little leaf (Fig. 1) were collected in Nida, in a garden neighboring the unique pine forest ecosystem of the Neringa peninsula. Nucleic acid for use as template in PCR was extracted from fresh tissue using the genomic DNA purification kit (Fermentas, Lithuania) according to manufacturer’s instructions. Nested PCR assays using extracted DNA and primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996) were carried out and the amplified products were analysed as previously described (Lee et al., 1998).

Phytoplasma 16S rDNA RFLP and nucleotide sequence analysis. Products from nested PCR primed by R16F2n/R16R2 were analysed by single enzyme digestion with AluI, BfaI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, MseI, RsaI, Sau3AI, TaqI, and ThaI (New England Biolabs, USA) according to manufacturer’s instructions. The restriction fragment length polymorphism (RFLP) profiles of digested DNA were analyzed by

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electrophoresis through 5% polyacrylamide gel; DNA fragment size standard was \( \Phi X174 \) DNA HaeIII digest (Life Technologies, USA). RFLP patterns were compared with those previously published (Lee et al., 1998; Marcone et al., 2000). The PCR product primed by R16F2n/R16R2 was cloned in \( E. coli \) using the TOPO-TA cloning kit (Invitrogan, USA) and sequenced. Other nucleotide sequences used in this study were obtained from the GenBank database. Putative restriction maps were constructed by the use of the DNASTAR program MapDraw option. For calculations of sequence similarities, sequences were aligned by using DNASTAR software MegAlign option. Virtual RFLP analysis was carried out by the use of the pDRAW program (AcaClone Software, http://www.acaclone.com) as previously described (Wei et al., 2007).

**Phylogenetic analysis.** For phylogenetic analysis, 16S rRNA gene sequences (1.2 kbp in size, representing the sequence between annealing sites of primer pair R16F2n/R16R2) from ChLL phytoplasma and 25 other phytoplasma strains were aligned using Clustal X version 1.63b (Thompson et al., 1997). A phylogenetic tree was constructed by the neighbor-joining method, and the tree was viewed by using TreeViewPPC (Page, 1996). GenBank accession numbers of nucleotide sequences used in study are given in Figs. 4 and 5.

**RESULTS**

Detection and classification of phytoplasma in cherry. In each of three separate experiments, DNA was extracted from the diseased cherry and used as template in three separate PCR assays. In all cases, a phytoplasma-specific 1.2 kb 16S rDNA product was amplified from DNA of diseased, but not from healthy cherry, using the phytoplasma universal primer pairs P1/P7 and R16F2n/R16R2 in nested PCR (data not shown). This was taken as an indication that the diseased plant hosted a phytoplasma which was referred to as cherry little leaf (ChLL) phytoplasma.

The 16S rDNA product from each of the three PCR runs was separately analysed by single enzyme digestion with \( AluI, BfaI, HbaI, HpaI, HpaII, HaeIII, HinfI, KpnI, MseI, RsaI, Sau3AI, TaqI, \) and \( TbaI \). All three products yielded the same collective RFLP patterns (Fig. 2 and data not shown). Comparison of these patterns with those previously published for 16S rDNA from other phytoplasmas (Lee et al., 1998; Marcone et al., 2000), showed

![Fig. 1. Sour cherry exhibiting disease symptoms associated with infection by cherry little leaf (ChLL) phytoplasma, the first described member of subgroup 16SrI-Q. S, symptomatic branch. A, asymptomatic branch.](image1)

![Fig. 2. RFLP analysis of 16S rDNA amplified, in nested PCR primed by oligonucleotide pair R16F2n/R16R2, from template DNA extracted from ChLL phytoplasma. DNA was subjected to single enzyme digestion by restriction nucleases. Lane 1, \( AluI \); lane 2, \( MseI \); lane 3, \( KpnI \); lane 4, \( HbaI \); lane 5, \( HaeIII \); lane 6, \( HpaI \); lane 7, \( HpaII \); lane 8, \( RsaI \); lane 9, \( HinfI \); lane 10, \( TgaI \); lane 11, \( Sau3AI \); lane 12, \( BfaI \); lane 13, \( TbaI \). Lanes S, \( \Phi X174 \) HaeIII digest size standard, fragment sizes from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp.](image2)
that the ChLL phytoplasma belongs to group 16SrI (aster yellows phytoplasma group) and is related to 'Candidatus Phytoplasma asteris'. However, the ChLL HaeIII and RsaI RFLP patterns were different from those previously published for group 16SrI phytoplasmal rDNAs.

### Nucleotide sequence of rDNA and putative restriction site analysis

A 1.2 kbp 16S rDNA sequence, amplified from ChLL phytoplasma in PCR primed by R16F2n/R16R2, was cloned in *E. coli* and sequenced, and the nucleotide sequence was deposited in the GenBank database under accession No. AY034089. The putative restriction site map of the sequenced ChLL 16S rDNA was in excellent agreement with the results from the enzymatic RFLP analysis (Fig. 4 and data not shown). Since the RsaI and/or HaeIII RFLP patterns of 16S rDNA distinguished ChLL phytoplasma (Fig. 2 and 3) from all group 16SrI phytoplasmas previously reported (Lee et al., 1998; Marcone et al., 2000), we classified the ChLL phytoplasma in group 16SrI, new subgroup I-Q.

### Phylogenetic analysis and alignments of 16S rDNA sequences

Based on 16S rDNA sequences, a phylogenetic tree was constructed (Fig. 5) that showed the cherry ChLL phytoplasma to be most closely related to phytoplasmas classified in subgroups I-B and I-M. Based on collective RFLP patterns and phylogenetic distance from other group 16SrI phytoplasmal rDNAs, ChLL phytoplasma possibly represents a distinct phytoplasma lineage.

### DISCUSSION

In this paper, we report a previously undescribed phytoplasma associated with a disease of cherry, as part of continued molecular investigations of phytoplasmal biodiversity in the Baltic region of Europe. In other parts of the world, phytoplasmas have been reported in association with several diseases of cherry and other *Prunus* species. In Europe, diseases including apricot...
chlorotic leaf roll, leptonecrosis and decline of Japanese plum (Prunus salicina), and declines of peach, almond, flowering cherry (Prunus serrulata), and European plum (Prunus domestica) are associated with a phytoplasma known as the European stone fruit yellows phytoplasma (Davies and Adams, 2000; Lorenz et al., 1994; Marcone et al., 1996). This phytoplasma is a member of group 16SrX (apple proliferation phytoplasma group) (Lee et al., 1998). A different phytoplasma associated with an apricot chlorotic leaf roll disease has been reported from Spain (Schneider et al., 1993). This phytoplasma, denoted apricot chlorotic leaf roll-AY phytoplasma by Lee et al. (1998), is a member of group 16SrI (aster yellows phytoplasma group), subgroup 16SrI-F. Molière's disease of cherry in Europe is associated with a phytoplasma belonging to group 16SrXII (stolbur phytoplasma group) (Schneider et al., 1993; Marcone et al., 1999). Phytoplasma strains belonging to subgroups 16SrX-B and 16SrI-B were identified in sour cherries in Hungary (Varga et al., 2001). In Italy, sweet cherries were found to be infected by phytoplasmas belonging to subgroups 16SrI-B, 16SrX-B, 16SrX-C, 16SrXII-A, and to group 16SrIII (Paltrinieri et al., 2001). Peach yellow leaf roll phytoplasma (PYLR, member of group 16SrX) and X-disease phytoplasma (member of group 16SrIII) were reported from Prunus spp. in North America (Gundersen et al., 1996; Kirkpatrick et al., 1994; Uyemoto et al., 2001). A phytoplasma associated with cherry lethal yellows disease in China is a member of group 16SrV (elm yellows phytoplasma group) (Lee et al., 1995). Taken together, the present record and the extant literature show that Prunus spp. around the world are susceptible to infection by a broad diversity of phytoplasma lineages and emphasize the need for adequate quarantine surveillance in intercontinental movement of fruit tree germplasm.

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